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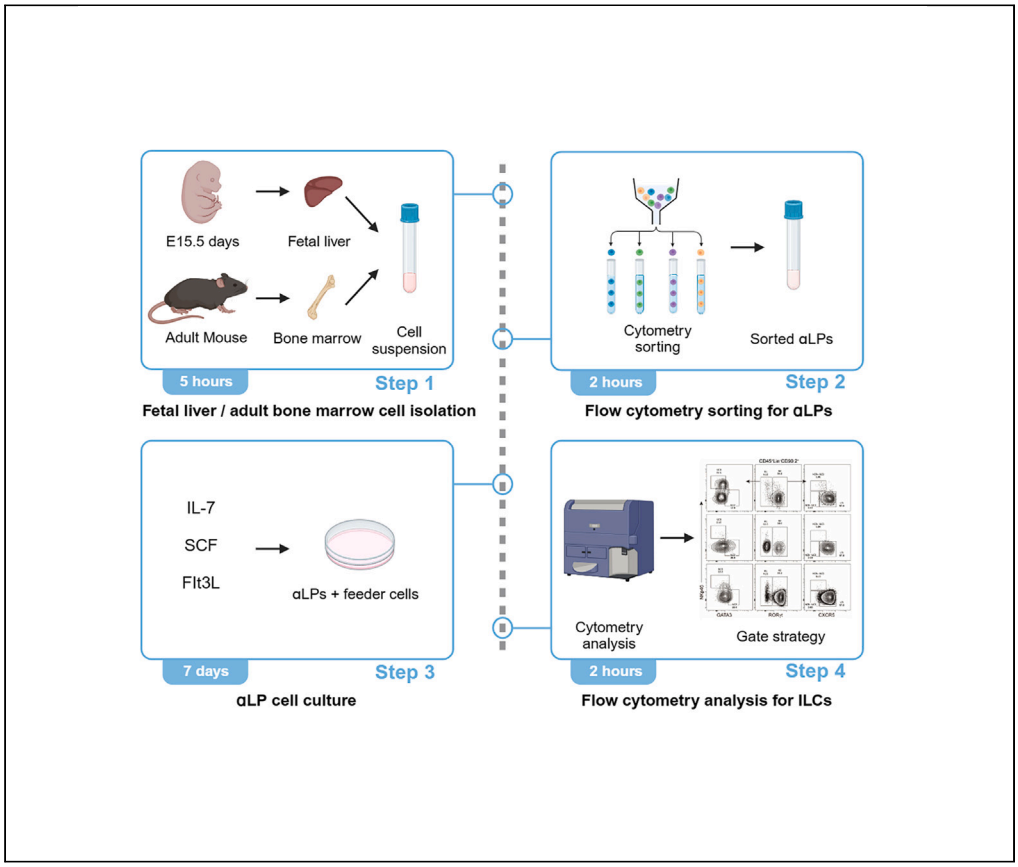
Protocol for *in vitro* generating innate lymphoid cells from mouse $\alpha_4\beta_7^+$ lymphoid progenitors



Building a simple and efficient *in vitro* differentiation system is crucial for studying the regulatory mechanisms during the development of innate lymphoid cells (ILCs). Here, we present a protocol for generating ILC subsets from $\alpha_4\beta_7^+$ lymphoid progenitors (αLPs). We describe steps for murine cell isolation from fetal liver and adult bone marrow, flow cytometry sorting for αLPs, and cell culture. We then detail procedures for flow cytometry analysis of ILCs. This protocol significantly simplifies the differentiation process through ILC differentiation *in vitro*.

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

Tao Wu, Yuanhao Wang, Li Wu

wutao13@tsinghua.org.cn (T.W.)
wuli@tsinghua.edu.cn (L.W.)

Highlights

Steps for isolating αLPs from mouse fetal liver and adult bone marrow

Steps for *in vitro* differentiation assay of mouse αLPs

A simplified protocol for mouse ILC differentiation *in vitro*

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Protocol for *in vitro* generating innate lymphoid cells from mouse $\alpha_4\beta_7^+$ lymphoid progenitors

Tao Wu,[1,2,4,*] Yuanhao Wang,[1] and Li Wu[1,2,3,5,*]

[1]School of Medicine, Institute for Immunology, Tsinghua University, Beijing 100084, China

[2]Tsinghua-Peking Center for Life Science, Beijing, China

[3]Beijing Key Laboratory for Immunological Research on Chronic Diseases, Beijing 100084, China

[4]Technical contact

[5]Lead contact

*Correspondence: wutao13@tsinghua.org.cn (T.W.), wuli@tsinghua.edu.cn (L.W.)
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SUMMARY

Building a simple and efficient *in vitro* differentiation system is crucial for studying the regulatory mechanisms during the development of innate lymphoid cells (ILCs). Here, we present a protocol for generating ILC subsets from $\alpha_4\beta_7^+$ lymphoid progenitors (αLPs). We describe steps for murine cell isolation from fetal liver and adult bone marrow, flow cytometry sorting for αLPs, and cell culture. We then detail procedures for flow cytometry analysis of ILCs. This protocol significantly simplifies the differentiation process through ILC differentiation *in vitro*.

For complete details on the use and execution of this protocol, please refer to Wu et al.[1]

BEFORE YOU BEGIN

This protocol described below was generated using adult mouse bone marrow or fetal liver, and all cells were cultured in a standard 5% CO_2 37°C humidified incubator, indoor air (about 20% oxygen), and treated in a sterile cell culture biosafety cabinet. All reagents should be prepared under sterile conditions.

Institutional permissions

Animal experiments described in this protocol were performed according to the animal protocol (No. 19-WL2) approved by the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University.

Before performing animal experiments as described in this protocol, the investigators need to get permission from the local IACUC institution.

Isolation and preparation of fetal liver cells

Timing: 30 min

1. Prepare pregnant mice to isolate fetal liver cells from mouse embryo.
   a. Prepare the adult male and female mice for timed mating.
   b. Female mice with detected vaginal plug the next morning was marked as pregnant mice at embryonic day 0.5 (E0.5).
   c. Isolate fetal liver cells from mouse embryo at E15.5∼E16.5.
2. Set up the dissecting station with the following materials:

a. A clean bench and stereomicroscope.
b. Autoclave sterilized scissors and tweezers.
c. Sterilized 1.5 mL, 50 mL tubes and 5 mL polystyrene round bottom tube.
d. 100 μm cell strainers.
e. 1.086 g/mL Nycodenz solution.
f. EDTA-containing balanced salt solution (EDTA-BSS).
g. Fetal bovine serum (FBS).

Isolation and preparation of adult bone marrow cells

Timing: 30 min

3. Preparing 8-week-old adult mice (C57BL/6J) for bone marrow precursor cells.
4. Set up the dissecting station with the following materials:
   a. A clean bench and stereomicroscope.
   b. Autoclave sterilized scissors and tweezers.
   c. Sterilized 1.5 mL, 50 mL tubes and 5 mL polystyrene round bottom tube.
   d. 70 μm cell strainers.
   e. 1.086 g/mL Nycodenz solution.
   f. KDS-balanced salt solution (KDS-BSS), a kind of buffer for bone marrow cell isolation optimized by Shortman, Kenneth Douglas.
   g. Fetal bovine serum (FBS).

Preparation of adult bone marrow cells as feeder cells

Timing: 30 min

5. Prepare bone marrow cells from adult C57BL/6J mice.
   a. Prepare C57BL/6J mice at 8–12 weeks old.
   b. Use CD45.1 mice for providing feeder cells.
   c. Use CD45.2 mice for isolating ILC progenitors.

   Note: Use mice with different backgrounds for distinguishing feeder cells and ILC progenitors during an *in vitro* differentiation assay.

6. Set up the dissecting station with the following materials:
   a. A clean bench and stereomicroscope.
   b. Autoclave sterilized scissors and tweezers.
   c. Sterilized 1.5 mL, 50 mL tubes and 5 mL polystyrene round bottom tube.
   d. 70 μm cell strainers.

Preparing OP9 and OP9-DL1 feeder cells

Timing: 30 min

7. Prepare Minimum Essential Medium (MEM) alpha complete medium for OP9 and OP9-DL1 cell lines.

For 500 mL of OP9 and OP9-DL1 culture medium, add:

a. 100 mL of heat-inactivated fetal bovine serum (FBS).
b. 5 mL of Penicillin- Streptomycin.
c. 500 μL of 2-Mercaptoethanol (β-ME).
d. 5 mL of 1M HEPES.

e. 5 mL of Sodium Pyruvate (100×).
f. 5 mL of L-Glutamine (100×).
g. 5 mL of MEM non-essential amino acid solution (100×) to 374.5 mL MEM alpha + Glutamax.

8. Prepare OP9 and OP9-DL1 cells as feeder cells.

Add 400 OP9 or OP9-DL1 cells into the round-button 96-well culture plate about 12 h before inputting the progenitors.[2]

Flow cytometer preparation

Timing: 2 h

9. Prepare 10 L of sterilized 1× PBS as sheath solution.
10. Clean the flow chamber with the FACS-Clean, FACS-Rinse Solution and ddH_2O.
11. Prepare the flow cytometer by switching on the machine and confirming the optimal settings.

Culture medium preparation

Timing: 1 h

12. Prepare RPMI 1640 complete medium.

For 500 mL RPMI 1640 complete medium, add:

a. 50 mL of heat-inactivated fetal bovine serum (FBS).
b. 5 mL of Penicillin- Streptomycin.
c. 500 μL of 2-Mercaptoethanol (β-ME).
d. 444.5 mL of RPMI 1640 medium.

13. Prepare culture medium for ILC differentiation by adding IL-7 (20 ng/mL), SCF (100 ng/mL) and Flt3L (100 ng/mL) to complete RPMI 1640 medium. Store at 4°C for a maximum 7 days.

Note: As αLP2 don't express Flt3, Flt3L is not required for αLP2 *in vitro* differentiation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD45.1 BV605 (A20)	BioLegend	Cat# 110738
Anti-mouse CD45.2 PE-Cy7 (104)	BioLegend	Cat# 109830
Anti-mouse CD3ε FITC (145-2C11)	BioLegend	Cat# 100306
Anti-mouse CD185 (CXCR5) Brilliant Violet 421™ (L138D7)	BioLegend	Cat# 145511
Anti-mouse TCR-β FITC (H57-597)	Therm Fisher Scientific	Cat# 11-5961-85
Anti-mouse Ly-6A/E (Sca-1) PE-Cy7 (D7)	Therm Fisher Scientific	Cat# 25-5981-82
Anti-mouse CD11b FITC (M1/70)	Therm Fisher Scientific	Cat# 11-0122-85
Anti-mouse CD19 FITC (6D5)	Therm Fisher Scientific	Cat# 115505
Anti-mouse NKp46 PerCP-eF710 (29A1.4)	Therm Fisher Scientific	Cat# 46-3351-82
Anti-mouse Thy1.2 APC-Cy7 (30-H12)	Therm Fisher Scientific	Cat# 105328
Anti-mouse GATA3 eFluor660 (TWAJ)	Therm Fisher Scientific	Cat# 50-9966-41
Anti-mouse RORγt PE (AFKJS-9)	Therm Fisher Scientific	Cat# 12-6988-82
Anti-mouse c-Kit BV605 (ACK2)	Therm Fisher Scientific	Cat# 135121
Anti-mouse CD127-biotin (A7R34)	Therm Fisher Scientific	Cat# 1312721-82
Anti-mouse $\alpha_4\beta_7$ APC (DATK-32)	Therm Fisher Scientific	Cat# 17-5887-82
Anti-mouse Flt3 PE (A2F10)	Therm Fisher Scientific	Cat# 12-1351-82
Chemicals, peptides, and recombinant proteins		
RPMI 1640	GIBCO	Cat# C22400500BT

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Penicillin- Streptomycin (10,000 U/mL)	GIBCO	Cat# 15140122
Fixable viability dye eFluor506	Therm Fisher Scientific	Cat# 65-0866-18
7-AAD	Therm Fisher Scientific	Cat# 00-6993-50
Streptavidin BV421	Biolegend	Cat# 405226
Rat Gamma Globulin	Jacksonimmuno	Cat# 012-000-002
BioMag anti-Rat IgG	Bangs Laboratories	Cat# BM560
Recombinant Murine IL-7	PeproTech	Cat# 217-17
Recombinant Murine SCF	PeproTech	Cat# 250-03
Recombinant Murine Flt3L	PeproTech	Cat# 250-31L
β-ME	GIBCO	Cat# 21985023
BioMag anti-Rat IgG	Bangs Laboratories	Cat# BM560
Nycodenz	Axis-Shield	Cat# 1002424
BioMag anti-Rat IgG	Bangs Laboratories	Cat# BM560
Heat-inactivated fetal bovine serum (FBS)	ExCell	Cat# FND500
Dulbecco's Phosphate-Buffered Sallines (DPBS)	GIBCO	Cat# C14190500BT
Critical commercial assays		
Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	Cat# 00-5523-00
Experimental models: Cell line		
OP9 and OP9-DL1 cell	Dr. Xiaohuan Guo laboratory	N/A
Experimental models: Organisms/strains		
Mouse: C57BL/6J mice (CD45.1)	Jackson lab	Jax002014
Mouse: C57BL/6J mice (CD45.2)	Jackson lab	Jax000664
Software and algorithms		
FACS Diva software	BD Biosciences	N/A
FlowJo V9.9.3 software	TreeStar	N/A
GraphPad Prism 7.0 software	GraphPad	N/A
BioRender	BioRender	https://www.biorender.com/
Other		
1.5 mL centrifuge tubes	Axygen	Cat# MCT-150-C
Falcon round bottom polystyrene tubes with cell strainer cap (5 mL)	Falcon	Cat# 352235
15 mL tube	Corning	Cat# 430052
50 mL tube	ExCell	Cat# CS015-0003
96-well culture plate, round button	Corning	Cat# 3799
Falcon® 70 μm Cell Strainer	Life Sciences	Cat# 352350
Falcon® 100 μm Cell Strainer	Life Sciences	Cat# 352360
BD™ Liquid Counting Beads	BD Bioscience	Cat# 335925
DynaMag™-15	Thermo Fisher Scientific	Cat# 12301D
FACSAria™ III	BD Bioscience	N/A
LSRFortessa™	BD Bioscience	N/A

MATERIALS AND EQUIPMENT

1× EDTA-BSS (pH = 7.12–7.15, Osmotic Pressure = 308–310 mOsm/kg)

Reagent	Final concentration	Amount
NaCl	150 mM	968 mL
EDTA	5 mM	560 mL
KCl	3.75 mM	24 mL
HEPES	14.5 mM	96 mL
DPBS	N/A	16 mL
Ultrapure Water	N/A	9424 mL
Total	N/A	11088 mL

Store at room temperature after sterilized for up to half a year.

1× KDS-BSS (pH = 7.12, Osmotic Pressure = 308–310 mOsm/kg)		
Reagent	Final concentration	Amount
$CaCl_2$	2.35 mM	21 mL
KCl	3.5 mM	21 mL
$MgSO_4$	1.18 mM	7 mL
NaCl	140 mM	847 mL
DPBS	N/A	14 mL
Ultrapure Water	N/A	8526 mL
Total	N/A	10 L
Store at room temperature after sterilized for up to half a year.		

3% FBS EDTA-BSS		
Reagent	Final concentration	Amount
FBS	3% (V/V)	15 mL
1× EDTA-BSS	N/A	485 mL
Total	N/A	500 mL
Store at 4°C for up to 1 week.		

3% FBS KDS-BSS		
Reagent	Final concentration	Amount
FBS	3% (V/V)	15 mL
1× KDS-BSS	N/A	485 mL
Total	N/A	500 mL
Store at 4°C for up to 1 week.		

Red cell removal buffer (RCRB) (pH = 7.0, Osmotic Pressure = 302–310 mOsm/kg)		
Reagent	Final concentration	Amount
$NaHCO_3$	1 g/L	5 g
NH_4Cl	0.168 M	41.7 g
EDTA	37 mg/L	0.185 g
Ultrapure Water	N/A	5 L
Total	N/A	5 L
Store at 4°C after sterilized for up to half a year.		

1.086 g/mL Nycodenz solution		
Reagent	Final concentration	Amount
Nycodenz stock	1.086 g/mL	According to stock
KDS-BSS	N/A	According to stock
Store at −20°C after sterilized for up to half a year.		

MEM alpha complete medium (500 mL)		
Reagent	Final concentration	Amount
Heat-inactivated FCS	20% (volvol)	100 mL
1M HEPES	10 mM	5 mL
Sodium Pyruvate (100×)	1×	5 mL
L-Glutamine (100×)	1×	5 mL
MEM non-essential amino acid solution (100×)	1×	5 mL
2-Mercaptoethanol (β-ME)	5×10^{-5} M	500 μL
MEM alpha + Glutamax		379.5 mL
Store at 4°C after sterilized for up to two months.		

Culture medium for ILC differentiation		
Reagent	Final concentration	Amount
RPMI 1640	N/A	443.65 mL
FBS	10% (V/V)	50 mL
β-ME	0.1% (V/V)	0.5 mL
Penicillin- Streptomycin (10,000 U/mL)	1% (V/V)	5 mL
SCF (100 μg/mL)	50 ng/mL	0.25 mL
IL-7 (100 μg/mL)	20 ng/mL	0.1 mL
Flt3L (100 μg/mL)	100 ng/mL	0.5 mL
Total	N/A	500 mL
Store at 4°C for up to 1 week. Warm at 37°C before use.		

STEP-BY-STEP METHOD DETAILS

Fetal liver cells isolation

Timing: 2 h

1. Keep a 6 cm dish of PBS-3%FBS on ice for the collection of fetal livers.
2. Euthanize the mice for aseptic operation.
   a. Euthanize the pregnant mice at 15.5–16.5 days gestation (E15.5–16.5) with CO_2.
   b. Sterilize the mice with 75% alcohol.
   c. Place the mice into the laminar flow hood for aseptic operation.
3. Dissect the pregnant mice and extract the uterus.
   a. Tear the uterine membrane with a sharp forceps.
   b. Squeeze out the fetus.
   c. Place the fetuses in PBS in a culture dish for washing.
   d. Place washed fetuses into a new dish with RPMI 1640.
4. Isolate the fetal liver cells.
   a. Carefully remove the surrounding tissue of the liver using microsurgical forceps, bluntly dissecting the fetal liver.
   b. Levigate fetal liver on 100 μm cell strainers with 40 mL EDTA-BSS-3%FBS into 50 mL tube.
   c. Centrifuge them for 5 min, at 4°C with 600 *g*.
5. Enrich the hematopoietic and immune cells in fetal liver.
   a. Remove supernatant.
   b. Add 5 mL of 1.086 g/mL Nycodenz solution to resuspend the cell pellet.
   c. Mix well.
   d. Gently overlay another 5 mL of 1.086 g/mL Nycodenz solution in a 15 mL centrifuge tube.
   e. And 1 mL FBS to the top layer.
   f. Centrifuge for 10 min at 4°C with 1700 *g* (Ascending 3, Descending 2).

   Note: Keep 1.086 g/mL Nycodenz on ice.

6. Remove the red blood cells.
   a. Add 1 mL red cell removal buffer (RCRB) to resuspend the cell pellet.
   b. Lyse the red cells at room temperature for 3 min.
   c. Add 20 mL EDTA-BSS-3%FBS to stop the red cell lysing.
   d. Centrifuge the remaining cells for 5 min at 4°C, with 600 *g*.
7. Resuspend the cell pellet for flow cytometry analysis.
   a. Remove the supernatant.
   b. Resuspend the cell pellet with 1 mL EDTA-BSS-3%FBS.
   c. Count the cell number.

Bone marrow cell isolation from adult mice

Timing: 40 min

8. Keep a 6 cm dish of KDS-BSS-3%FBS on ice for the collection of bones.
9. Euthanize the mice for aseptic operation.
   a. Euthanize the pregnant mice with CO_2.
   b. Sterilize the mice with 75% alcohol.
   c. Place the mice into the laminar flow hood for aseptic operation.
10. Flush the bone marrow cells.
   a. Dissect both sides of the femur and tibia.
   b. Cut both ends of the bone.
   c. Insert the needle into the end of the bone.
   d. Flush the bone marrow cells out with KDS-BSS-3%FBS by using a syringe.
   e. Centrifuge for 5 min at 4°C, with 600 *g*.
11. Remove the red blood cells.
   a. Remove supernatant.
   b. Resuspend cell pellet with 1 mL RCRB (for one mouse).
   c. Lyse red cells for 2 min at room temperature.
   d. Add 20 mL KDS-BSS-3%FBS to terminate the lysis of red cells.
12. Filter cell suspension through a 70 μm strainer and centrifuge the cells.
13. Resuspend the cell pellet for further use.
   a. Resuspend the cell pellet with KDS-BSS-3%FBS.
   b. Count the cell number.

Note: We suggest resuspending bone marrow cells at a cell concentration of 1×10^7 cells/mL, when used as feeder cells.

Bone marrow lineage negative (Lin^-) cells isolation from adult mice

Timing: 5 h

14. Prepare a single cell suspension of bone marrow cells following the steps above.
15. Use density gradient centrifugation to enrich the bone marrow Lin^- cells.
   a. Remove supernatant.
   b. Add 5 mL of 1.086 g/mL Nycodenz solution to resuspend the cell pellet.
   c. Mix well.
   d. Gently overlay another 5 mL of 1.086 g/mL Nycodenz solution in a 15 mL centrifuge tube.
   e. And 1 mL FBS to the top layer.
   f. Centrifuge for 10 min at 4°C with 1700 *g* (Ascending 3, Descending 2).
16. Acquire the cells after density gradient centrifugation.
   a. Gently aspirate the cell layer between FBS and Nycodenz solution.
   b. Transfer the cell layer into 40 mL of KDS-BSS-3%FBS in the tube.
   c. Centrifuge the cells for 5 min at 4°C, with 600 *g*.
17. Add antibody cocktail of lineage surface markers.
   a. Prepare the antibody cocktail of lineage surface markers by adding CD2 (1/50), CD3 (1/50), CD8 (1/50), CD45R (1/100), CD11b (1/100), TER119 (1/100) and Ly6G (1/50) into KDS-BSS-3%FBS.
   b. Remove supernatant.
   c. Count the cell number.
   d. Resuspend cell pellet with antibody cocktail of lineage surface markers (10 μL antibody cocktail for each 10^6 cells).
   e. Incubate at 4°C for 40 min.

18. Add 40 mL of KDS-BSS-3%FBS and wash to fully remove free antibodies.
19. Prepare the anti-rat IgG-magnetic beads.
    a. Aspirate the corresponding number of anti-rat IgG-magnetic beads (number of beads: number of cells = 10:1) to 5 mL tube.
    b. Add 2 mL KDS-BSS-3%FBS to the tube to wash the beads.
    c. Put the tube in the magnetic stand for 3 min.
    d. Remove the supernatant.
    e. Repeat step b ∼ d twice.
    f. Resuspend the beads with about 400 μL KDS-BSS-3%FBS for one mouse.
20. Incubate bone marrow cells with anti-rat IgG-magnetic beads.
    a. Resuspend the cell pellet with about 400 μL KDS-BSS-3%FBS for one mouse in 5 mL tube.
    b. Add 400 μL magnetic beads.
    c. Mix thoroughly using a 1 mL pipette.
    d. Incubate for 25 min with shaking in ice.
21. Use magnetic grate to delete the lineage cells.
    a. Add 2 mL KDS-BSS-3%FBS to the tube.
    b. Mix thoroughly using a pipette.
    c. Place the tube in a magnetic grate for 2 min.
    d. Aspirate the supernatant into a new 5 mL tube.
    e. Repeat step c ∼ d twice.
    f. Centrifuge the final supernatant.
22. Resuspend the cells for further use.
    a. Remove the supernatant.
    b. Add 1 mL KDS-BSS-3%FBS to resuspend cell pellet.
    c. Count the cell number.

Flow cytometry sorting for $\alpha_4\beta_7^+$ lymphoid progenitors (αLPs)

Timing: 2 h

23. Aspirate cells for flow cytometry antibody staining.
    a. Aspirate fetal liver cells or bone marrow Lin^- cells.
    b. Centrifuge for 5 min at 4°C, 600 *g* (centrifugation conditions, the same below).

Note: The number of cells used for sorting need to be adjusted according to the appropriate stain protocol.

24. Use Rat-IgG to block non-specific binding.
    a. Remove the supernatant.
    b. Resuspend the cell pellet with 50 μL Rat-IgG (50 μL for 1 × 10^7 cells).
    c. Incubate at 4°C for 10 min.
25. Prepare the surface marker staining antibody cocktail.
    a. Aspirate the needed volume of staining buffer into a 1.5 mL centrifuge tube (50 μL for 1 × 10^7 cells).
    b. Add CD45-APC-Cy7 (1/100), CD3ε-FITC (1/50), CD19-FITC (1/100), CD11b-PerCP-Cy5.5 (1/100), CD117-BV605 (1/50), CD127-Biotin (1/150), Flt3-PE (1/50), $\alpha_4\beta_7$-APC (1/100), Fixable viability dye eFluor506 (1/500) to EDTA-BSS-3%FBS.
    c. Streptavidin BV421 (1/300) was used for the second staining of CD127-Biotin (100 μL for 1 × 10^7 cells).
26. Surface marker staining.
    a. Add 50 μL of the surface staining antibody cocktail directly to the 50 μL Rat-IgG suspended cells.
    b. Incubate in dark at 4°C for 30 min.

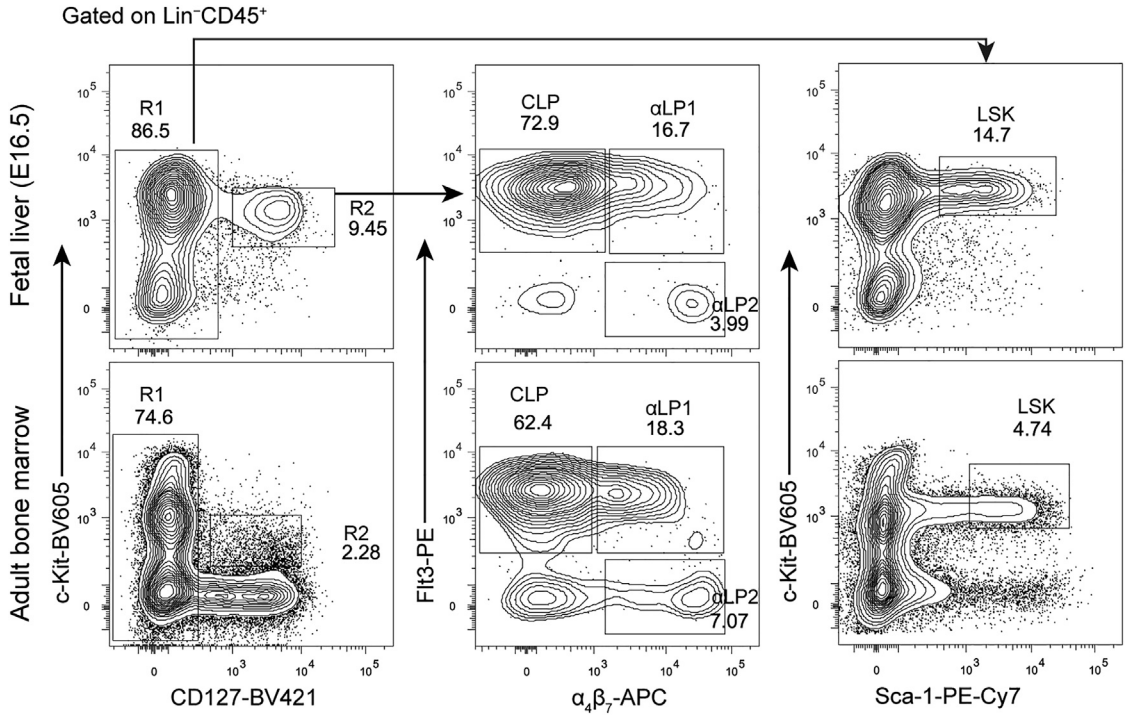

Figure 1. Gating strategy for hematopoietic progenitors in fetal liver and adult bone marrow

c. Wash the cells with 2 mL buffer.
d. Resuspend cell pellet with buffer containing Streptavidin BV421.
e. Incubate in dark at 4°C for 30 min.

27. Resuspend cells for flow cytometry sorting.
    a. Wash the cells with 2 mL buffer.
    b. Resuspend the cell pellet with 1 mL buffer (EDTA-BSS-3%FBS for fetal liver cells, KDS-BSS-3%FBS for bone marrow cells).
28. Flow cytometry analysis.
    a. Analyze the cell suspension on the FACSAria™ III.
    b. Gate αLPs as $CD45^+Lin^-CD117^+CD127^+\alpha_4\beta_7^+$ cells.
    c. αLPs can be further divided into αLP1 ($Flt3^+$ αLPs) and αLP2 ($Flt3^-$ αLPs).

Note: Lineage markers included CD3ϵ, CD19 and CD11b. The gating strategy was showed in Figure 1.

29. Sort αLPs using FACS.
    a. Prepare a sterile 5 mL tube containing 1 mL of RPMI 1640 complete medium as the collecting tube.
    b. Sort the target cells to the collecting tube for further *in vitro* culture.

Note: We can sort the need number of αLPs directly into the collecting tube with ILC culture medium (RPMI 1640 complete medium supplemented with IL-7 and SCF, and feeder cells). The volume of the collecting medium is consistent with the number of sorted αLPs (e.g., 5×10^3 αLPs/1×10^4 feeder cells/200 μL medium for each well of 96-well culture plate). Then, the sorted cells could directly put into the culture plate for *in vitro* differentiation.

In vitro culture assay

Timing: 7 days

30. Add adult bone marrow cells as feeder cells for *in vitro* culture assay.
    a. Prepare a 96-well round-bottom plate.
    b. Prepare the ILC culture medium by adding IL-7 (20 ng/mL), SCF (100 ng/mL), and Flt3L (100 ng/mL) to RPMI 1640 complete medium.[3]
    c. Count the number of sorted αLPs (CD45.2, 2,000–5,000/well).
    d. Add indicated number of adult bone marrow cells as feeder cells (CD45.1 mice, 1×10^4/well).

    Note: CD45.1 and CD45.2 are used in this study to distinguish between cells of different origins.

    e. Centrifuge for 5 min at 4°C, with 600 *g*.
    f. Resuspend the cell pellet with indicated volume of ILC culture medium.
    g. Put the cells into 96-well culture plate, 200 μL/well.
    h. Add PBS to the wells surrounding the cell culture wells to minimize the effect of medium evaporation on *in vitro* development and differentiation.
31. Add OP9 or OP9-DL1 as feeder cells for *in vitro* culture assay.
    a. Prepare a 96-well round-bottom plate.
    b. Add 400 OP9 or OP9-DL1 cells into the round-button 96-well culture plate about 12 h before inputting the progenitors.[2]
    c. Aspirate indicated number of sorted progenitor cells.
    d. Centrifuge for 5 min at 4°C, with 600 *g*.
    e. Resuspend cell pellet in ILC culture medium (1000 cells/200 μL/well).
    f. Remove the supernatant of the well with OP9 or OP9-DL1 cells.
    g. Add 200 μL cell suspension of the sorted cells to each well.
    h. Add PBS to the wells surrounding the cell culture wells to minimize the effect of medium evaporation on *in vitro* development and differentiation.
32. Incubate the cells at 37°C in a constant temperature incubator with 5% CO_2.
33. After 7 days, collect the cells for flow cytometric analysis.

Flow cytometry analysis for ILCs in the progeny

Timing: 2 h

34. Collect the cells by centrifuge the cells for 5 min at 4°C, 600 *g*.

    Note: We suggest directly centrifuging the 96-well plate and conduct the flow cytometry staining in this plate, to minimize the cell lose.

35. Block the non-specific staining.
    a. Remove the supernatant.
    b. Resuspend the cell pellet with 10 μL Rat-IgG to block the non-specific binding.
    c. Incubate at 4°C for 10 min.
36. Stain the surface marker.
    a. Prepare the surface marker staining antibody cocktail. Add CD45-BV605 (1/100), CD90.2-APC-Cy7 (1/100), CCR6-BV421 (1/50), NKp46-PerCPeF710 (1/50), and Fixable viability dye eFluor506 (1/500) to EDTA-BSS-3%FBS buffer (10 μL buffer for one sample).
    b. Add 10 μL of the antibody cocktail directly to the cells in step 2.
    c. Incubate in dark at 4°C for 40 min.

Note: For intracellular staining analysis, Fixable Viability Dye should be used to stain dead cells, do not use 7-AAD.

37. Cell fixation and permeabilization.
    a. Prepare the Fixation/Permeabilization solution (Fixation/Permeabilization Concentrate: Diluent = 1:3, 50 μL for one sample).
    b. Add 200 μL PBS-3%FBS to wash the cell.
    c. Centrifuge the cells for 5 min at 4°C, 600 *g*.
    d. Resuspend the cell pellet with 50 μL of Fixation/Permeabilization solution.
    e. Incubate for more than 1 h in dark at 4°C.
38. Intracellular staining.
    a. Prepare washing buffer (Permeabilization Buffer: ddH_2O = 1:10, V/V).
    b. Prepare the intracellular staining antibodies (RORγt-PE 1/200, GATA3-eFluor660 1/200 in washing buffer).
    c. Add 200 μL washing buffer for one sample.
    d. Centrifuge the cells for 5 min at 4°C, 600 *g*.
    e. Resuspend the cell pellet with 15 μL intracellular staining antibodies.
    f. Incubate in dark at 4°C for 1 h.

Note: Fixation/Permeabilization Concentrate, Fixation/Perm Diluent and Permeabilization Buffer are included in Foxp3 Transcription Factor Staining Kit.

39. Flow cytometry analysis by BD LSRFortessa™.
    a. Add 200 μL washing buffer to wash the cells twice.
    b. Add counting beads to the resuspending buffer (2,000 beads/200 μL EDTA-BSS-3%FBS for one sample).
    c. Resuspend the cell pellet with 200 μL resuspending buffer.
    d. Identify and gate the bead population based on forward and side scatter properties.
    e. Analyze the ILC subsets among the progeny.

EXPECTED OUTCOMES

Referring to the gating strategy from the previous study,[4] we isolated αLP in both fetal liver and adult bone marrow as follow (Figure 1).

After 7 days of *in vitro* differentiation, we assessed the composition of ILC subsets that developed from progenitor cells using flow cytometry analysis. As shown Figures 2 and 3, using adult bone marrow cells as feeder cells can efficiently promote the differentiation of major ILC subsets by both fetal and adult αLPs. Notably, using adult bone marrow as feeder cells presented dramatically differences when compared with OP9-DL1, which showed higher capacity of ILC2 generation but relatively lower capacity of LTi cell generation, especially at the fetal state, consistent with the role of Notch signaling in ILC subset fate determination.[4–6] However, the effect of adult bone marrow as feeder cells was more akin to that of OP9. Moreover, using adult bone marrow as feeder cells showed even higher efficiency of RORγt$^+$ ILCs differentiation, including both ILC3 and LTi cells, especially at the αLP1 stage. This suggests that adult bone marrow cells can function as more efficient feeder cells for the differentiation assay of RORγt$^+$ ILCs *in vitro*.

Furthermore, we noticed that this disparity was more pronounced at the αLP1 stage, potentially due to the retained strong differentiation capabilities of αLP1 cells towards multiple lineages. Indeed, our analysis did suggest that αLP1 cells harbor developmental potential for T cells, B cells, ILCs, and DCs.[1] However, it was not efficient to induce the differentiation of ILC subsets by earlier progenitors, like LSKs or CLPs.[1]

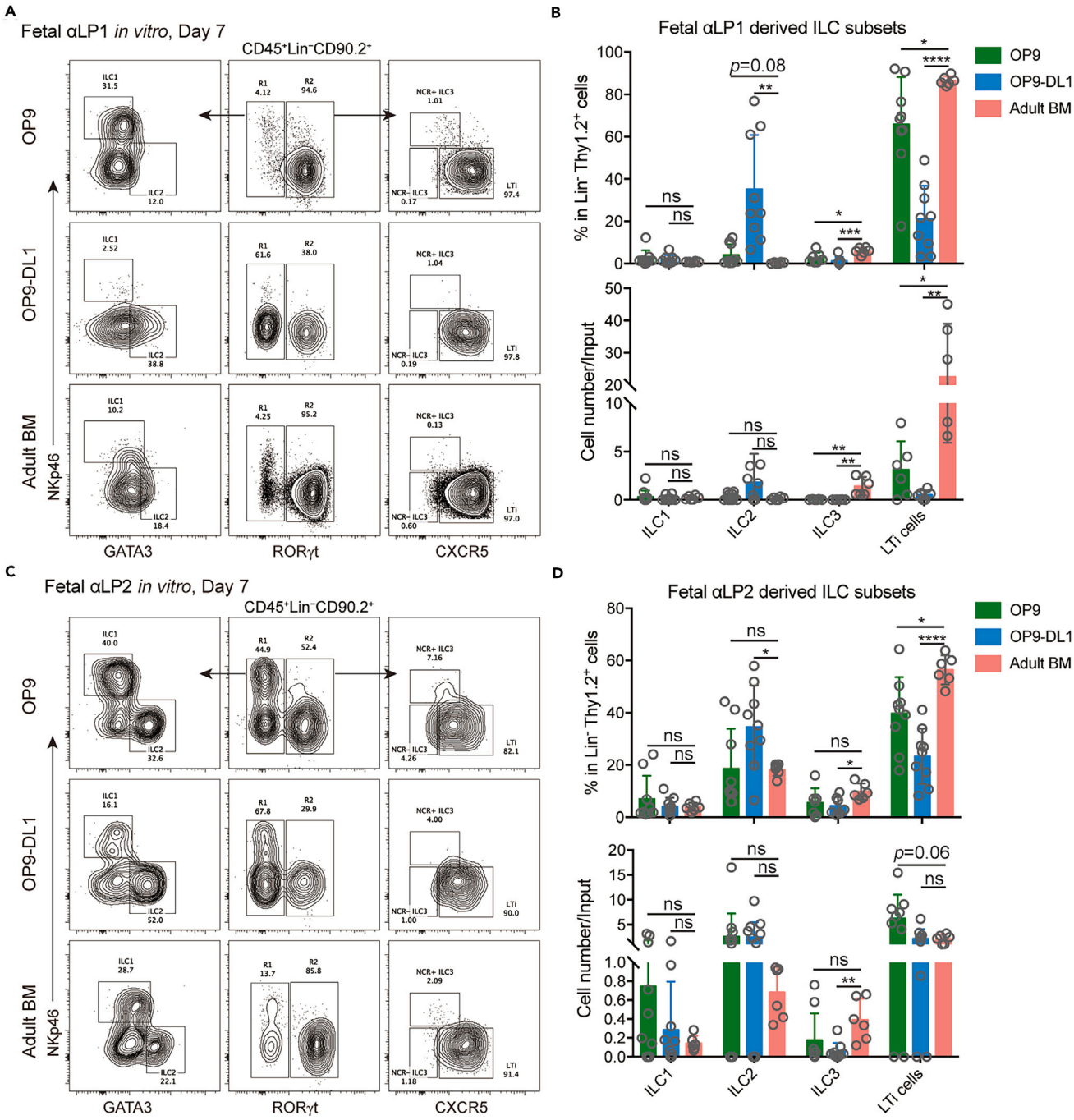

Figure 2. ILC subsets derived from fetal αLP1 and αLP2

(A) Comparing the effects of different feeder cells on the differentiation of fetal αLP1 into ILCs on day 7.

(B) Percentages of each fetal αLP1 derived ILC subsets among Lin$^-$Thy1.2$^+$cells and the culture efficiency revealed by the ratio of each ILC subsets' cell number to αLP1 cell number input.

(C) Comparing the effects of different feeder cells on the differentiation of fetal αLP2 into ILCs on day 7.

(D) Percentages of each fetal αLP2 derived ILC subsets among Lin$^-$Thy1.2$^+$cells and the culture efficiency revealed by the ratio of each ILC subsets' cell number to αLP2 cell number input. Statistical significance was tested using multiple *t*-tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$.

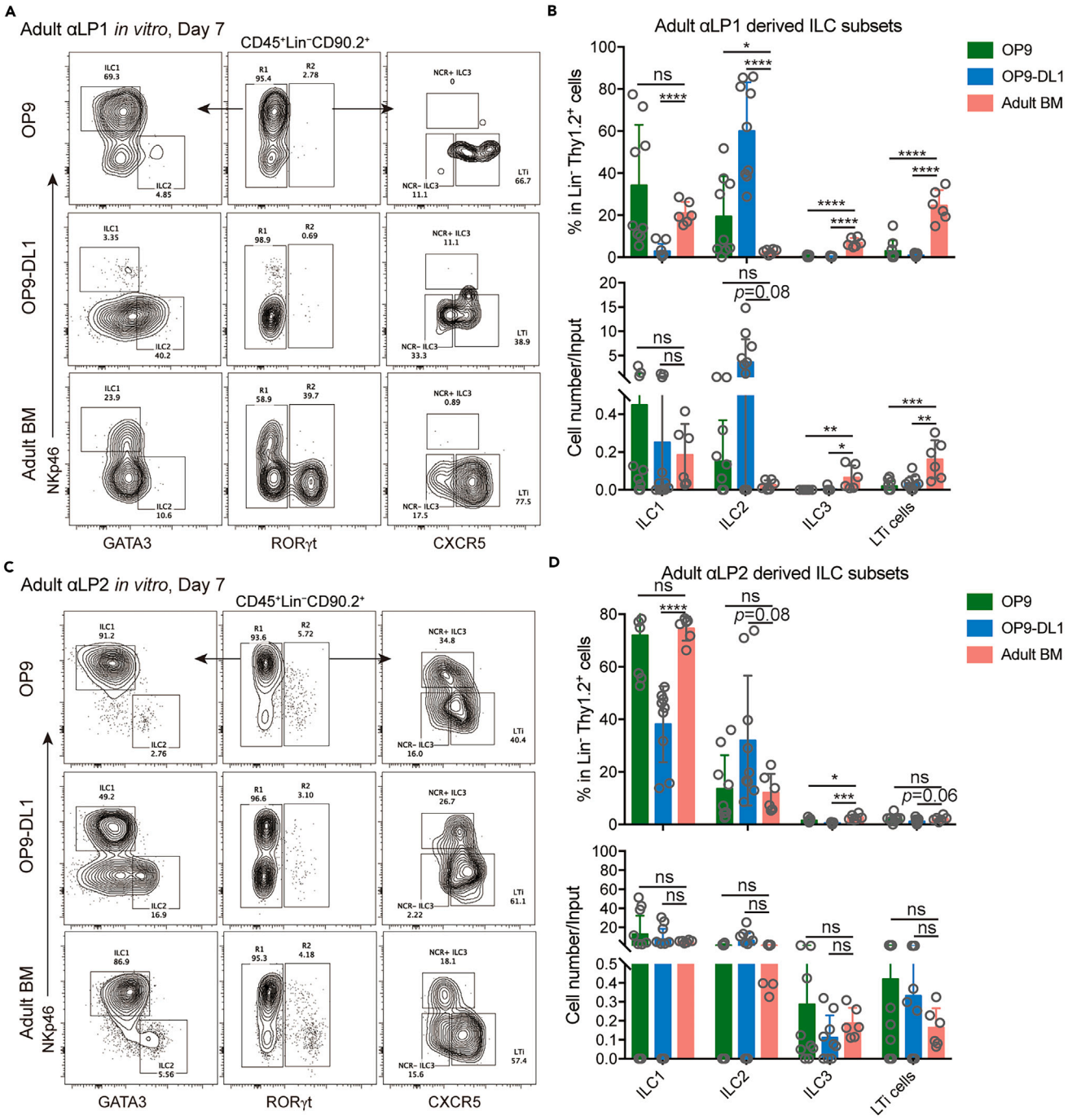

Figure 3. ILC subsets derived from adult αLP1 and αLP2

(A) Comparing the effects of different feeder cells on the differentiation of adult αLP1 into ILCs on day 7.

(B) Percentages of each adult αLP1 derived ILC subsets among Lin$^-$Thy1.2$^+$ cells and the culture efficiency revealed by the ratio of each ILC subsets' cell number to αLP1 cell number.

(C) Comparing the effects of different feeder cells on the differentiation of adult αLP2 into ILCs on day 7.

(D) Percentages of each adult αLP2 derived ILC subsets among Lin$^-$Thy1.2$^+$ cells and the culture efficiency revealed by the ratio of each ILC subsets' cell number to αLP2 cell number. All data are presented as mean ± SEM. Statistical significance was tested using multiple *t*-tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$.

Hence, we provide a method for *in vitro* differentiation assay for ILC progenitor cells by utilizing adult bone marrow cells as feeder cells. Applying this method can obtain the main ILC subsets, but compared with the classic method of using OP9 or OP9-DL1 as feeder cells, each has advantages and disadvantages. Our method is more suitable for analyzing the development of RORγt$^+$ ILCs, while OP9-DL1 may better reflect ILC2 development, and OP9 serves as an intermediate. Therefore, we believe that our method is an important alternative for ILC *in vitro* differentiation, particularly suitable for studying the development of RORγt$^+$ ILCs.

LIMITATIONS

While using whole bone marrow cells as feeder cells efficiently promotes the differentiation of late-stage ILC progenitor cells such as αLP1 and αLP2, their support for early lymphoid progenitor cells like CLP is significantly limited.[1] Furthermore, ILC2 differentiation using BM feeder cells is poorer compared to using OP9-DL1. Finally, αLP1 and αLP2 are relatively scarce among lymphoid progenitor cells, thus additional steps like flow cytometry sorting are necessary to obtain a sufficient number of αLP1 and αLP2.

TROUBLESHOOTING

Problem 1

The female mice were not pregnant at 15.5–16.5 days post mating.

Potential solution

In this method, we set timed mating in the first night, and detect copulation plug in the next morning. Mark the mice with plug as embryonic day 0.5 (E0.5). Mice should be confirmed to be pregnant at E10. Then the mice are analyzed at E15.5–16.6. Moreover, the fetal liver is reported to be one of the main leukocytes producing tissues in mice between days E12.5 and E17.5.[7] Therefore, fetal livers from E12.5 to E16.5 embryo can be used for sorting precursor cells.

Problem 2

The cell count observed under the microscope is prone to being influenced by subjective human factors.

Potential solution

As the ILC cells obtained from *in vitro* differentiation are relatively small, microscopic counting by Trypan Blue may lead to serious calculation errors. Therefore, BD™ Liquid Counting Beads can be used to mix with the stained cells which then are counted via BD LSRFortessa™ Cell Analyzer.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Li Wu (wuli@tsinghua.edu.cn).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to the technical contact, Tao Wu (wutao13@tsinghua.org.cn).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

This study did not generate original code.

Statistical analysis

GraphPad Prism 7 was used for statistical analysis with the specific tests and the number of samples used for each analysis (n) shown in the figure legends.

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AUTHOR CONTRIBUTIONS

L.W. conceptualized and supervised the project. T.W. and Y.H.W. performed the experiments. L.W., T.W. and Y.H.W. wrote the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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