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Protocol

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Highlights

Isolation of ILC from mouse bone marrow

Lymphoid cell reconstitution in mice

Applicable to study the development and function of ILCs *in vivo*

Innate lymphoid cells (ILCs) and adaptive T cells remain a challenge to study because of a significant overlap in their transcriptomic profiles. Here, we describe the adoptive transfer of ILC progenitors into mice genetically deficient in innate and adaptive immune cells to allow detailed study of the development and function of ILCs and gene regulation in an *in vivo* setting.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

A protocol to isolate bone marrow innate lymphoid cells for alymphoid mouse reconstitution

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SUMMARY

Innate lymphoid cells (ILCs) and adaptive T cells remain a challenge to study because of a significant overlap in their transcriptomic profiles. Here, we describe the adoptive transfer of ILC progenitors into mice genetically deficient in innate and adaptive immune cells to allow detailed study of the development and function of ILCs and gene regulation in an *in vivo* setting.

For complete details on the use and execution of this protocol, please refer to Jacquelot et al. (2021) and Seillet et al. (2016).

BEFORE YOU BEGIN

Innate lymphoid cells (ILC) are critical contributors to host defense and tissue homeostasis. They are the innate counterparts of T cells, but in contrast to adaptive lymphocytes, they are devoid of antigen specific receptor expression. Similar to T cells, ILC exhibit subset specific expression of key transcription factors and cytokines. These features, together with their distinct developmental pathways, have led to the current classification of this family into five distinct subsets, namely natural killer (NK) cells, ILC1, ILC2, ILC3 and lymphoid tissue-inducer (LTi) cells (Vivier et al., 2018). In contrast to T cells, ILC activity is not restricted to a specific antigen but does rely on the integration of environmental signals driving their effector functions (Klose and Artis, 2020). Despite apparent redundancy, ILC and T cells play complementary roles with distinct temporal activity that enables them to eliminate pathogens and preserve tissue homeostasis (Rankin et al., 2016; Serafini et al., 2022). Due to a significant overlap between T cell and ILC transcriptomic profiles (Ercolano et al., 2020) and effector functions, it remains challenging to unravel the actions of individual genes regulators in ILC homeostasis through gene deletional targeting (Cording et al., 2018). Complementary to existing mouse models (Cording et al., 2018), we outline a protocol to study the role of specific genes on ILC development, maintenance and function through the reconstitution of adaptive and innate alymphoid $Rag2^{-/-}Il2r\gamma^{-/-}$ mice (Colucci et al., 1999; Jacquelot et al., 2021; Seillet et al., 2016).

The protocol below describes step-by-step the purification of bone marrow-derived ILC2 progenitors (ILC2p) from wildtype mice, their injection into alymphoid $Rag2^{-/-}Il2r\gamma^{-/-}$ mice to reconstitute ILC2, and the analysis of the intestinal lamina propria in recipient mice to determine the specificity and efficacy of ILC2 replenishment. This protocol can be easily adapted to isolate other lymphoid progenitors aimed at reconstituting either all lymphocytes or individual innate lymphoid cell subsets (Jacquelot et al., 2021; Seillet et al., 2016).

Mice

Before commencing this approach, ideally obtain age and sex matched donor and recipient mice. Mice from 6 to 10 weeks old are most suitable. Normally, cells purified from one donor mouse can reconstitute up to two to three recipient (or host) mice. This ratio will depend on the donor bone marrow and factors such as whether the genes that are examined affect the differentiation of the lymphoid cell progenitors, their maintenance or survival. On the day of the experiment, $Rag2^{-/-}Il2r\gamma^{-/-}$ recipient mice are sublethally irradiated, receiving a single total body irradiation of 4.5 Gray, to promote progenitor engraftment (Vély et al., 2016). Mice are allowed to rest for a minimum of two hours after irradiation prior to the adoptive transfer of progenitor cells. Alternate background mouse strains, several of which are available with congenic markers, can also be used.

Institutional permissions

Animals were handled according to the guidelines of the Australian Code for the Care and Use of Animals of the National Health and Medical Research Council of Australia. Experimental procedures were approved by the Animal Ethics Committee of the Walter and Eliza Hall Institute of Medical Research.

> **Note:** All mouse experiments must be approved by an Animal Care Committee in your research institution.

Cell enrichment and reagents

This protocol is performed using a MidiMACS™ Separator attached to a MACS® Multistand. These components are necessary to hold the LS column (Miltenyi Biotec) and perform cell enrichment prior flow cytometry cell sorting. Alternate enrichment approaches using optimally pre-titrated unconjugated antibodies and magnetic beads (e.g., Dynabeads™, sheep anti-mouse IgG, 1 bead/cell, ThermoFisher Scientific Cat#11031 combined with Dynal magnet) may be used but will require optimization. It is important to keep all the reagents cold and work on ice to maximize cell viability and recovery.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Biotin anti-mouse IL-7Rα (CD127), clone A7R34, 5 μg/mL	eBioscience	Cat# 13-1271-82; RRID: AB_466588
PerCP-eFluor 710 anti-mouse c-kit, clone 2B8, 1:300	eBioscience	Cat# 46-1171-82; RRID: AB_1834421
PE-Cy7 anti-mouse IL-7Rα (CD127), clone SB/199, 1 :100	BD Bioscience	Cat# 560733; RRID: AB_1727424
V500 anti-mouse Ly6A/E (Sca-1), clone D7, 1:200	BD Bioscience	Cat# 561228; RRID: AB_10584334
PE anti-mouse Flt3 (CD135), clone A2F10.1, 1:50	BD Bioscience	Cat# 553842; RRID: AB_395079
APC anti-mouse α4β7 (LPAM-1), clone DATK32, 1:300	eBioscience	Cat# 17-5887-82; RRID: AB_1210577
eFluor450 anti-mouse CD25, clone PC61.5, 1:200	eBioscience	Cat# 48-0251-82; RRID: AB_10671550
PE-Cy7 streptavidin, 1:200	BD Bioscience	Cat# 557598
AF700 anti-mouse CD3ε, clone 500A2, 1:300	BioLegend	Cat# 152316; RRID: AB_2632713
AF700 anti-mouse B220 (CD45R), clone RA3-6B2, 1:300	eBioscience	Cat# 56-0452-82; RRID: AB_891458
AF700 anti-mouse CD11b, clone M1/70, 1:300	eBioscience	Cat# 56-0112-82; RRID: AB_657585
AF700 anti-mouse TER119, clone TER-119, 1:300	eBioscience	Cat# 56-5921-82; RRID: AB_2815252
AF700 anti-mouse NKp46 (CD335), clone 29A1.4, 1:200	BD Bioscience	Cat# 561169; RRID: AB_10561840
APC-eFluor 780 anti-mouse F4/80, clone BM8, 1:200	eBioscience	Cat# 47-4801-82; RRID: AB_2735036
APC-eFluor780 anti-mouse CD19, clone 1D3, 1:200	eBioscience	Cat# 47-0193-82; RRID: AB_10853189
APC-eFluor780 anti-mouse TCRβ, clone H57-597, 1:200	eBioscience	Cat# 47-5961-82; RRID: AB_1272173
APC-Cy7 anti-mouse NK1.1, clone PK136, 1:200	BD Bioscience	Cat# 560618; RRID: AB_1727569
BV786 anti-mouse CD45, clone 30-F11, 1:1200	BD Bioscience	Cat# 564225; RRID: AB_2716861
BUV395 anti-mouse CD3ε, clone 145-2C11, 1:200	BD Bioscience	Cat# 563565; RRID: AB_2738278

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BUV737 anti-mouse CD19, clone 1D3, 1:800	BD Bioscience	Cat# 612781; RRID: AB_2870111
BV510 anti-mouse CD11b, clone M1/70, 1:800	BD Bioscience	Cat# 562950; RRID: AB_2737913
BV650 anti-mouse NK1.1, clone PK136, 1:400	BD Bioscience	Cat# 564143; RRID: AB_2738617
PE anti-mouse NKp46, clone 29A1.4, 1:100	eBioscience	Cat# 12-3351-82; RRID: AB_1210743
AF647 anti-mouse KLRG1, clone 2F1, 1:200	eBioscience	Cat# 51-5893-82; RRID: AB_2744744
BV711 anti-mouse GATA3, clone L50-823, 1:100	BD Bioscience	Cat# 565449; RRID: AB_2739242
BV421 anti-mouse RORγt, clone Q31-378, 1:300	BD Bioscience	Cat# 562894; RRID: AB_2687545
Chemicals, peptides, and recombinant proteins		
Phosphate buffered saline (PBS) pH 7.4	GIBCO	Cat# 10010023
10× PBS, pH 7.4	GIBCO	Cat# 70011044
HBSS without Ca^{2+} and $Mg2^+$	GIBCO	Cat# 14170161
RPMI-1640 medium	GIBCO	Cat# 11875176
Fixation concentrate	eBioscience	Cat# 00-5123-43
Fixation/Permeabilization diluent	eBioscience	Cat# 00-5223-56
Permeabilization buffer (10× solution)	eBioscience	Cat# 00-8333-56
Fetal Bovine Serum (FBS) (heat inactivated)	GIBCO	Cat# 10099141
Percoll®	GE Healthcare	Cat# 17-0891-01
Ethylenediaminetetraacetic acid disodium salt solution (EDTA)	Sigma-Aldrich	Cat# E7889-100ML
ACK Lysing Buffer	Sigma-Aldrich	Cat# A1049201
BD Horizon™ Fixable Viability Stain 700	BD Bioscience	Cat# 564997
DNAse I	Roche	Cat# 10104159001
Collagenase IV	Worthington Biochemical	Cat# LS004189
Dispase	Sigma-Aldrich	Cat# D4693-1G
Bovine Serum Albumin, heat shock fraction, pH 7, ≥98%	Sigma-Aldrich	Cat# A7906-10G
Anti-Biotin MicroBeads	Miltenyi Biotec	Cat# 130-090-485
Trypan Blue Solution, 0.4%	GIBCO	Cat# 15250061
Experimental models: Organisms/strains		
Mouse: C57BL/6J (donor), female, 8–12 weeks old or genetically modified mice on background similar to recipient (host) mice.	Vendor	https://www.jax.org/strain/000664
Mouse: $Rag2^{-/-} Il2r\gamma^{-/-}$ (host), female 8–12 weeks old	Vendor	https://www.jax.org/strain/014593
Software and algorithms		
FlowJo v. 10.8	FlowJo, LLC	https://www.flowjo.com/solutions/flowjo
Other		
BD® FACS ARIA III Cell Sorter*	BD Biosciences	N/A
BD® LSR Fortessa Flow Cytometer*	BD Biosciences	N/A
LS Column	Miltenyi Biotec	Cat# 130-042-401
MidiMACS™ Separator	Miltenyi Biotec	Cat# 130-042-302
MACS® MultiStand	Miltenyi Biotec	Cat# 130-042-303
Centrifuge*	Beckman Coulter	N/A
Fisherbrand™ Disposable Borosilicate Glass Pasteur Pipets, 9in	Fisher scientific	Cat# 13-678-20C
Microcentrifuge*	Eppendorf	N/A
Microcentrifuge tubes with attached lid*	Eppendorf	Cat# T6649
Coors™ porcelain mortar	Sigma-Aldrich	Cat# Z247464
Coors™ porcelain pestle	Sigma-Aldrich	Cat# Z247502
Corning® 70 μm cell strainer*	Corning	Cat# 431751
50 mL Conical Sterile Polypropylene Centrifuge Tubes*	Thermo Scientific™	Cat# 339653
15 mL Conical Sterile Polypropylene Centrifuge Tubes*	Thermo Scientific™	Cat# 339651

*Similar reagents or equipment with similar characteristics from other companies can be used for this protocol.

MATERIALS AND EQUIPMENT

MACS buffer

Reagent	Final concentration	Amount
PBS, pH 7.4	1×	489 mL
Bovine Serum Albumin	1% (w/v)	5 g
EDTA (0.5 M)	1 mM	1 mL
Total	n/a	500 mL

Keep sterile and store the filtered solution at 4°C for up to 2–3 months.

Sorting buffer

Reagent	Final concentration	Amount
PBS, pH 7.4	1×	48.9 mL
FBS	2%	1 mL
EDTA (0.5 M)	1 mM	100 μL
Total	n/a	50 mL

Keep sterile and store the solution at 4°C for up to 2–3 months.

Flow cytometric (FACS) buffer

Reagent	Final concentration	Amount
PBS, pH 7.4	1×	980 mL
FBS	2%	20 mL
Total	n/a	1 L

Keep sterile and store the solution at 4°C for up to 2–3 months.

Dissociation media

Reagent	Final concentration	Amount
HBSS without Ca^{2+} and Mg^{2+}	1×	19.4 mL
FBS	2%	400 μL
EDTA (0.5 M)	5 mM	200 μL
Total	n/a	20 mL

Prepare fresh. This is for one sample – adjust volumes accordingly.

Digestion media

Reagent	Final concentration	Amount
RPMI-1640	1×	37.2 mL
FBS	2%	800 μL
Collagenase IV (40 mg/mL)	1 mg/mL	1 mL
Dispase (8 U)	0.2 U/mL	1 mL
DNAse I	0.2 mg/mL	8 mg
Total	n/a	40 mL

Prepare fresh. This is for 4–5 samples – adjust volumes and weight accordingly. Stock solutions for Collagenase IV and Dispase are prepared in PBS and milliQ Water, respectively, and stored at −20°C for maximum 6 months.

100% Percoll

Reagent	Final concentration	Amount
10× PBS	n/a	5 mL
Percoll	n/a	45 mL
Total	n/a	50 mL

Prepare Fresh. To make 40% Percoll, dilute 20 mL 100% Percoll in 30 mL 1× PBS. To make 80% Percoll, dilute 20 mL 100% Percoll in 5 mL 1× PBS.

STEP-BY-STEP METHOD DETAILS
Isolation of bone marrow progenitors from donor mice

⊙ Timing: 3–4 h

Sex and age-matched wildtype C57BL/6J (CD45.2$^{+/+}$) or similar compatible background bone marrow donor mice are used to isolate bone marrow progenitors. Preferentially use mice between 6–10 weeks old.

1. Bones collection.
   a. Euthanize mice using either CO_2 (<20% displacement/minute) asphyxiation or cervical dislocation.

   Note: Methods of euthanasia should be appropriate consistent with animal ethics regulations. Tissues should be harvested immediately after euthanasia of mice using aseptic procedures.

   b. Remove the mouse hind limb femur and tibia from both legs. After removing muscles and connective tissue, place the bones in sterile PBS on ice.

   Note: Hips and sternum can be also collected to improve cellular yields.

2. Extraction of bone marrow cells.
   a. Under a PSP2 cell culture hood, transfer the bones into a mortar and crush the bones using a pestle in fresh sterile PBS. Continue to crush the bones until the bone debris becomes completely white, indicating that all the bone marrow cells have been released.
   b. Filter the suspension through a 70 μm cell strainer in a 50 mL tube to remove the bone fragments. Rinse the mortar and the strainer using fresh sterile PBS.
   c. Centrifuge the cell suspension at 400 g for 5 min at 4°C and discard supernatant.
   d. Perform red blood cell lysis using 5 mL of ACK buffer.
   e. After 2 min of incubation at 20°C, wash the cells by adding 10 times of the volume of ACK used of sterile PBS (final volume = 50 mL) to quench the lysis.
   f. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.
   g. Resuspend the pellet in 10 mL and count cells using Trypan Blue exclusion cell viability staining using appropriate dilutions.
   h. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.
   i. Resuspend the pellet with 1 mL per 10^7 cells of sterile cold MACS buffer.
   j. Filter the suspension through a 70 μm cell strainer in a 15 mL tube to remove red blood cell debris.
3. Enrichment of IL-7Rα expressing bone marrow cells.
   a. Centrifuge the bone marrow cells at 400 g for 5 min at 4°C, discard supernatant, and resuspend cells in fresh MACS buffer at a concentration of 25×10^7 cells/mL containing an anti-CD16/CD32 antibody (5 μg/mL).
   b. Incubate cells on ice for 20 min, then wash by filling the tube with MACS Buffer.
   c. Centrifuge the cell suspension at 400 g for 5 min at 4°C and discard supernatant.
   d. Resuspend the pellet in MACS Buffer at a concentration of 50×10^7 cells/mL containing IL-7Rα -biotin antibody (CD127, A7R34; 5 μg/mL). Rely on the count performed in 2.f. Incubate for 40 min at 4°C.
   e. After incubation, wash cells by adding 1 mL of MACS buffer per 5×10^7 cells (rely on the count performed in 2.f.) to the cell suspension.
   f. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.

g. Resuspend cells in MACS buffer at a concentration of 50×10^7 cells/mL (rely on the count performed in 2.f). Add 10 μL of α-biotin magnetic microbeads (Miltenyi Biotec) per 10^7 cells.

h. Incubate cells with the microbeads for 30 min at 4°C.

i. After incubation, wash cells by adding 1 mL of MACS buffer per 5×10^7 cells (rely on the count performed in 2.f.).

j. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.

k. Resuspend up to 10^8 cells (rely on the count performed in 2.f.) in 500 μL of MACS buffer.

l. Proceed to magnetic separation using LS columns (Miltenyi Biotec).

m. Attach the MidiMACS Separator to the MACS MultiStand and place LS Column in the separator. Place a 15 mL collection tube under the LS Column to collect the negative (unlabeled) fraction.

n. Equilibrate the LS column by rinsing it with 3 mL of MACS Buffer. Wait until the buffer completely run through the column without letting the column dry.

o. Add the cell suspension on the column. Wash the columns three times with 3 mL of MACS Buffer.

p. After washing, add 5 mL of MACS Buffer on the column, remove the column from the MACS separator and place it on a new 15 mL collection tube to collect IL-7Rα⁺ cells. Use the plunger to flush out the labeled cells.

⚠ CRITICAL: Cells are positively selected and IL-7Rα⁺ cells are retained within the column until their release via removal from the magnetic column stand and washing while using the plunger. Cells that flow through while the column is attached to the magnet during successive washes should be IL-7Rα negative. Keep these flow-through cells on ice as they can be used for single stained controls to appropriately set PMT voltages and compensation on the FACS.

q. Wash the recovered IL-7Rα⁺ cells in MACS buffer and centrifuge the cells at 400 g for 5 min at 4°C.

r. Discard supernatant. Cells are ready to be stained with surface antibodies (Table 1).

Flow cytometric cell sorting of bone marrow progenitors

⏱ Timing: 3–4 h

Enriched single cell suspension(s) will be stained with antibodies and a lineage cocktail (Table 1) to discriminate different bone marrow progenitors using a FACS ARIA III Cell Sorter or a comparable Cell Sorter with similar characteristics. Minimal requirements consist of a FACS Cell Sorter with four lasers – violet (405 nm), blue (488 nm), yellow (561 nm) and red (640 nm) – and appropriate filters to optimally detect the fluorochromes detailed in Table 1. Prepare collection tubes (e.g., 1.5 mL Eppendorf tubes) containing 1 mL of sorting buffer to collect sorted cells. Keep all the tubes, solutions and sorted cells on ice (4°C) during the sort to maintain cell viability.

4. Staining of bone marrow progenitors.

a. Resuspend enriched cells in the antibody cocktail (Table 1) together with the fixable viability stain 700 (100 μL/10^7 cells final volume in MACS Buffer) and stain for 30 min on ice. Protect from light from this point forward.

b. After incubation, wash cells by adding 10 times the volume of the antibody cocktail of MACS buffer.

c. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.

d. Resuspend the cells at 1×10^7/mL of MACS buffer and keep the cell suspension on ice until cell sorting.

5. Isolation of ILC2p.

Table 1. Bone marrow lymphoid progenitor staining panel

Fluorochrome	Marker	Clone	Final dilution
Fixable Viability Stain 700	Live_Dead marker	N/A	1:1000
AF700	CD3ε	500A2	1:300
AF700	B220 (CD45R)	RA3-6B2	1:300
AF700	CD11b	M1/70	1:300
AF700	TER119	TER-119	1:300
AF700	NKp46 (CD335)	29A1.4	1:200
APC-eFluor 780	F4/80	BM8	1:200
APC-eFluor 780	CD19	1D3	1:200
APC-eFluor 780	TCRβ	H57-597	1:200
APC-Cy7	NK1.1	PK136	1:200
PerCP-eFluor 710	c-kit	2B8	1:300
PE-Cy7	IL-7Rα (CD127)	SB/199	1:100
PE-Cy7	streptavidin	N/A	1:200
V500	Ly6A/E (Sca-1)	D7	1:200
PE	Flt3 (CD135)	A2F10.1	1:50
APC	α4β7 (LPAM-1)	DATK32	1:300
eFluor450	CD25	PC61.5	1:200

Prepare fresh antibody cocktails using MACS Buffer.

a. Before acquiring samples, set appropriate PMT voltages and compensation by using single stained controls. Adjust compensation using the same set of antibodies.

Note: Alternatively, antibodies against different markers can be used but they need to be coupled to the same fluorochromes for appropriate compensation. Commercially available beads for compensation can be used (e.g., UltraComp eBeads Plus compensation beads, Invitrogen™, Cat# 01-3333-42) but the autofluorescence signal won't be captured.

b. Perform cell sorting using a 70 μm nozzle with a sorting speed of 5,000–8,000 events/second.

⚠ CRITICAL: Use aseptic procedures to keep progenitor cells sterile for *in vivo* injections.

Note: Where possible, a FACS Cell Sorter placed under a hood should be used to maintain complete sterility.

c. Isolate ILC2 progenitors based on the expression of the following parameters and the absence of expression of lineage markers (AF700 and APC-Cy7 antibody coupled fluorochromes): Lineage⁻IL-7Rα⁺Flt3⁻α4β7⁺c-kit⁻CD25⁺Sca-1⁺ (Figure 1).

Note: It is expected to isolate between 2,000 – 4,000 of ILC2 progenitors from a single wild-type mouse donor.

Optional: The common and alpha lymphoid bone marrow progenitors can be isolated based on the expression of the following markers: Lineage⁻IL-7Rα⁺Flt3⁺α4β7⁻c-kit⁺ and Lineage⁻IL-7Rα⁺Flt3⁻α4β7⁺c-kit⁺CD25⁻, respectively (Figure 1).

d. Verify the purity of the sorted population by reanalysing 10 μL of cells, transferred into a new tube (Figure 2).

⚠ CRITICAL: It is essential to evaluate the purity of isolated progenitors (Figure 2) as contamination by other lymphoid progenitors can give rise to unwanted lymphoid cell subsets in recipient mice.
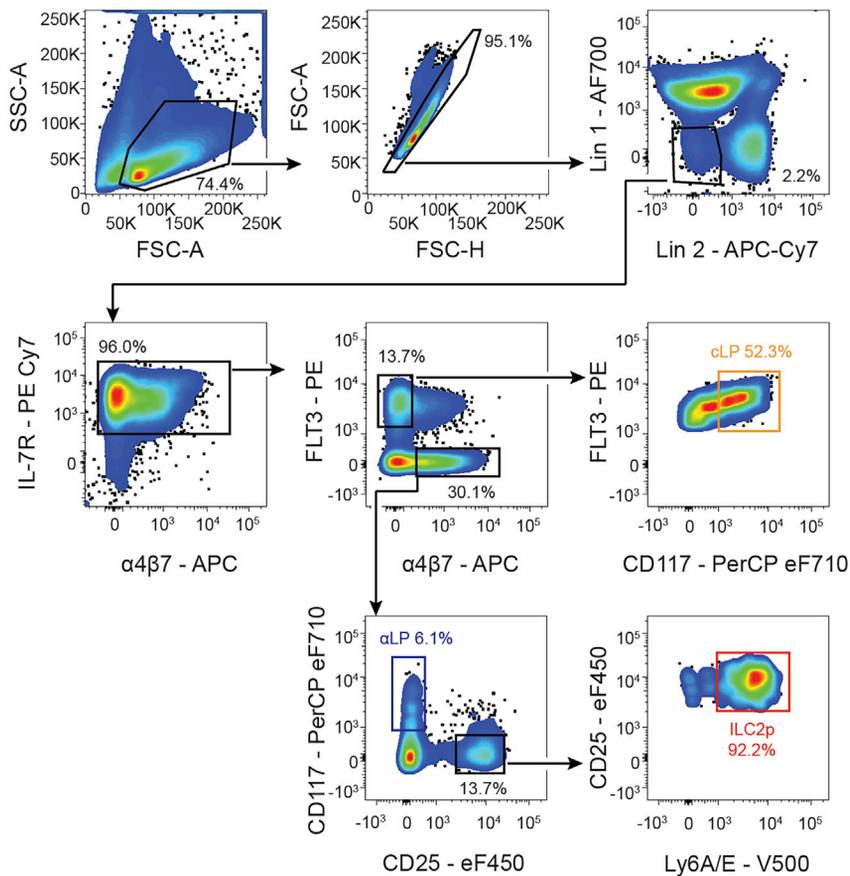
Figure 1. Gating strategy to isolate bone-marrow ILC progenitors

e. Wash isolated cell progenitors by adding 1 mL of sterile PBS/10^4 of cells.

f. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.

g. Repeat steps 5e–f.

h. Resuspend cells in 200 μL and count cells using Trypan Blue exclusion cell viability staining using appropriate dilutions.

i. Resuspend cells in sterile PBS at 7.5–10 × 10^3 cells/mL and proceed immediately to the injections of progenitor cells in irradiated $Rag2^{-/-}Il2r\gamma^{-/-}$ mice.

Lymphoid cell reconstitution in $Rag2^{-/-}Il2r\gamma^{-/-}$ recipient mice

⏱ Timing: 6–8 weeks

The generation of bone marrow chimeras requires reconstitution of sublethally irradiated recipient mice with bone marrow-derived donor immune cells.

Note: The immunodeficient $Rag2^{-/-}Il2r\gamma^{-/-}$ mice receive a unique dose of 4.5 Gray irradiation to induce inflammation, promoting progenitor cell engraftment (Vély et al., 2016).

6. Under a laminar flow hood (class II) cell culture hood, inject each $Rag2^{-/-}Il2r\gamma^{-/-}$ mouse intravenously with 200 μL of the donor cell suspension.
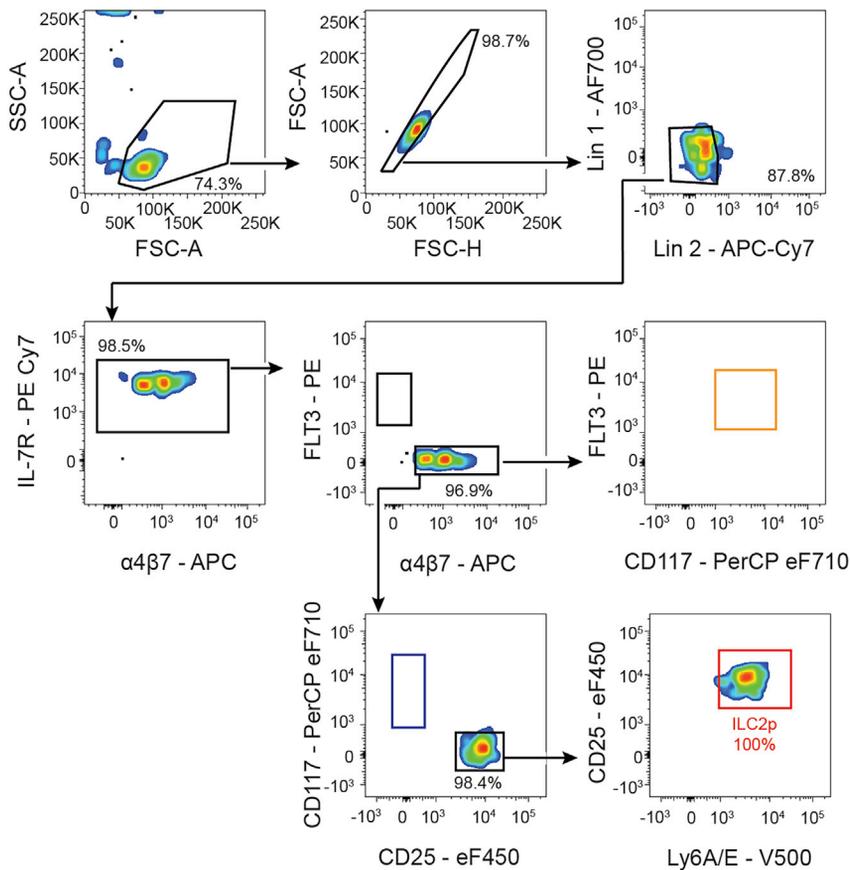
Figure 2. Purity check of the ILC2 progenitors

Note: Mice are monitored during 30 min to 1 h after injections and then daily by experienced animal technicians as per local ethics guidelines to ensure good animal welfare. Prophylactic antibiotics (e.g., neomycin sulfate) can be used to prevent infection during the two first weeks after transplantation. However, this is not usually necessary as the low dose irradiation does not induce complete myeloablation.

7. Perform ILC2 *in vivo* function assessment or *ex vivo* assays 6–8 weeks after bone marrow progenitor injection.

Note: This time-lapse is necessary to allow full reconstitution of the lymphoid compartment. While it is possible to analyze mice earlier than 6 weeks, the full development of ILC function requires 6–8 weeks. In ILC2-reconstituted $Rag2^{-/-} Il2r\gamma^{-/-}$ mice, we found a progressive increase of eosinophil frequency over time in the peripheral blood compared with control animals (Jacquelot et al., 2021). These transplanted mice achieved similar frequencies of eosinophils to ILC2 sufficient mice at ± 6 weeks post ILC2p injections. These observations highlight that at least 6 weeks is required prior to analysis of ILC2 function in bone marrow-reconstituted $Rag2^{-/-} Il2r\gamma^{-/-}$ mice for accurate functional analyses.

Evaluation of reconstitution efficiency

⏱ Timing: 6–8 h

It is critical to establish the efficacy of ILC2 reconstitution in alymphoid $Rag2^{-/-}Il2r\gamma^{-/-}$ recipient mice to make sure that the observed phenotype is directly attributable to ILC2. This will distinguish ILC2 activity from other potential lymphoid populations which could result from contamination of ILC2p with other progenitor cells. Although carefully performed, this is rare, but can occur when limited separation of markers is observed during cell sorting affecting the purity of the ILC2p subset. For every reconstituted $Rag2^{-/-}Il2r\gamma^{-/-}$ recipient mice, we suggest analysing the frequency and number of lymphoid cells in the intestinal lamina propria which is known to be home of large numbers of both innate and adaptive immune cells, facilitating the evaluation of the reconstitution of the lymphoid cell compartment. Non-reconstituted non-irradiated $Rag2^{-/-}Il2r\gamma^{-/-}$ and C57BL/6J mice should be analyzed in parallel and serve as controls. The protocol below will precisely describe the step-by-step isolation (Figure 3) and analysis of intestinal immune cells.

8. Isolation of intestinal immune cells from the lamina propria.
    a. Euthanize mice and collect small intestines in 4 mL PBS in 6 well plate on ice.
    b. Separate the duodenum and ileum from the stomach and the caecum, respectively.
    c. Remove successively the fat tissue and Peyer's patches, when present, using tweezers and scissors.

    Note: On average, there are between 6 to 9 Peyer's patches per mouse that are distributed along the small intestine (Figure 3A, as indicated by *).

    △ CRITICAL: It is essential to ensure the small intestine is kept moist to avoid tissue dehydration and associated cell death. This may strongly impact cell recoveries and analysis.

    d. Cut the small intestine into 4 parts, open them longitudinally (Figure 3B), and remove the intestinal contents by gently scraping the contents away from the mucosal surface.
    e. Wash the small intestine in PBS (Figure 3C).

    △ CRITICAL: It is important to gently remove the intestinal contents to avoid any damage to the underlying tissues.

    f. Cut the tissue into 2 mm pieces (Figure 3D), transferred into 50 mL tubes containing 10 mL HBSS + 2% FBS.
    g. Vortex vigorously for 10–20 s (Figure 3E) and filter the solution through a 100 µm cell strainer (Figure 3F).
    h. Wash intestinal pieces with 20 mL HBSS + 2% FBS and transfer the pieces into a new 50 mL tube containing 20 mL of the dissociation solution.
    i. Vortex the tube for 10–20 s and incubate for 40 min at 37°C under gentle shaking (230 rpm).

    Note: The dissociation media allows the separation of the epithelial layer from the lamina propria.

    j. After incubation, vortex the tubes vigorously for 10–20 s and filter the solution through a 100 µm cell strainer.

    △ CRITICAL: After vortex, the solution should appear cloudy (Figure 3G). This means that the epithelial layer has been effectively separated from the lamina propria of the small intestine.

    k. Wash the intestinal pieces with 20 mL PBS.
    l. Transfer intestinal pieces in a new 50 mL tube containing 8–10 mL of the digestion solution (Figure 3H).

Dissociation and digestion of the small intestine
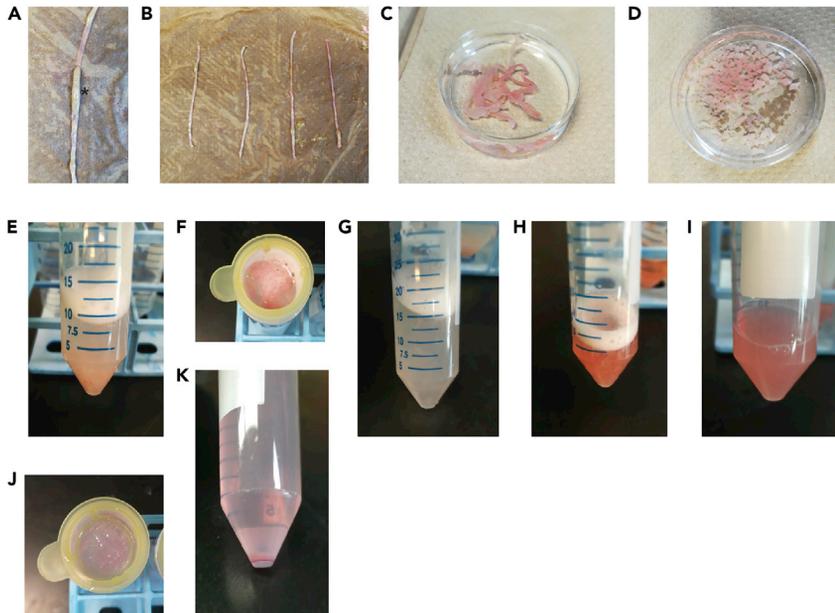
Percoll
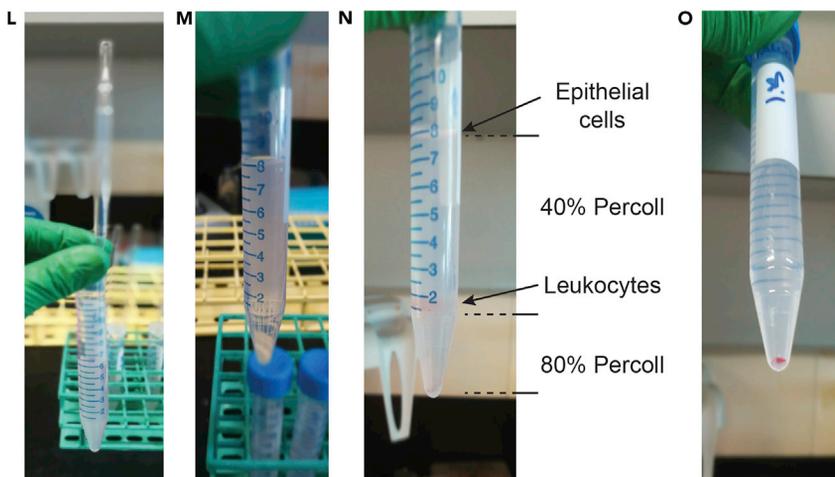
Figure 3. Pictures to illustrate the step-by-step isolation of intestinal immune cells

(A) Identify Peyer's patch (indicated by *) on the small intestine.

(B) The intestinal contents are gently removed.

(C) Small intestine is opened longitudinally and kept in PBS in a Petri dish.

(D) Small intestine is cut in small pieces.

(E) Tissue pieces are transferred in dissociation buffer.

(F and G) Tissues are filtered from dissociation buffer and washed.

(H) Tissue pieces are transferred in digestion buffer.

(I) After incubation, most of the tissues should have been digested.

(J) Remove remaining tissues through a cell strainer.

(K) Pellet the single cell suspension.

(L) Load the 80% Percoll from the bottom of the tube.

(M) Clear separation between the 40% Percoll containing cell suspension in top and the 80% Percoll below.

(N) After centrifugation the leukocyte are at the interface of the 40% and 80% Percoll.

(O) Leukocytes collected at the interface is rinsed in 10 mL of cold PBS to remove any Percoll from the solution and spun down to pellet.

Optional: Cells from the epithelial layer can be collected and analyzed separately. Keep the solution at 4°C until step 8.o. Intraepithelial immune cells can be enriched by centrifugation on a 40%–80% Percoll gradient. Flow cytometric analysis of the immune cells can be performed after surface and intracellular staining using desired antibodies.

⚠ CRITICAL: The enzymes are sensitive to freeze thaw cycles. The enzymatic cocktail must be prepared freshly every time you perform a gut digestion.

m. Vortex the tube vigorously for 20 s and incubate for 45 min at 37°C with gentle shaking (230 rpm).
n. After incubation, vortex the tubes vigorously for 20 s and filter the solution through a 100 μm cell strainer. Rinse the cell strainer with 30 mL PBS.

⚠ CRITICAL: The solution should appear cloudy (Figure 3I). Only minimal residual tissue should be seen on the cell strainer (Figure 3J).

o. Centrifuge cells at 400 g for 7 min.

Note: A pellet should be clearly seen (Figure 3K).

p. Discard supernatant and resuspend cells in 6 mL of 40% Percoll.
q. Transfer cells into a 15 mL tube and underlay 2 mL of 80% Percoll using a glass Pasteur pipette (Figure 3L).

Note: A clear separation of the two layers should be observed (Figure 3M).

r. Centrifuge cells at 950 g for 20 min at 20°C without brake.

Note: A ring which contains lymphocytes should be visible at the interface (Figure 3N).

s. Using a vacuum aspiration system, carefully remove and discard the top layer constituted by remaining epithelial cells to avoid contamination of your immune cell preparation.
t. Collect the lymphocytes at the interface and transfer them into 15 mL tube containing 10 mL of cold PBS.
u. Centrifuge the cells at 400 g for 5 min at 4°C.

Note: A pellet should be visible (Figure 3O).

v. Discard supernatant and resuspend the cells in 5 mL cold PBS, count the cells using Trypan Blue, and transfer 1–2 × 10^6 cells in V-bottom 96 well plate for staining.
w. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.
x. Incubate the cells with an anti-CD16/32 antibody (2.4G2 5 μg/mL in 50 μL of FACS buffer) for 20 min on ice.
y. Wash cells with 150 μL of FACS Buffer.
z. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.
9. Surface antibody staining.
   a. Resuspend the cells in the antibody cocktail together with the fixable viability stain 700 (50 μL final volume in FACS Buffer) (Table 2) and stain for 30 min on ice.

Note: Protect samples from light from all subsequent steps.

b. Wash cells with 150 μL of FACS Buffer.
c. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.

Table 2. Antibody cocktail for surface staining panel

Fluorochrome	Marker	Clone	Final dilution
Fixable viability stain 700	Live/Dead marker	N/A	1:1000
BV786	CD45	30-F11	1:1200
BUV395	CD3ϵ	145-2C11	1:200
APC eF780	TCRβ	H57-597	1:200
BUV737	CD19	1D3	1:800
BV510	CD11b	M1/70	1:800
BV650	NK1.1	PK136	1:400
PE	NKp46	29A1.4	1:100
AF647	KLRG1	2F1	1:200

Prepare fresh using FACS Buffer.

10. Cell fixation and permeabilization.
    a. Prepare fixation buffer using the Fixation concentrate diluted (1 in 4) in the Fixation/Permeabilization diluent and fix/permeabilize cells (50 μL per sample) for 30 min on ice.
    b. Prepare the permeabilization buffer (1×) by adding 9 volumes of distilled water to 1 volume of Permeabilization buffer (10×).
    c. Wash cells with 150 μL of 1× permeabilization buffer.
    d. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.
11. Intracellular antibody staining.
    a. Resuspend cells in the antibody cocktail (50 μL final volume prepared in 1× permeabilization buffer) (Table 3) and incubate for 45–60 min on ice.
    b. Wash cells with 200 μL 1× permeabilization buffer.
    c. Centrifuge the cells at 400 g for 5 min at 4°C and discard supernatant.
    d. Resuspend cells in 200 μL of 1× permeabilization buffer and store your samples at 4°C for no greater than 2–3 days prior to FACS acquisition.
12. Acquire your samples on a flow cytometer after appropriate compensation to detect your population of interest (Figure 4).

EXPECTED OUTCOMES

Restored ILC2 frequencies and numbers in the small intestine of reconstituted $Rag2^{-/-}Il2r\gamma^{-/-}$ mice should be observed with similar levels to that of C57BL/6J or $Rag1^{-/-}$ mice (Figure 5).

LIMITATIONS

This system does not recapitulate a full mature immune system. Lymphocytes that develop in this system reflect the potential of the bone marrow progenitor cells that were adoptively transferred into the $Rag2^{-/-}Il2r\gamma^{-/-}$ mice. In addition, $Rag2^{-/-}Il2r\gamma^{-/-}$ mice lack lymph nodes which normally develop during embryogenesis. Thus, it is important to take into consideration the potential interactions and impact of other innate or adaptive lymphoid cells that may influence ILC2 development, maintenance, and function in tissues, and that some tissues, such as secondary lymphoid organs, may provide a specific microenvironment and niche that may be overlooked in ILC2 reconstituted $Rag2^{-/-}Il2r\gamma^{-/-}$ mice. Thus, this model, while powerful, complements other models of analyses of ILC development and activity.

Table 3. Antibody cocktail for intracellular staining panel

Fluorochrome	Marker	Clone	Final dilution
BV711	GATA3	L50-823	1:100
BV421	RORγt	Q31-378	1:300

Prepare antibody cocktail for intracellular staining using fresh 1× Permeabilization Buffer.
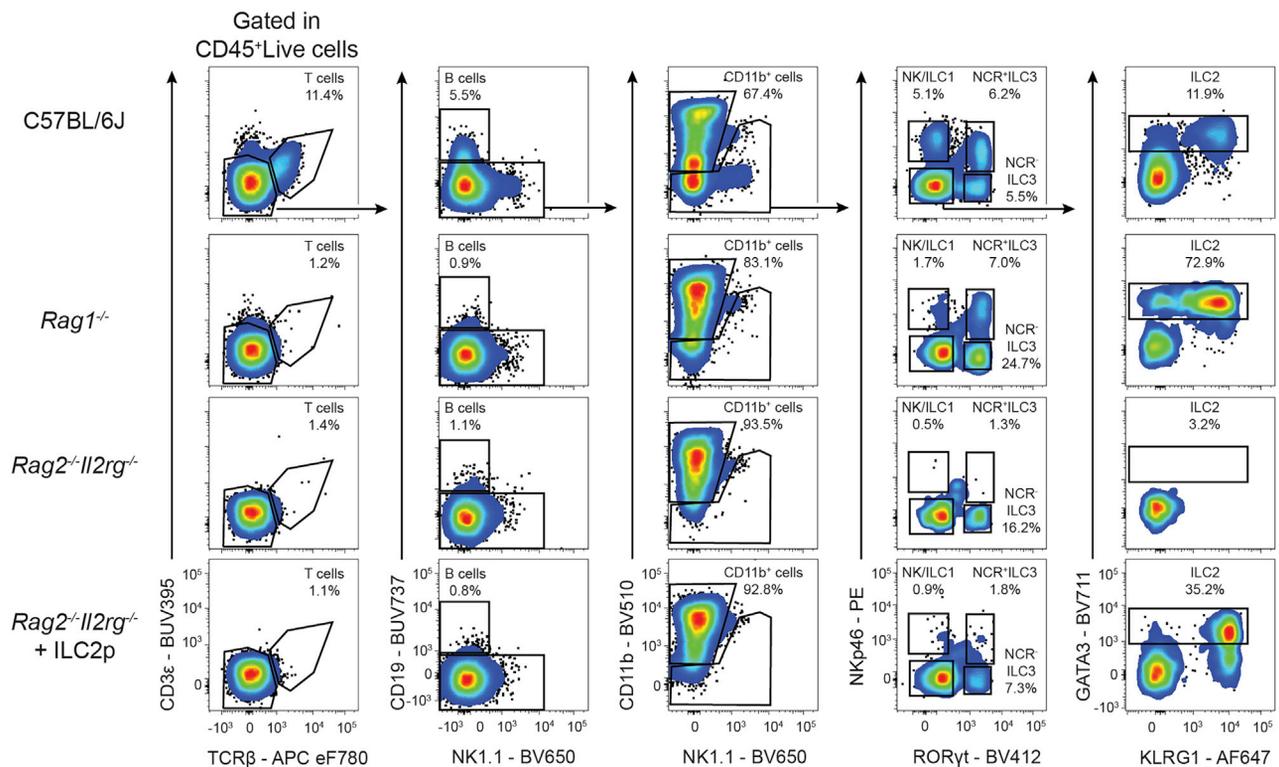
Figure 4. Representative FACS contour plots showing the gating strategy used to assess the frequency of intestinal lymphocytes isolated from C57BL/6, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice reconstituted or not with ILC2p

TROUBLESHOOTING

Problem 1
Step 4. Suboptimal antibody staining.

Potential solution
It is possible that antibodies were not optimally titrated. It is important to titrate each lot of antibodies as some variations can occur. If too concentrated, your negative population will appear positive. If too diluted, you will not be able to distinguish your positive cells. In Figure 6 is an example of poor discrimination of the different IL-7Rα$^+$ lymphoid progenitors due to suboptimal Flt3 and α4β7 staining. You need to calculate the stain index and identify the best dilution for each antibody that gives you the higher stain index.

Problem 2
Step 5. Absence of IL-7Rα$^+$ progenitors during cell sorting.

Potential solution
It is possible that you stained the negative fraction after cell enrichment. Make sure you stained the positive fraction which contains IL-7Rα$^+$ progenitors. It may be also due to the omission of antibodies or microbeads. Finally, it might be important to use appropriate FMO controls to make sure of the positivity of your staining when insufficient separation of your populations is observed.

Problem 3
Step 8o. Absence of cells after enzymatic digestion of the intestine.
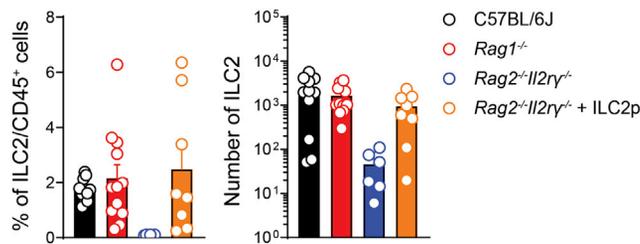
Figure 5. Frequency and enumeration of intestinal ILC2 isolated from C57BL/6J, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice reconstituted or not with ILC2p

Data show the pool of two independent experiments with n=11 C57BL/6J, n=12 *Rag1*$^{-/-}$, n=6 *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$, and n=8 *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ + ILC2p. Mean+SEM.

Potential solution

They are many steps during the preparation of the small intestine at which the quality of the sample can be impacted.

Loss after the dissociation: the solution must appear cloudy. If not, there are two potential explanations: i) the dissociation did not work indicating issues with the EDTA solution, or ii) the mucosal surface of the intestine has been scraped too vigorously during cleaning, resulting in separation of the epithelial layer from the underlying tissues.

Loss after digestion: the tissue pieces should be digested, resulting in the formation of a pellet of cells following centrifugation. If not, ensure the small intestine is cut into sufficiently small pieces. This will improve the digestion of the samples. In addition, ensure the appropriate enzymes are at the right concentration. The enzymes are sensitive to freeze thaw cycles. The enzymatic cocktail must be prepared freshly every time you perform a gut digestion. Enzymes are aliquoted and stored at $-20°C$ for no greater than 6 months. Ensure that the enzymes are added to the digestion buffer and that the aliquots have been prepared recently and stored appropriately.

Problem 4
Step 8r. Absence of cells after Percoll despite the presence of cells after digestion.

Potential solution
For the separation of leukocytes from remaining epithelial cells and other contaminants using Percoll gradient, ensure you have removed the brake when centrifuging the cells.

Problem 5
Step 12. Cell markers have been cleaved off after enzymatic digestion.

Potential solution
To avoid such scenario, the optimal concentration of the different enzymes should be tested for each new lot. This is particularly true for the Dispase as this enzyme, at high concentration, mediates the cleavage of some key surface molecules (e.g., CD4 in Figure 7). Ensure that the appropriate concentration for each enzyme has been determined prior to its use.

Problem 6
Step 12. Other lymphoid cell subsets are present in the intestinal lamina propria of ILC2 reconstituted *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice.

Potential solution
Progenitors are multipotent cells, having the capacity to give rise to multiple cell types while maintaining self-renewal. Rigorous gating of progenitor cells during the sorting process is important
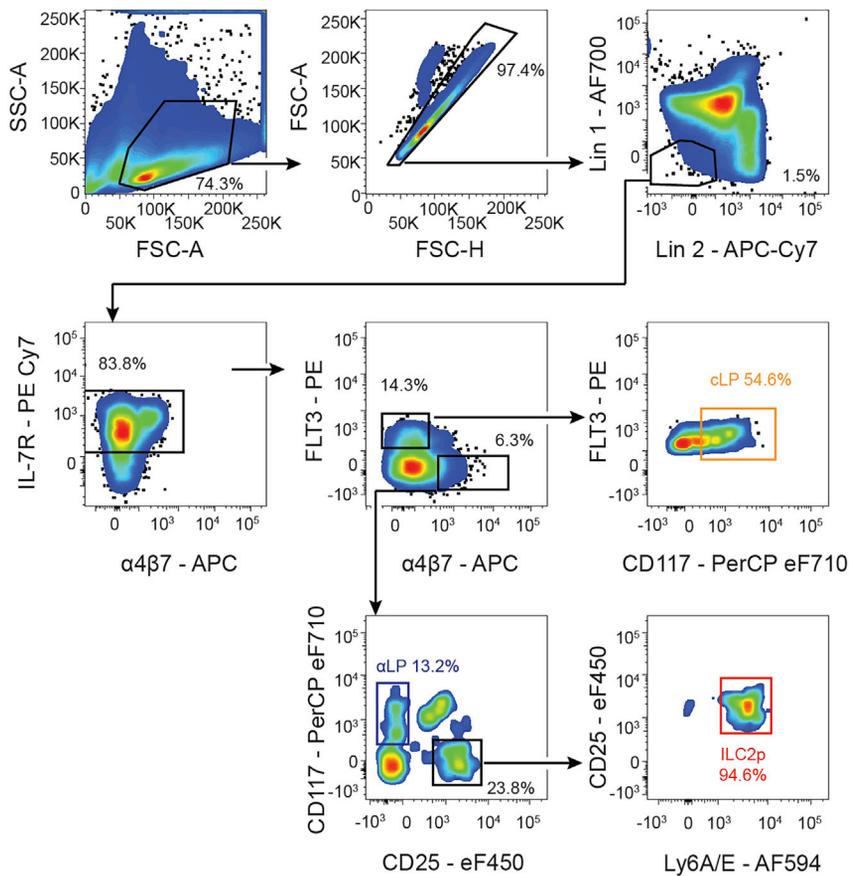
Figure 6. Flow-cytometric contour plots showing IL-7Rα enriched bone marrow cells stained with suboptimal concentrations of anti-Flt3 and α4β7 antibodies
An anti-Ly6A/E AF594 antibody (in house) was used.

together with verification of the purity of sorted populations post sort. These steps are necessary to exclude the possibility of a contamination of ILC2p with other lymphoid cell progenitors. To avoid such scenario, the PMT voltages should be adjusted, and stringent gates should be set based on single stained and FMO controls to ensure a good discrimination of the different progenitor subsets.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr Cyril Seillet (seillet@wehi.edu.au).

Materials availability
All the resources or reagents used in this study are commercially available.

Data and code availability
The published article includes all datasets generated or analyzed during this study. This study did not generate code.

ACKNOWLEDGMENTS
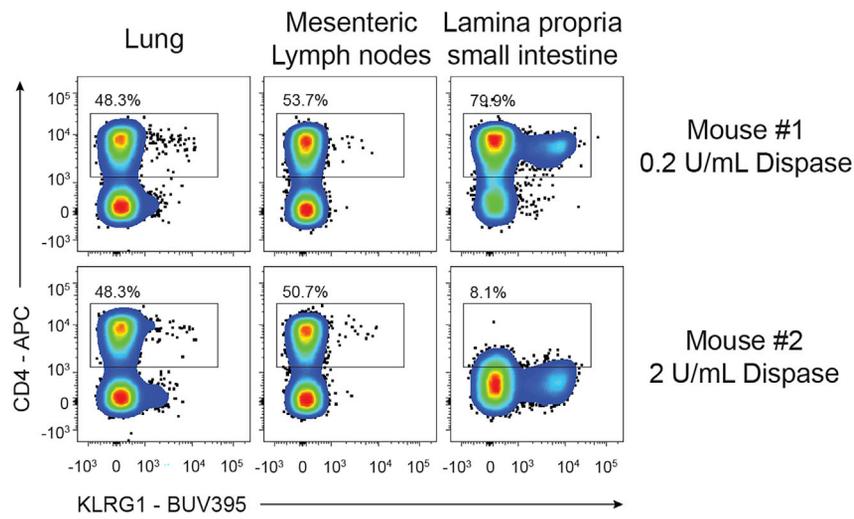
Figure 7. Representative FACS contour plots showing Live CD45$^+$CD3$^+$TCRβ^+ cells and the consequences of excessive concentration of Dispase used for gut digestion on the CD4 marker

Identical protocols were used to isolate intestinal immune cells from mouse #1 and #2 except for the concentration of Dispase as indicated. Lung and mesenteric lymph nodes were used as controls. Anti-CD45 BV786 (30-F11, BD Bioscience, CAT# 564225, 1/1000), anti-KLRG1 BUV395 (2F1, BD Bioscience, CAT# 740279, 1/200), anti-CD3 APC-eF780 (17A2, eBioscience, CAT# 47-0032-82, 1/200), anti-TCRβ PerCP-Cy5.5 (H57-597, BioLegend, CAT# 109228, 1/200), and anti-CD4 APC (GK1.5, eBioscience, CAT# 17-0041-83, 1/200) antibodies together with the fixable viability dye eF506 (eBioscience, CAT# 65-0866-14, 1/800) were used.

AUTHOR CONTRIBUTIONS

Conceptualization, N.J., G.T.B., and C.S.; methodology, N.J., G.T.B., and C.S.; investigation, N.J., Q.H., and C.S; writing – original draft, N.J.; writing – review and editing, N.J., Q.H., G.T.B., and C.S.; funding acquisition, N.J., G.T.B., and C.S.; supervision, G.T.B. and C.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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