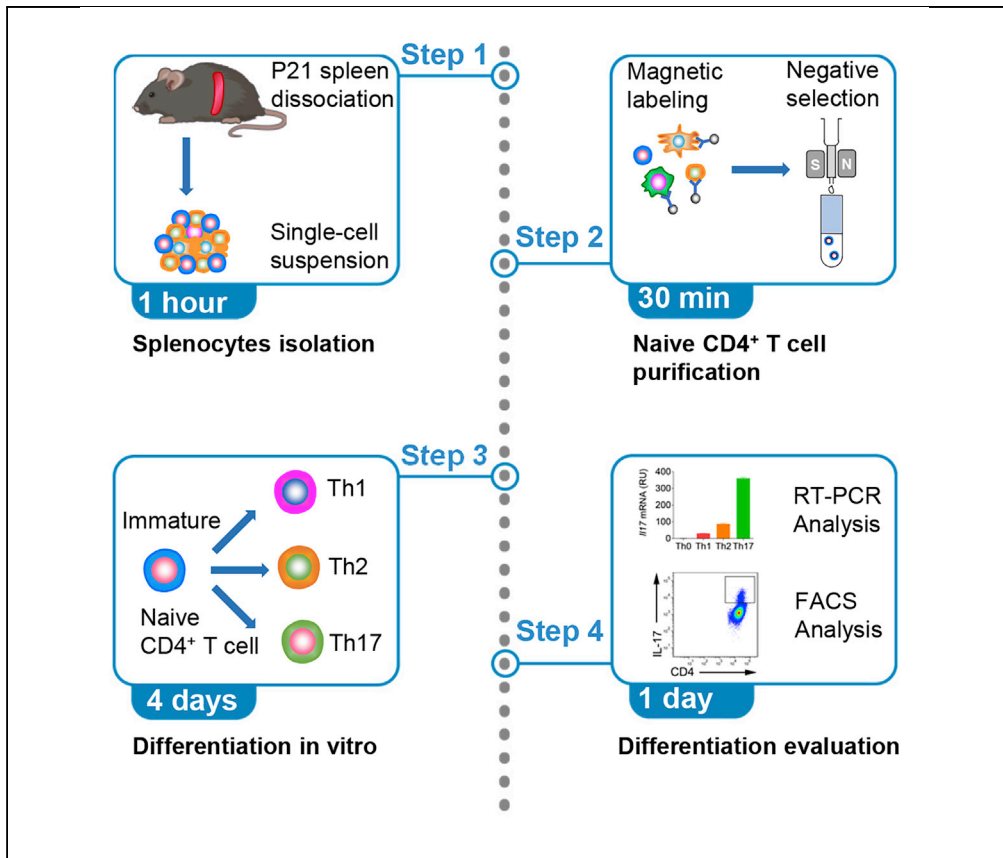


## Protocol

# Primary culture of immature, naïve mouse CD4<sup>+</sup> T cells



Primary T-cell culture is an invaluable model for investigating mechanisms underlying T-cell differentiation and function in health and disease. However, different culture conditions are required for immature versus mature CD4<sup>+</sup> T cells. Here, we provide an improved culture protocol for immature naïve mouse CD4<sup>+</sup> T cells, including details for splenocyte isolation, naïve CD4<sup>+</sup> T-cell purification and differentiation, and functional evaluation via flow cytometry. This protocol can also be applied for immature human CD4<sup>+</sup> T cells.

Wei Wang, Xingbin Ai

wwang@mgh.harvard.edu (W.W.)  
xai@partners.org (X.A.)

### Highlights

We provide a protocol of immature naïve CD4<sup>+</sup> T cell culture

This protocol can be used for mouse and human immature CD4<sup>+</sup> T cells

We give details of cytokine and transcriptional factor detection by flow cytometry

## Protocol

Primary culture of immature, naïve mouse CD4<sup>+</sup> T cellsWei Wang<sup>1,2,\*</sup> and Xingbin Ai<sup>1,3,\*</sup><sup>1</sup>Division of Neonatology, Department of Pediatrics, Massachusetts General Hospital, Boston, MA 02115, USA<sup>2</sup>Technical contact<sup>3</sup>Lead contact\*Correspondence: [wwang@mgh.harvard.edu](mailto:wwang@mgh.harvard.edu) (W.W.), [xai@partners.org](mailto:xai@partners.org) (X.A.)  
<https://doi.org/10.1016/j.xpro.2021.100756>

## SUMMARY

Primary T-cell culture is an invaluable model for investigating mechanisms underlying T-cell differentiation and function in health and disease. However, different culture conditions are required for immature versus mature CD4<sup>+</sup> T cells. Here, we provide an improved culture protocol for immature naïve mouse CD4<sup>+</sup> T cells, including details for splenocyte isolation, naïve CD4<sup>+</sup> T-cell purification and differentiation, and functional evaluation via flow cytometry. This protocol can also be applied for immature human CD4<sup>+</sup> T cells.

For complete details on the execution of this protocol, please refer to Wang et al. (2019).

## BEFORE YOU BEGIN

The protocol below describes specific steps for the isolation and culture of naïve CD4<sup>+</sup> T cells from neonatal mouse (both male and female) spleen at postnatal day 21 (P21) and provides details of suboptimal differentiation conditions for effector Th cells such that neurotransmitter modulators of differentiation can be identified. Because of a limited number of splenocytes from each spleen at this age, we pool cells from three P21 mice in one batch of isolation. Investigators should be proficient with the use of a flow cytometer with large multi-parameter capabilities (e.g., LSRFortessa) and data analysis by FlowJo. Slight modification of this protocol permits primary culture of CD4<sup>+</sup> T cells isolated from lymph nodes associated with lung samples from human donors under 13 years of age.

## Culture medium and buffer preparation

⌚ Timing: 30 min

1. See [materials and equipment](#) for preparation of the required culture medium and buffers.
2. Store the medium and buffers at 4°C for up to 1 month.

## Recombinant protein stock preparation

⌚ Timing: 30 min

3. Dissolve all the recombinant proteins in sterile 1×PBS (pH 7.4) at 100 × working concentration and make aliquots. The working concentration for each recombinant protein is listed in [Tables 5, 6, 7, and 8](#).
4. Keep one aliquot of each recombinant protein at 4°C for up to 1 month and store the rest at –80°C for up to 1 year.



### Chemical stock solution preparation

⌚ Timing: 30 min

- Dissolve the Concanavalin A (ConA, in water), Phorbol 12-myristate 13-acetate (PMA, in dimethyl sulfoxide) and Ionomycin calcium salt (in dimethyl sulfoxide) at 100 × working concentration and make aliquots. The working concentration for each recombinant protein is listed in [Tables 5, 6, 7, and 8](#).
- Store them at −20°C for up to 1 year.

### Pre-coat the 48-well tissue culture plate with αCD3

⌚ Timing: 10 min

- Dilute αCD3e in sterile 1×PBS (pH 7.4) to a final concentration of 1 μg/mL.
- Add 300 μL to one well of a 48-well tissue culture plate and seal the plate with parafilm.
- Incubate in the fridge at 4°C overnight (15–18 h).

**Alternatives:** The coating can also be done by incubating at room temperature (20°C–22°C) for 1 h or in a cell incubator for 2–3 hrs.

### Disinfection

⌚ Timing: 30 min

- Autoclave scissors and forceps and keep them sterile.
- Mix 350 mL ethanol with water to make a 500 mL of 70% ethanol solution and store at room temperature.
- Turn on the UV light in the tissue culture cabinet for at least 15 min.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Mouse monoclonal anti-Stat5 (Y694), FITC, clone SRBCZX, 1:200 dilution	eBioscience	Cat#11-9010-41; RRID: AB_2572520
Rat monoclonal anti-CD4, APC, clone RM4-5, 1:200 dilution	eBioscience	Cat#17-0042-82; RRID: AB_469323
Rat monoclonal anti-CD62L, PE, clone MEL-14, 1:200 dilution	eBioscience	Cat#12-0621-81; RRID: AB_465720
Rat monoclonal anti-CD25, Alexa Fluor® 488, clone eBio3C7 (3C7), 1:200 dilution	eBioscience	Cat#53-0253-82; RRID: AB_763471
Rat monoclonal anti-CD44, APC, clone IM7, 1:200 dilution	eBioscience	Cat#17-0441-81; RRID: AB_469389
Rat monoclonal anti-IL-13, PE, clone eBio13A, 1:200 dilution	eBioscience	Cat#12-7133-41; RRID: AB_10852712
Rat monoclonal anti-IL-17, PerCP-Cy5.5, clone eBio17B7, 1:200 dilution	eBioscience	Cat#45-7177-80; RRID: AB_925754
Rat monoclonal anti-CD4, PE-Cy7, clone GK1.5, 1:200 dilution	BioLegend	Cat#100421; RRID: AB_312706
Rat monoclonal anti-CD45, PE-Cy7, clone 30-F11, 1:200 dilution	BioLegend	Cat#103114; RRID: AB_312979
Rat monoclonal anti-IFN-γ, FITC, clone XMG1.2, 1:200 dilution	BD Biosciences	Cat#562019; RRID: AB_10893998
Armenian hamster monoclonal anti-CD3e, clone 145-2C11	BD Biosciences	Cat#553058; RRID: AB_394591
Armenian hamster monoclonal anti-CD28, clone 37.51	BD Biosciences	Cat#553295; RRID: AB_394764
<b>Chemicals, peptides, and recombinant proteins</b>		
Concanavalin A (ConA)	Sigma-Aldrich	Cat#L7647-100MG
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	Cat#P8139-1MG
Ionomycin calcium salt	Sigma-Aldrich	Cat#I0634-1MG

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
2-Mercaptoethanol	Sigma-Aldrich	Cat#M6250-100ML
Dimethyl sulfoxide	Sigma-Aldrich	Cat# D8418-500ML
Methanol	Fisher Scientific	Cat#AC326950010
Ethanol	Fisher Scientific	Cat#BP2818-4
Ethylenediamine tetraacetate (EDTA)	Fisher Scientific	Cat#BP2483500
Bovine serum albumin (BSA)	Fisher Scientific	Cat#BP1605100
Mouse rIL-4	BioLegend	Cat#574302
Mouse rIL-12	R&D Systems	Cat#419-ML-010
Mouse rIL-6	R&D Systems	Cat#406-ML-005
Mouse rIL-23	R&D Systems	Cat#1887-ML-010
Human rTGF- $\beta$	R&D Systems	Cat#240-B-002
Human rIL-2	R&D Systems	Cat#202-IL-010
RPMI 1640 with L-glutamine	Gibco	Cat#11875-119
Fetal bovine serum	Gibco	Cat#10437-028
pH7.4 1 $\times$ PBS w/o Ca and Mg	Gibco	Cat#10010-072
Penicillin-streptomycin solution	VWR	Cat#16777-164
RNaseOUT™ Recombinant RNase Inhibitor	Thermo Fisher	Cat#10777-019
DTT (0.1 M, in Superscript III Reverse Transcriptase Kit)	Thermo Fisher	Cat#18080-044
dNTPs	NEB	Cat# N0446S
<b>Critical commercial assays</b>		
Red blood cell lysis buffer	Sigma-Aldrich	Cat#R7757
Paraformaldehyde 4% in PBS	VWR	Cat#AAJ61899-AP
Cell Stimulation Cocktail (plus protein transport inhibitors)	eBioscience	Cat#00-4975-03
Fixable Viability Dye eFluor 780	eBioscience	Cat#65-0865-14
Fixation/permeabilization solution	BD Biosciences	Cat#554722
Perm/Wash solution	BD Biosciences	Cat#554723
Naive CD4 <sup>+</sup> T cells isolation kit, mouse	Miltenyi Biotec	Cat#130-104-453
LS columns	Miltenyi Biotec	Cat#130-042-401
MidiMACS Separator	Miltenyi Biotec	Cat#130-042-302
RNeasy Kit	QIAGEN	Cat#74106
QIAshredder	QIAGEN	Cat#79656
Superscript III Reverse Transcriptase Kit	Thermo Fisher	Cat#18080-044
TaqMan® Fast Universal PCR Master Mix (2 $\times$ )	Thermo Fisher	Cat#4366073
<b>Experimental models: organisms/strains</b>		
Mouse: C57BL/6	The Jackson Laboratory	Cat#000664
<b>Oligonucleotides</b>		
Mouse <i>Il13</i> TaqMan primers with probe	Thermo Fisher	Cat#Mm00434204_m1
Mouse <i>Ifng</i> TaqMan primers with probe	Thermo Fisher	Cat#Mm01168134_m1
Mouse <i>Il17</i> TaqMan primers with probe	Thermo Fisher	Cat#Mm00439618_m1
Mouse <i>18s</i> TaqMan primers with probe	Thermo Fisher	Cat#Mm03928990_g1
Oligo dT	Invitrogen	Cat#18418-020
<b>Software and algorithms</b>		
BD FACSDiva Software v8.0	BD	<a href="https://www.bdbiosciences.com/cn/instruments/research/software/flow-cytometry-acquisition/bdfacsddiva-software/m/111112/resourcestools">https://www.bdbiosciences.com/cn/instruments/research/software/flow-cytometry-acquisition/bdfacsddiva-software/m/111112/resourcestools</a>
FlowJo v10.7.0	Tree Star	<a href="https://www.flowjo.com">https://www.flowjo.com</a>
<b>Other</b>		
15 mL Centrifuge tubes	Corning	Cat#352196
50 mL Centrifuge tubes	Corning	Cat#430291
48-Well tissue culture plate (flatten bottom)	Corning	Cat#353230
96-Well round bottom plate	Corning	Cat#3788
5 mL Round-bottom tubes	Corning	Cat#352235
100 mm Petri dish	Fisher Scientific	Cat#FB0875712
96-well Unskirted qPCR Plates, Low-Profile	Bio-Rad	Cat#MLL-9601

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Optical Flat 8-Cap Strips for qPCR plate	Bio-Rad	Cat#TCS-0803
0.22 µm Vacuum filter	EMD Millipore	Cat#SCGPU01RE
70 µm Cell strainer	CELLTREAT	Cat#229483
Hemocytometer	Bulldog Bio	Cat#DHC-N51
1.5 mL Centrifuge tubes	USA Scientific	Cat#1615-5500
Centrifuge	Eppendorf	5810R
Cell culture CO <sub>2</sub> incubator	Thermo Fisher	HEPA Class100
Thermal cycler	Bio-Rad	C1000 Touch
NanoDrop™ 2000 Spectrophotometer	Thermo Fisher	ND-2000
Real-time PCR System	Bio-Rad	CFX96
BD LSRFortessa flow cytometer	BD	N/A

## MATERIALS AND EQUIPMENT

⚠ **CRITICAL:** 2-mercaptoethanol is toxic. It can cause irritation to the nasal passageway and respiratory tract upon inhalation, irritation to the skin upon contact, vomiting and stomach pain through ingestion, and potential death if severe exposure occurs. 2-mercaptoethanol should be handled under the fume hood.

## STEP-BY-STEP METHOD DETAILS

### Neonatal mouse spleen isolation and single splenocyte suspension preparation

⌚ **Timing:** 45–55 min

This step contains information on how to isolate the mouse spleen and how to prepare single splenocyte suspension.

1. Euthanize P21 mice by CO<sub>2</sub> inhalation.
2. Lay a mouse on its back on a dissection mat, pin the paws on the mat, and spray with 70% ethanol to wet the fur.
3. Make an incision in the peritoneum and cut the inferior vena cava to bleed the mouse to reduce the red blood cells left in the spleen.
4. Gently lift the spleen by a pair of forceps and remove the attached pancreatic tissue with scissors. Transfer the spleen to a 15 mL collection tube containing 5 mL of T cell culture medium (Table 3) with forceps.
5. Pour the spleen with T cell culture medium into a 100 mm Petri dish.

⚠ **CRITICAL:** To maintain the aseptic condition, work in the tissue culture hood.

6. Put a 70 µm cell strainer on top of a 50 mL conical tube and rinse the cell strainer with 1 mL ice cold 1 × PBS (pH 7.4).
7. Smash the spleen on the cell strainer using a sterile plunger of a 1 mL syringe and flush the cell strainer with 10 mL ice cold 1 × PBS (pH 7.4).
8. Discard the cell strainer and centrifuge the sample at 400 × g for 15 min at 4°C.
9. Discard the supernatant by gently flipping the 50 mL tube. Add 2 mL red blood cell lysis buffer to the tube and resuspend the splenocytes by gentle pipetting. Incubate for 3 min on ice.

**Note:** Optimal lysis of erythrocytes is usually achieved by incubating at room temperature. However, for subsequent naïve CD4<sup>+</sup> T cell purification, the manufacturer suggests that cells always be kept cold. So, we keep the cells on ice.

10. Add 5 mL of T cell culture medium to stop the lysis of red blood cells and centrifuge the sample at  $400 \times g$  for 5 min at  $4^{\circ}\text{C}$ .
11. Discard the supernatant. Resuspend the splenocytes with 10 mL of ice cold pH 7.4  $1 \times \text{PBS}$  and keep the cells on ice until finishing the cell counting.

**Note:** Repeat step 9–10 if the cell pellet is still red.

12. Take out 20  $\mu\text{L}$  of the cell suspension, dilute 10 times in 0.1% trypan blue, and determine the splenocyte number with hemocytometer.
13. Take  $7 \times 10^6$  splenocytes prior to naïve  $\text{CD4}^+$  T cell purification for staining of the compensation control and for the evaluation of naïve  $\text{CD4}^+$  T cells percentage before purification by flow cytometry.

### Neonatal naïve $\text{CD4}^+$ T-cell purification via magnetic bead-based negative selection

⌚ Timing: 25–30 min

This step contains information on how to purify naïve  $\text{CD4}^+$  T cells from the splenocyte suspension using a magnetic bead-based isolation kit. More information about this procedure can be found on manufacturer's website: <https://www.miltenyibiotec.com/US-en/products/naive-cd4-t-cell-isolation-kit-mouse.html#gref>

14. Centrifuge the sample at  $400 \times g$  for 5 min at  $4^{\circ}\text{C}$  and discard the supernatant completely.
15. Resuspend the cell pellet in 400  $\mu\text{L}$  of purification buffer (Table 1) per  $10^8$  total cells and then add 100  $\mu\text{L}$  of Biotin-Antibody Cocktail per  $10^8$  total cells. Mix well and incubate for 5 min in the refrigerator ( $2\text{--}8^{\circ}\text{C}$ ).
16. Add 200  $\mu\text{L}$  of purification buffer, 200  $\mu\text{L}$  of Anti-Biotin MicroBeads and 100  $\mu\text{L}$  of CD44 MicroBeads per  $10^8$  total cells in sequence. Mix them well and incubate for 10 min in the refrigerator ( $2\text{--}8^{\circ}\text{C}$ ).
17. Place a LS column in the magnetic field of MidiMACS Separator and prepare the LS column by rinsing with 3 mL of purification buffer.
18. Apply the cell suspension onto the column and collect the flow-through that contain unlabeled cells enriched in naïve  $\text{CD4}^+$  T cells.
19. Wash the column with 3 mL of purification buffer. Collect unlabeled cells in the flow-through and combine with the flow-through from step 18.
20. Determine the cell number with hemocytometer. Take  $1 \times 10^6$  cells for staining to evaluate the purity of naïve  $\text{CD4}^+$  T cells by flow cytometry.

**Alternatives:** Here, we used the magnetic bead-based negative selection isolation kit to purify naïve  $\text{CD4}^+$  T cells because it is effective and time saving. Magnetic bead-based positive selection isolation kit can also be used for purification. Another alternative approach is to FACS-sort naïve  $\text{CD4}^+$  T cells that yields slightly higher purity; however, it takes longer time and requires a cell sorter. As few as 0.2 million cells can be used for staining to evaluate the purity.

### Evaluate naïve $\text{CD4}^+$ T-cell purity by flow cytometry

⌚ Timing: 2 h

This step contains information on antibody staining to evaluate the purity of the isolated naïve  $\text{CD4}^+$  T cells by flow cytometry. In total,  $7 \times 10^6$  splenocytes (before purification) and  $1 \times 10^6$  purified naïve  $\text{CD4}^+$  T cells will be used. Figure 1 shows the allocation of these cells for different staining in a 96-well plate.

**Table 1. Purification buffer**

Component	Final concentration	Amount
1×PBS (pH 7.4)	n/a	49.8 mL
BSA	0.5% (w/w)	0.25 g
EDTA (0.5 M)	2 mM	200 μL
<b>Total</b>	<b>n/a</b>	<b>50 mL</b>

Sterilize with 0.22 μm filter and store at 4°C for up to 1 month.

21. Take  $1 \times 10^6$  splenocytes and fix them with 4% PFA for 10 min on ice to generate a positive control for dead cell staining.
22. Wash twice of the fixed cells by adding 300 μL of FACS buffer (Table 2). Centrifuge at  $400 \times g$  for 5 min at 4°C to collect the cell pellet.
23. Mix fixed cells with  $1 \times 10^6$  unfixed splenocytes and use this sample as a single-stained cell compensation control for Fixable Viability Dye.
24. Transfer  $1 \times 10^6$  unfixed cells to each allocated well of a 96-well round bottom plate and centrifuge the plate at  $400 \times g$  for 5 min at 4°C. Discard the supernatant completely.

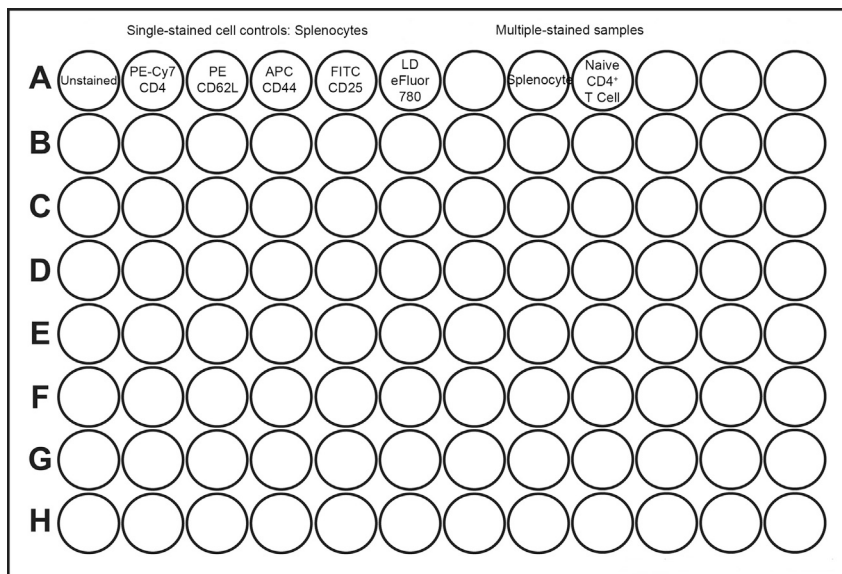
**△ CRITICAL:** During the following steps, cells have to be protected from light with aluminum foil.

25. During the spin, prepare Live/Dead Fixable Viability Dye eFluor 780 staining solution by diluting 1 μL of a concentrated stock with 2 mL of 1×PBS (pH 7.4).
26. Resuspend the cell pellet in one well as the compensation control for Fixable Viability Dye staining and the two wells for multiple staining with 100 μL of diluted Live/Dead Fixable Viability Dye eFluor 780. Resuspend the cell pellet in other wells with 200 μL 1×PBS (pH 7.4) and incubate for 30 min at 4°C.
27. During the incubation, prepare multiple staining antibody solution as shown in Table 4 and single staining solutions.
28. Centrifuge the plate at  $400 \times g$  for 5 min at 4°C and discard the supernatant. Wash the cell pellet twice using 300 μL of FACS buffer.
29. Resuspend the cell pellet in the two wells for multiple staining with 100 μL of antibody mix 1 solution and cell pellets in other wells for single staining with 100 μL of respective single staining solution. Resuspend the cell pellet in the well for Fixable Viability Dye staining with 100 μL of FACS buffer. Incubate for 30 min at 4°C.

**Note:** According to the manufacturer's protocol, it is best to stain with Live/Dead Fixable Viability Dye in azide and protein-free PBS for the brightest staining.

30. Wash twice by adding 300 μL of FACS buffer, centrifuge the plate at  $400 \times g$  for 5 min at 4°C, and discard the supernatant.
31. Resuspend the cell pellet in 300 μL of FACS buffer. Passage the cell suspension through the 40 μm cell strainer on top of the 5 mL Falcon® tube before analysis with Fortessa 405/488/561/640 (BD Biosciences) flow cytometer.
32. See Figure 2 for the gating strategy of naïve CD4<sup>+</sup> T cell analysis with Flowjo software. Purity over 95% is considered acceptable. If the purity is less than 95%, another round of enrichment is suggested.

**Alternatives:** OneComp eBeads™ (01-1111-42, Invitrogen) can also be used for compensation purposes. A mouse, rat or hamster origin antibody with APC-Cy7 labeling should be used for the compensation of Fixable Viability Dye eFluor 780.



**Figure 1.** Layout of a 96-well round bottom plate setup for the staining of compensation controls, splenocytes and naïve CD4<sup>+</sup> T cells after enrichment

△ **CRITICAL:** Since some surface markers, like CD62L, are temperature sensitive, always keep the plate on ice during the process of staining and use a centrifuge setting at 4°C.

### Neonatal naïve CD4<sup>+</sup> T-cell differentiation *in vitro*

⌚ **Timing:** 4 days

This step contains information on differentiation conditions of neonatal naïve CD4<sup>+</sup> T cells.

33. Centrifuge the purified naïve CD4<sup>+</sup> T cells at 400 × g for 5 min at 4°C and discard the supernatant completely.
34. During the spin, wash the αCD3 pre-coated, 48-well tissue culture plate with 1mL 1×PBS (pH 7.4).
35. Resuspend the cell pellet with T cell culture medium (Table 3) and adjust the cell density to 1 × 10<sup>6</sup> cells/mL.
36. Seed 5 × 10<sup>5</sup> cells in 0.5 mL cell suspension in each well of the 48-well tissue culture plate.
37. For Th0, Th1, Th2 and Th17 cultures, add respective antibodies, chemicals and recombinant proteins following the instructions in Tables 5, 6, 7, and 8.

**Note:** Prepare immediately before use and add the reagents individually to the culture medium. As human IL-2 stimulates proliferation of mouse T cells at similar concentration, we used human IL-2 in our protocol. Alternatively, mouse IL-2 can also be used at the same concentration.

**Table 2.** FACS buffer

Component	Final concentration	Amount
1×PBS (pH 7.4)	n/a	49 mL
FBS	2% (v/v)	1 mL
<b>Total</b>	<b>n/a</b>	<b>50 mL</b>

Store at 4°C for up to 1 month.



**Table 3. T cell culture medium**

Component	Final concentration	Amount
RPMI 1640 with L-glutamine	n/a	445 mL
Penicillin-streptomycin solution (10×)	1×	5 mL
2-mercaptoethanol (1 M)	50 μM	25 μL
FBS	10% (v/v)	50 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

Store at 4°C for up to 1 month.

**Note:** Prepare immediately before use and add reagents individually to the culture medium. As human IL-2 stimulates proliferation of mouse T cells at similar concentration, we used human IL-2 in our protocol. Alternatively, mouse IL-2 can also be used at the same concentration.

**Note:** Prepare immediately before use and add reagents individually to the culture medium. As human IL-2 stimulates proliferation of mouse T cells at similar concentration, we used human IL-2 in our protocol. Alternatively, mouse IL-2 can also be used at the same concentration.

**Note:** Prepare immediately before use and add reagents individually to the culture medium. As human IL-2 stimulates proliferation of mouse T cells at similar concentration, we used human IL-2 in our protocol. Alternatively, mouse IL-2 can also be used at the same concentration. The optimal working concentration of TGF-β should be titrated by the operator, since it can vary among different batches. As naive CD4<sup>+</sup> T cells do not express IL-23R and IL-23 promotes maintenance but not commitment to the Th17 lineage (Stritesky et al., 2008), add IL-23 after 2 days. Only adding the 5 μL of IL-23 to each well with the already existing culture medium containing the other recombinants/reagents.

**Note:** Add IL-23 after 2 days.

38. Put the plate into a CO<sub>2</sub> incubator and culture for 4 days at 37°C with 5% CO<sub>2</sub>.

**Alternatives:** we have employed a suboptimal differentiation condition in our screen for neurotransmitters that can modulate CD4<sup>+</sup> T cell differentiation. Standard T cell differentiation can be achieved by adding anti-mIL-4 antibody (10 μg/mL) to Th1 culture, anti-mIFN-γ antibody (10 μg/mL) to Th2 culture, anti-mIL-4 antibody (10 μg/mL) and anti-mIFN-γ antibody (10 μg/mL) to Th17 culture at the beginning of each culture.

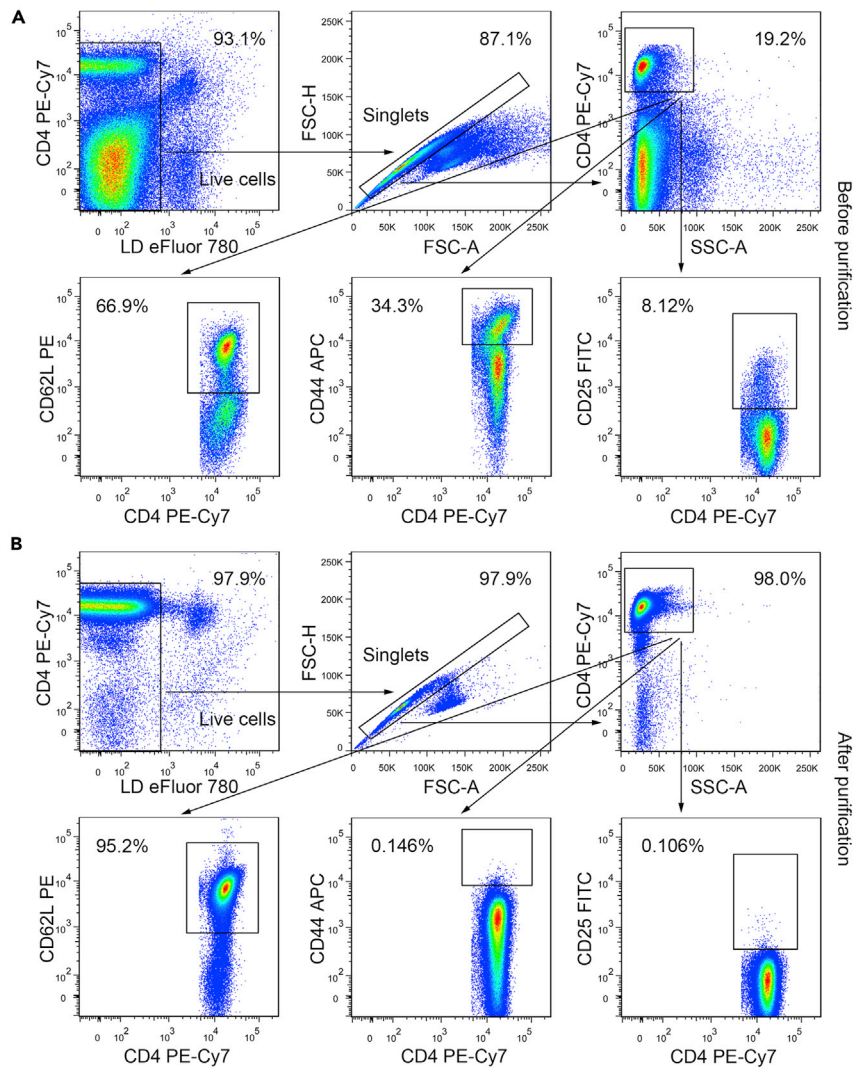
### Evaluate T-cell differentiation by cytokine gene expression assays via qPCR

⌚ Timing: 3.5–4 h

**Table 4. Antibody mix for cell surface marker staining of splenocytes**

Fluorophore	Marker	Final dilution	Volume per sample
PE-Cy7	CD4	1/200	0.5 μL
PE	CD62L	1/200	0.5 μL
FITC	CD25	1/200	0.5 μL
APC	CD44	1/200	0.5 μL
FACS buffer	n/a	n/a	98 μL
<b>Total</b>	<b>n/a</b>	<b>n/a</b>	<b>100 μL</b>

Prepare immediately before use.



**Figure 2. Gating strategy used to evaluate the purity of naïve CD4<sup>+</sup> T cells before and after enrichment**

(A) Representative result of naïve CD4<sup>+</sup> T cells purity within splenocytes from C57BL/6J mouse at P21.

(B) Representative result of naïve CD4<sup>+</sup> T cells purity after enrichment.

This step contains information on RNA extraction and quantification of cytokine gene expression by qPCR to evaluate T cell differentiation.

39. RNA extraction. More information about RNA extraction can be found on manufacturer's website: <https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en>

- a. Determine the cell number at the end of T cell culture. Collect cells (no more than  $5 \times 10^6$  cells) in a 1.5 mL centrifuge tube by spinning for 5 min at  $400 \times g$ . Carefully discard all the supernatant.

**Note:** Incomplete removal of the supernatant will inhibit lysis and dilute the lysate. These factors may reduce the RNA yield.

**Pause point:** Proceed with RNA extraction following manufacturer's protocol or snap freeze the cell pellet in liquid nitrogen before storage at  $-80^{\circ}\text{C}$ .

**Table 5. Th0 culture recipe (suboptimal condition)**

Component	Stock concentration	Final concentration	Volume per sample
PMA	100 nM	1 nM	5 $\mu$ L
ConA	50 $\mu$ g/mL	0.5 $\mu$ g/mL	5 $\mu$ L
Ionomycin	25 $\mu$ M	250 nM	5 $\mu$ L
$\alpha$ CD28	500 $\mu$ g/mL	1 $\mu$ g/mL	1 $\mu$ L
Human rIL-2	500 ng/mL	5 ng/mL	5 $\mu$ L

- b. Flick the tube to thoroughly loosen the cell pellet and add 350  $\mu$ L Buffer RLT. Mix well by pipetting.
- c. Transfer the lysate into a QIAshredder spin column placed in a 2 mL collection tube and centrifuge for 2 min at 8000  $\times$  g.
- d. Add 1 volume of 70% ethanol to the homogenized lysate, and pipet up and down to mix well.

**△ CRITICAL: Do not centrifuge.**

- e. Transfer up to 700  $\mu$ L of the sample to an RNeasy spin column placed in a 2 mL collection tube. Centrifuge for 15 s at 8000  $\times$  g. Discard the flow-through.

*Optional:* If performing optional on-column DNase digestion to eliminating genomic DNA contamination, follow Appendix D in manufacturer's protocol after performing this step.

- f. Add 700  $\mu$ L Buffer RW1 to the column and centrifuge for 15 s at 8000  $\times$  g to wash the column. Discard the flow-through.
- g. Add 500  $\mu$ L Buffer RPE to the column and centrifuge for 15 s at 8000  $\times$  g to wash the column. Discard the flow-through.
- h. Add 500  $\mu$ L Buffer RPE to the column and centrifuge for 2 min at 8000  $\times$  g to wash the column.

*Optional:* Place the column into a new 2 mL collection tube and centrifuge at 8000  $\times$  g for 1 min to completely remove Buffer RPE.

- i. Place the RNeasy spin column into a new 1.5 mL collection tube. Add 30  $\mu$ L RNase-free water to the spin column membrane and centrifuge for 1 min at 8000  $\times$  g to elute the RNA.

40. First-strand cDNA synthesis. More information about the first-strand cDNA synthesis procedure can be found on manufacturer's website: [https://assets.thermofisher.com/TFS-Assets/LSG/manuals/superscriptIII\\_man.pdf](https://assets.thermofisher.com/TFS-Assets/LSG/manuals/superscriptIII_man.pdf)
  - a. Determine the RNA concentration of each sample by NanoDrop™ 2000 spectrophotometer.
  - b. Follow Table 9 to make the mix for step 1.

**Note:** According to the manufactory's protocol, the amount of starting material can vary from 1 pg–5  $\mu$ g of total RNA. In our protocol, we used 1  $\mu$ g of total RNA.

**Table 6. Th1 culture recipe (suboptimal condition)**

Component	Stock concentration	Final concentration	Volume per sample
PMA	100 nM	1 nM	5 $\mu$ L
ConA	50 $\mu$ g/mL	0.5 $\mu$ g/mL	5 $\mu$ L
Ionomycin	25 $\mu$ M	250 nM	5 $\mu$ L
$\alpha$ CD28	500 $\mu$ g/mL	1 $\mu$ g/mL	1 $\mu$ L
Human rIL-2	500 ng/mL	5 ng/mL	5 $\mu$ L
Mouse rIL-12	1 $\mu$ g/mL	10 ng/mL	5 $\mu$ L

**Table 7. Th2 culture recipe (suboptimal condition)**

Component	Stock concentration	Final concentration	Volume per sample
PMA	100 nM	1 nM	5 $\mu$ L
ConA	50 $\mu$ g/mL	0.5 $\mu$ g/mL	5 $\mu$ L
Ionomycin	25 $\mu$ M	250 nM	5 $\mu$ L
$\alpha$ CD28	500 $\mu$ g/mL	1 $\mu$ g/mL	1 $\mu$ L
Human rIL-2	500 ng/mL	5 ng/mL	5 $\mu$ L
Mouse rIL-4	1 $\mu$ g/mL	10 ng/mL	5 $\mu$ L

- c. Add the above components to a nuclease-free microcentrifuge tube. Heat mixture to 65°C for 5 min and incubate on ice for at least 1 min.
  - d. During the incubation, follow Table 10 to prepare the mix for step 2.
  - e. Collect the contents of the tubes by brief centrifugation and add cDNA mix 2 to the tubes.
  - f. Mix by gently pipetting up and down and incubate at 50°C for 50 min.
  - g. Inactivate the reaction by heating at 85°C for 5 min and hold at 4°C.
41. PCR reaction. More information about PCR reaction procedure can be found on manufacturer's website: [https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms\\_042252.pdf](https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_042252.pdf)
- a. Dilute the cDNA template to 10 ng/ $\mu$ L with distilled water.
  - b. Follow Table 11 to make the mix for PCR reaction. Mix TaqMan® PCR Master Mix with different TaqMan® primers first and transfer them to a 96 well Unskirted qPCR Plate. Add diluted cDNA templates to respective wells.
  - c. Cover the qPCR plate with optical flat 8-cap strips and centrifuge at 600  $\times$  g for 1 min.
  - d. Follow Table 12 to set up the PCR program in a real-time PCR machine.
  - e. See Figure 4 for representative relative expression levels of *Il13*, *Ifng*, and *Il17* in CD4<sup>+</sup> T cells after differentiation for 4 days.

**Alternatives:** Here we used two-step RT-PCR. Alternatively, a one-step RT-PCR assay may be used to save time and reagent cost.

### Evaluate T-cell differentiation by cytokine expression assays via flow cytometry

⌚ Timing: 7–7.5 h

This step contains information on fixation, permeabilization, antibody staining for different cytokines in differentiated CD4<sup>+</sup> T cells by flow cytometry.

42. Cell stimulation
  - a. Dilute the 500 $\times$  Cell Stimulation Cocktail (plus protein transport inhibitors) in the cultures to 1 $\times$ .
  - b. Put the cultures back to CO<sub>2</sub> incubator and incubate for 4 h.
  - c. Collect the cells by centrifuge at 400  $\times$  g for 5 min at 4°C and discard the supernatant.

**Table 8. Th17 culture recipe (suboptimal condition)**

Component	Stock concentration	Final concentration	Volume per sample
PMA	100 nM	1 nM	5 $\mu$ L
ConA	50 $\mu$ g/mL	0.5 $\mu$ g/mL	5 $\mu$ L
Ionomycin	25 $\mu$ M	250 nM	5 $\mu$ L
$\alpha$ CD28	500 $\mu$ g/mL	1 $\mu$ g/mL	1 $\mu$ L
Human rIL-2	500 ng/mL	5 ng/mL	5 $\mu$ L
Human rTGF- $\beta$	200 ng/mL	2 ng/mL	5 $\mu$ L
Mouse rIL-6	2 $\mu$ g/mL	20 ng/mL	5 $\mu$ L
Mouse rIL-23	1 $\mu$ g/mL	10 ng/mL	5 $\mu$ L

**Table 9. cDNA mix 1**

Component	Stock concentration	Amount per sample
Total RNA	n/a	1 µg (Volume may vary among samples)
oligo(dT) <sub>20</sub>	50 µM	1 µL
dNTP Mix	10 mM	1 µL
Distilled water	n/a	To 12 µL

d. Resuspend the cell pellet in 300 µL of FACS buffer and transfer to each allocated well of a 96-well round bottom plate and centrifuge the plate at 400 × g for 5 min at 4°C. Discard the supernatant completely.

43. Live/Dead cell staining and surface marker staining

a. During the spin, prepare Live/Dead Fixable Viability Dye eFluor 780 staining solution by diluting 1 µL of original solution with 2 mL of 1 × PBS (pH 7.4).

**Note:** During the following steps, cells have to be protected from light.

b. Resuspend the cell pellet with 100 µL of diluted Live/Dead Fixable Viability Dye eFluor 780 and incubate 30 min at 4°C. Keep extra cells for other compensation single staining.

△ **CRITICAL:** Surface marker staining should be performed here with Live/Dead cell staining before fixation as fixatives can cause antigen epitope structures to be altered, which might render the antibodies unable to bind to their targets. This effect depends on the clone of antibody you're using. The antibodies we used are compatible with fixation and the surface markers staining can be performed with intracellular cytokines staining after fixation. Check this website (<https://www.biolegend.com/en-us/fixation>) or consult the literature to confirm whether the antibodies you used are compatible with fixation.

c. Wash twice by adding 300 µL of FACS buffer, centrifuge at 400 × g for 5 min at 4°C and discard the supernatant.

44. Fixation and permeabilization

a. Resuspend cells in 300 µL Fixation/permeabilization solution per 1 million cells. Mix well to dissociate the cell pellet and prevent cross-linking of individual cells.

b. Fix for 30 min at 4°C.

45. Immunostaining

a. Wash twice by adding 300 µL of 1 × Perm/Wash solution, centrifuge at 400 × g for 5 min at 4°C and discard the supernatant.

b. During the spin, prepare multiple staining antibody solution as shown in Table 13 and single staining solution.

c. Resuspend the cell pellet in the well for multiple staining with 100 µL of antibody mix solution shown in Table 13. Resuspend the cell pellets in the wells for single staining with 100 µL of respective single staining solution. Resuspend the cell pellet in the well for no staining and Fixable Viability Dye staining with 100 µL of 1 × Perm/Wash solution. Incubate for 30 min at 4°C.

d. Wash twice by adding 300 µL of 1 × Perm/Wash solution, centrifuge at 400 × g for 5 min at 4°C and discard the supernatant.

**Table 10. cDNA mix 2**

Component	Stock concentration	Amount per sample
First-Strand Buffer	5 X	4 µL
DTT	0.1 M	2 µL
RNaseOUT™ Inhibitor	40 units/µL	1 µL
SuperScript™ III RT	200 units/µL	1 µL

**Table 11. PCR reaction mix**

Component	Stock concentration	Amount per sample
TaqMan® Fast Universal PCR Master Mix (2X)	2x	10 µL
TaqMan® primers with probe	20 x	1 µL
cDNA template + RNase-free water	10 ng/µL	9 µL
<b>Total</b>	n/a	20 µL

e. Resuspend the cell pellet in 300 µL of FACS buffer. Passage the cell suspension through the 40 µm cell strainer on top of the 5 mL Falcon® tube before analysis with Fortessa 405/488/561/640 (BD Biosciences) flow cytometer.

f. See [Figure 5](#) for the gating strategy of cytokines analysis in CD4<sup>+</sup> T cell with Flowjo software.

**Alternatives:** Splenocytes or OneComp eBeads™ (01-1111-42, Invitrogen) can be used for compensation. If OneComp eBeads™ are used, a mouse, rat or hamster origin antibody with APC-Cy7 labeling should be used for compensation of Fixable Viability Dye eFluor 780.

**△ CRITICAL:** Always keep the plate on ice during the staining procedure and use a centrifuge set at 4°C.

### Evaluate the level of p-STAT5 in differentiated CD4<sup>+</sup> T cells by flow cytometry

⌚ Timing: 4–4.5 h

This step contains information on fixation, permeabilization, antibody staining for phosphorylated STAT5 in differentiated CD4<sup>+</sup> T cells by flow cytometry.

#### 46. Live/Dead cell staining

- Take  $1 \times 10^6$  cells and fix them with 4% PFA for 10 min on ice to generate a positive cell control for dead cells staining.
- Wash twice by adding 300 µL of FACS buffer, centrifuge at  $400 \times g$  for 5 min at 4°C and discard the supernatant.
- Mix fixed cells with  $1 \times 10^6$  unfixed cells and use this sample as a single-stained cell compensation control for the Fixable Viability Dye.
- Transfer  $1 \times 10^6$  cells to each labeled 1.5 mL centrifuge tube and centrifuge the sample at  $400 \times g$  for 5 min at 4°C. Discard the supernatant completely.

**Note:** To avoid excessive cell loss, we used 1.5 mL centrifuge tube instead of 96-well round bottom plate in this section.

- During the spin, prepare Live/Dead Fixable Viability Dye eFluor 780 staining solution by diluting 1 µL of original solution with 2 mL of  $1 \times$  PBS (pH 7.4).

**Note:** During the following steps, cells have to be protected from light.

- Resuspend the cell pellet in the tube for multiple staining and single staining compensation control for the Fixable Viability Dye with 100 µL of diluted Live/Dead Fixable Viability Dye

**Table 12. PCR program**

Step	Temperature	Time	Cycles
Hot start	95°C	3 min	1
Denaturation	95°C	10 s	40
Annealing + Extension	60°C	30 s	
Hold	4°C	∞	1

**Table 13. Antibody mix for the cytokines staining of CD4<sup>+</sup> T cells**

Fluorophore	Marker	Final dilution	Volume per sample
PE-Cy7	CD45	1/200	0.5 $\mu$ L
APC	CD4	1/200	0.5 $\mu$ L
FITC	IFN- $\gamma$	1/200	0.5 $\mu$ L
PE	IL-13	1/200	0.5 $\mu$ L
PerCP-Cy5.5	IL-17	1/200	0.5 $\mu$ L
1 $\times$ Perm/Wash solution	n/a	n/a	97.5 $\mu$ L
<b>Total</b>	<b>n/a</b>	<b>n/a</b>	<b>100 <math>\mu</math>L</b>

eFluor 780. Resuspend the cell pellets in other tubes with 1  $\times$  PBS (pH 7.4) and incubate 30 min at 4°C.

- g. Wash twice by adding 300  $\mu$ L of FACS buffer, centrifuge at 400  $\times$  g for 5 min at 4°C and discard the supernatant.

#### 47. Fixation

- a. Resuspend cells in 100  $\mu$ L 4% formaldehyde per 1 million cells. Mix well to dissociate the cell pellet and prevent cross-linking of individual cells.
- b. Fix for 30 min at 4°C.

**Note:** Due to the nature of fixatives, they can cause antigen epitope structures to be altered, which might render the antibodies unable to bind to their targets. Surface marker staining before fixation is suggested. This effect depends on the clone of antibody you're using. The antibodies we used are compatible with fixation and the surface markers staining can be performed with intracellular cytokines staining after fixation. Check this website (<https://www.biolegend.com/en-us/fixation>) or consult the literature to confirm whether the antibodies you used are compatible with fixation.

#### 48. Permeabilization

- a. Permeabilize cells by slowly adding ice-cold 100% methanol to pre-chilled cells while gently vortexing to a final concentration of 90% methanol.
- b. Permeabilize for 45 min on ice.

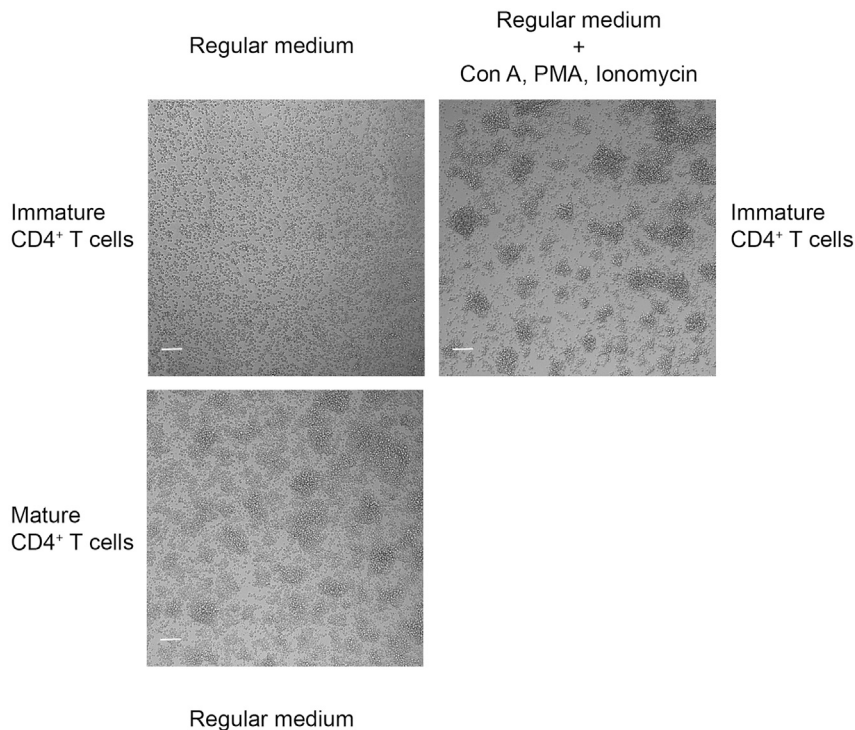
**▮▮ Pause point:** Proceed with immunostaining or store cells at –20°C in 90% methanol.

#### 49. Immunostaining

- a. Wash twice by adding 300  $\mu$ L of FACS buffer, centrifuge at 400  $\times$  g for 5 min at 4°C and discard the supernatant.
- b. During the spin, prepare multiple staining antibody solution as shown in Table 14 and single staining solution.
- c. Resuspend the cell pellet in the well for multiple staining with 100  $\mu$ L of antibody mix solution. Resuspend the cell pellets in the wells for single staining with 100  $\mu$ L of respective single staining solution. Resuspend the cell pellet in the well for no staining and Fixable Viability Dye staining with 100  $\mu$ L of FACS buffer. Incubate for 30 min at 4°C.

**Table 14. Antibody mix for the p-STAT5 staining of CD4<sup>+</sup> T cells**

Fluorophore	Marker	Final dilution	Volume per sample
PE-Cy7	CD45	1/200	0.5 $\mu$ L
FITC	p-STAT5	1/200	0.5 $\mu$ L
APC	CD4	1/200	0.5 $\mu$ L
FACS buffer	n/a	n/a	98.5 $\mu$ L
<b>Total</b>	<b>n/a</b>	<b>n/a</b>	<b>100 <math>\mu</math>L</b>



**Figure 3. Representative images of P21 and adult mouse CD4<sup>+</sup> T cells cultured under Th0 condition with/without Con A, PMA and ionomycin for 4 days**

Scale bar, 100  $\mu$ m.

- d. Wash twice by adding 300  $\mu$ L of FACS buffer, centrifuge at 400  $\times$  g for 5 min at 4°C and discard the supernatant.
- e. Resuspend the cell pellet in 300  $\mu$ L of FACS buffer. Passage the cell suspension through the 40  $\mu$ m cell strainer on top of the 5 mL Falcon® tube before analysis with Fortessa 405/488/561/640 (BD Biosciences) flow cytometer.
- f. See [Figure 6](#) for the gating strategy of p-STAT5 analysis in CD4<sup>+</sup> T cell with Flowjo software.

**Alternatives:** Splenocytes or OneComp eBeads™ (01-1111-42, Invitrogen) can be used for compensation. If OneComp eBeads™ are used, a mouse, rat or hamster origin antibody with APC-Cy7 labeling should be used for compensation of Fixable Viability Dye eFluor 780.

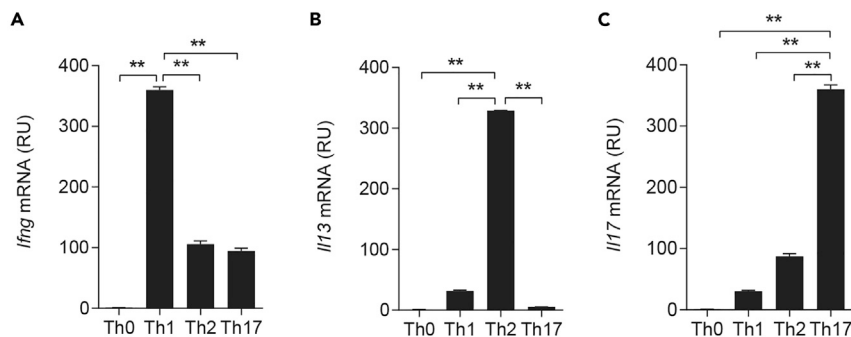
**△ CRITICAL:** Always keep the plate on ice during the staining procedure and use a centrifuge set at 4°C.

### EXPECTED OUTCOMES

If successful, using the gating schema displayed in [Figure 2B](#), over 95% of live cells after purification should be naïve CD4<sup>+</sup> T cells. Complete activation of T cells results in CD25 (Interleukin-2 receptor alpha chain) expression ([Schlegel, 1997](#)). CD44 localizes in lipid rafts with the T cell receptor (TCR) complex and is also up-regulated early after TCR engagement ([DeGrendele et al., 1997](#)). Therefore, a large majority of T cells (~98%) after isolation should be CD25<sup>-</sup> and CD44<sup>-</sup> compared to a significant number of CD25<sup>+</sup> and CD44<sup>+</sup> cells before purification. L-selectin, also known as CD62L, is a cell adhesion molecule found on naïve T cells ([Goldstein et al., 1989](#)). Over 95% of cells after purification should be CD62L<sup>+</sup>.

After 4 days culture under Th0 condition, immature CD4<sup>+</sup> T cells from P21 mice with Con A, PMA and ionomycin stimulation should proliferate very well and form big clusters ([Figure 3](#)) like mature CD4<sup>+</sup>





**Figure 4. Representative results of relative expression level of signature cytokines in differentiated CD4<sup>+</sup> T cells of neonatal C57BL/6J mouse**

(A) Representative result of *Ifng* mRNA level in differentiated CD4<sup>+</sup> T cells of neonatal C57BL/6J mouse.

(B) Representative result of *Il13* mRNA level in differentiated CD4<sup>+</sup> T cells of neonatal C57BL/6J mouse.

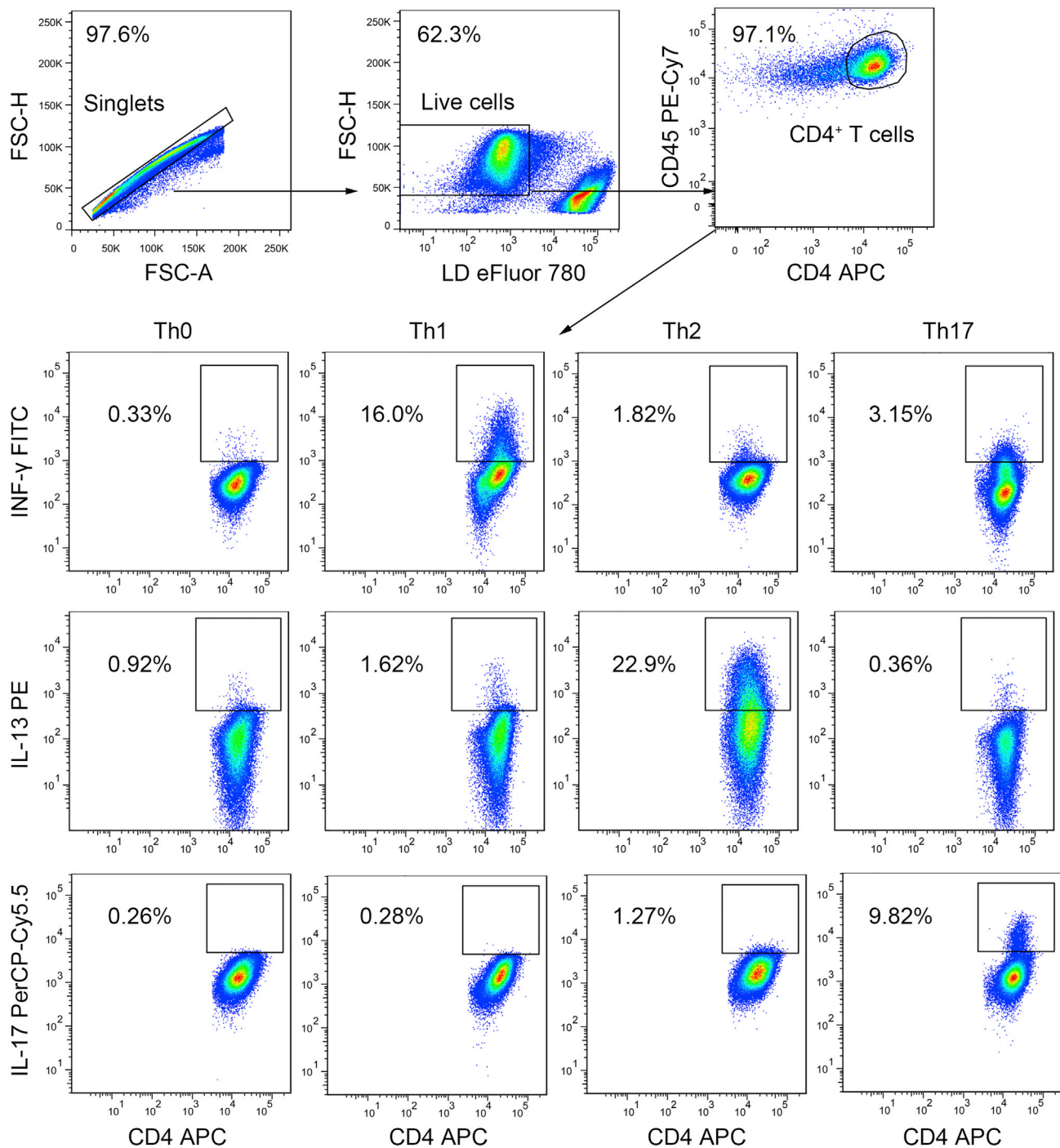
(C) Representative result of *Il17* mRNA level in differentiated CD4<sup>+</sup> T cells of neonatal C57BL/6J mouse. Data are represented as mean ± SEM.

T cells from adult mice without Con A, PMA and ionomycin (Figure 3). Immature CD4<sup>+</sup> T cells from P21 mouse without Con A, PMA and ionomycin stimulation couldn't proliferate and failed to form big clusters (Figure 3).

During TCR activation and in the presence of a particular cytokine milieu, naïve CD4<sup>+</sup> T cells differentiate into one of several lineages of T helper (Th) cells, including Th1, Th2, Th17, and iTreg, that can be defined by signature cytokine production and their unique function. Th1 cells make IFN- $\gamma$  as their signature cytokine. Th2 cells predominately produce IL-4, IL-5, and IL-13 (Killar et al., 1987; Mosmann et al., 1986). Th17 cells are characterized by the production of IL-17A, IL-17F, and IL-22 as signature cytokines (Aggarwal et al., 2003). After 4 days in culture, neonatal mouse CD4<sup>+</sup> T cells should proliferate well and form clusters after adding low dosages of PMA, ConA and Ionomycin. Under the listed culture conditions in Tables 5, 6, 7, and 8, suboptimal Th1, Th2 and Th17 differentiation should be achieved. As shown in Figure 4, robust *Ifng*, *Il13* and *Il17* mRNA elevation should be detected by q-PCR in Th1, Th2 and Th17 culture conditions, respectively. As shown in Figure 5, a substantial IFN- $\gamma$ , IL-13 and IL-17 positive population should also be detected by flow cytometry in Th1, Th2 and Th17 culture conditions, respectively. However, since the differentiation condition in our assays is suboptimal, spontaneous differentiation of naïve CD4<sup>+</sup> T cells may occur at a low level. As such, signature genes of other types of Th cells may be found in a specific differentiation condition. For example, *Il13* expression may be detectable in Th1 culture, since the anti-IL-4 antibody is not added to the Th1 culture to completely block Th2 differentiation (Figure 4). A small portion of cells from Th17 condition are also IFN- $\gamma$  positive as detected by flow cytometry as INF- $\gamma$  antibody was not added to Th17 condition (Figure 5).

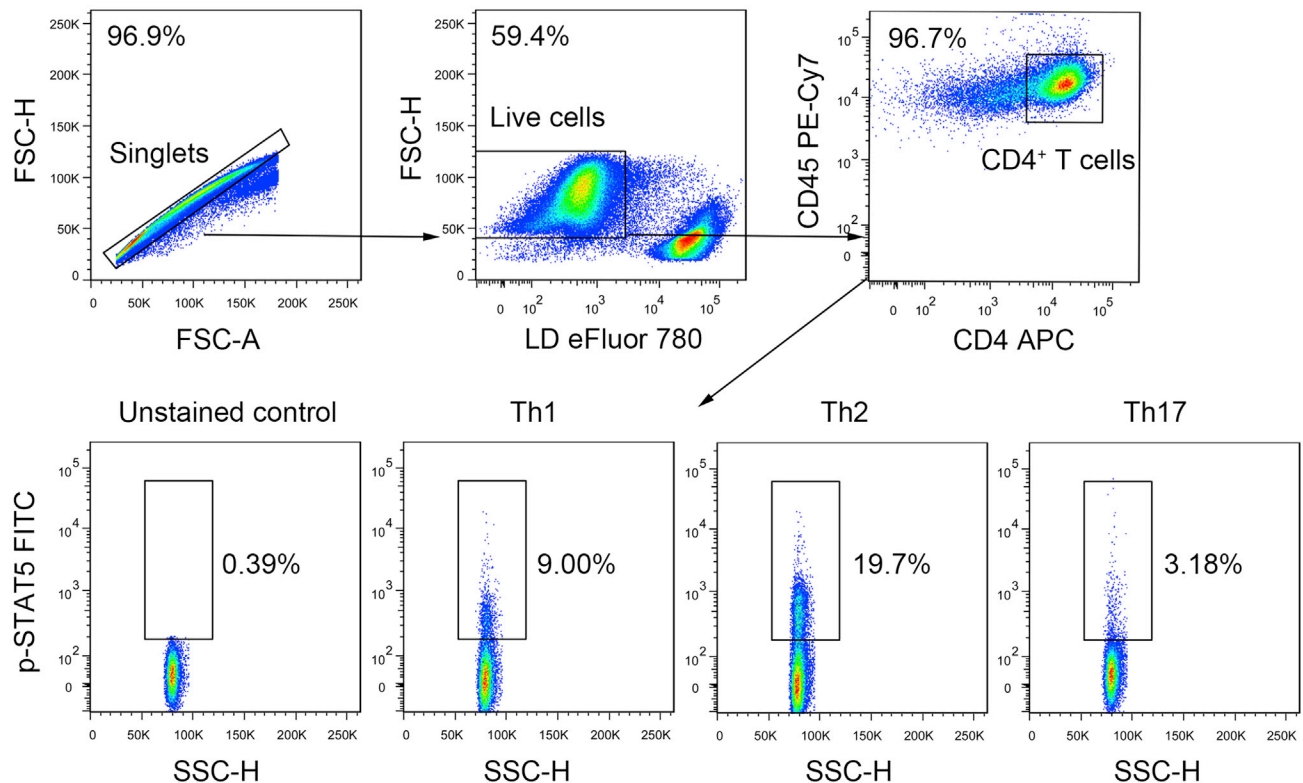
IL-2 promotes T cell proliferation and Th2 differentiation by transcriptionally activating the expression of the *Il2ra*, *Il4*, and *Il4ra* genes via phosphorylating STAT5 (Cote-Sierra et al., 2004; Zhu et al., 2003). The differentiated Th2 cells can produce more IL-2 to maintain the proliferation and enhance the differentiation (Zhu et al., 2010). After 4 day's differentiation under Th2 condition, a robust signal of phosphorylated STAT5 should be detected in live CD4<sup>+</sup> T cell singlets (Figure 6). IL-2 signaling via STAT5 activation can also potentiate the Th1 fate by inducing IL12R $\beta$ 2 and T-bet expression, thereby allowing the cell to respond to IL-12 and polarize toward the Th1 fate (Liao et al., 2011). IL-2/STAT5 signaling can also affect Th17 development by downregulating expression of the IL6R, which is required to activate STAT3 (Laurence et al., 2007). Therefore, some levels of phosphorylated STAT5 can also be detected in Th1 cells, but very few phosphorylated STAT5 positive cells can be detected in Th17 cells (Figure 6).

By slight modification, this protocol is adaptable to the establishment of primary cultures of human naïve CD4<sup>+</sup> T cells isolated from lymph nodes associated with lung samples from donors under



**Figure 5. Gating strategy for analysis of signature cytokines in neonatal mouse T helper cells cultured for 4 days**

13 years of age. These modifications include the method of enzymatic dissociation of the dissected lymph nodes and the antibodies against specific human epitopes. For dissociation, lymph nodes are digested with 400 U/mL Collagenase Type 4 (LS004188, Worthington-Biochem), 0.1% Dispase II (4942078001, Sigma-Aldrich) and 20 mg/mL DNase I (439807, NEB) for 30 min at 37°C. After enzymatic digestion, the sample is subjected to mechanical dissociation by pushing through a syringe with 18-gauge needle 10 times before passage through 70  $\mu$ m cell strainer. Naïve CD4<sup>+</sup> T cells



**Figure 6. Gating strategy for analysis of phosphorylated STAT5 positive population in neonatal mouse T helper cells cultured for 4 days**

are enriched using a human naïve CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotec, 130-094-131). The 48-well plate was pre-coated with 2 µg/mL  $\alpha$ hCD3 (eBioscience, 16-0037-85). To induce Th2 differentiation, naïve CD4<sup>+</sup> T cells are cultured in the presence of 2 µg/mL  $\alpha$ hCD28, 5 ng/mL rhIL-2 (R&D Systems, 202-IL-010) and 10 ng/mL rhIL-4 (BioLegend, 204-IL-010). All the other reagents, including ConA, PMA, and ionomycin, should be added similarly as in the primary culture of immature mouse CD4<sup>+</sup> T cells. The Th2 differentiation is similar to mice samples.

## LIMITATIONS

This protocol provides the basics to neonatal mouse CD4<sup>+</sup> T cell culture and differentiation *in vitro*. We only cultured naïve CD4<sup>+</sup> T cells from mice as early as P21 (Wang et al., 2019). Younger CD4<sup>+</sup> T cells culture were never tested in our system and adjustments may be needed to achieve good culture for younger CD4<sup>+</sup> T cells.

Here, we only tested signature cytokines production and phosphorylated STAT5 in the nuclei by flow cytometry. Other function assays, including cell proliferation and transcription factor activation, can also be performed using this protocol with slight adjustments.

## TROUBLESHOOTING

### Problem 1

The purity of naïve CD4<sup>+</sup> T cells after enrichment is low (step 31).

### Potential solution

A potential reason for this problem might be that the number of splenocytes is not accurately determined such that there are more cells than the capacity of the added antibody cocktail and magnetic

beads. To improve the efficacy of naïve CD4<sup>+</sup> T cell purification, please ensure that cell counting is accurate. In addition, 10% more of the antibody cocktail and magnetic beads than the recommended amount in this protocol may be used.

### Problem 2

There aren't many T cell clusters formed in culture and the majority of the cells are dead during the process of T cell differentiation (step 38).

### Potential solution

The potential reason for this problem might be that cell number determination for naïve CD4<sup>+</sup> T cells before seeding is not correct and the cell density is too low. Activated T cells expand rapidly at a high cell density but undergo apoptosis at a low cell density (Ma et al., 2010). A better counting method should be adapted to accomplish an accurate cell density at seeding. The final cell density should be around  $1 \times 10^6$  cells/mL. If the total cell number is too low, a flat 96-well plate can be used instead of the 48-well plate. Another potential reason for this problem might be technical issues with CO<sub>2</sub> incubator. Make sure the temperature and the CO<sub>2</sub> concentration is correct. Unproper storage (e.g., repeating freeze/thaw) can also lead to culture failure.

### Problem 3

The yield and the quality of the extracted RNA are low (step 40a).

### Potential solution

The concentration of the extracted RNA tested by Nanodrop should be over 100 ng/μL. The potential reason for low concentration might be that cultured CD4<sup>+</sup> T cells are not healthy and majority of them are dead or dying. Check the potential solution for problem 2 to make sure the culture is in good condition before RNA isolation. Another potential reason for this problem might be that the final cell number for RNA extraction is too low. In case of a low cell number (such as in 96-well culture condition), use PicoPure RNA isolation kit (KIT0204, Thermo Fisher) to extract RNA. The PicoPure® RNA Isolation Kit is designed to recover high-quality total RNA consistently from fewer than ten cells, even from a single cell. The OD260/OD280 ratio tested in Nanodrop should be around 1.9–2.1. If it is too high, there may be protein contamination. Increasing Buffer RW1 washing may help. If it is too low, there may be RNA degradation. RNase decontamination, for example, using RNase free water and 1.5 mL tubes, is suggested.

### Problem 4

The relative expression level of signature cytokine genes is low (step 41e).

### Potential solution

The potential reason for this problem might be that the purity of naïve CD4<sup>+</sup> T cells is low and the sample is contaminated with already differentiated T cells that may affect *in vitro* differentiation. Check the potential solution for problem 1 to make sure the purity of naïve CD4<sup>+</sup> T cells is over 95%. Respective antibodies can also be added to the cultures to achieve better differentiation. Another potential reason for this problem might be that the quality of the extracted RNA is poor. Check the potential solution for problem 3 to make sure high quality of the extracted RNA. Unproper storage (e.g., repeating freeze/thaw) can also lead to culture failure.

### Problem 5

There are few cells left after wash and spin before the detection of p-STAT5 by flow cytometry (step 49d).

### Potential solution

The potential reason for this problem might be that too many cells get lost during wash and spin. The solution is to increase the initial cell number for staining and/or increase the spinning speed to ensure that all the cells are pelleted down.

### Problem 6

The signal of phosphorylated STAT5 is too weak (step 49e).

#### Potential solution

The potential reason for this problem might be that fixation and cell permeabilization are not ideal. The solution to this problem may be to try different fixation and permeabilization methods. These include the intracellular staining kit with saponin (554722 and 554723, BD Biosciences) and transcription factor staining buffer set (00–5523, Invitrogen). In our hands, fixation with 4% PFA followed by methanol permeabilization generates the best staining result.

### Problem 7

The separation between p-STAT5 positive and negative population is not clear (step 49e).

#### Potential solution

The potential reason for this problem might be that dead cells are not excluded. Dead cells have high autofluorescence and will lead to false positivity, which may make the identification of weakly positive samples and rare populations difficult. Adding the Fixable Viability Dye to the staining panel, as we have described, will help reduce the background and make the separation clearer. Another potential reason is the antibody concentration is not optimal. Antibody titration will help to solve this problem.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xingbin Ai ([xai@partners.org](mailto:xai@partners.org)).

### Materials availability

This study did not generate new unique reagents

### Data and code availability

This study did not generate or analyze any datasets.

## ACKNOWLEDGMENTS

We would like to thank the MGH Pathology, Flow, Image and Mass Cytometry Cores for providing training, support, and access to the instruments. Research in the authors' laboratory is supported by NIH grant (1R01HL132991) to X.A.

## AUTHOR CONTRIBUTIONS

Conceptualization, W.W. and X.A.; methodology, W.W.; investigation, W.W.; writing –original draft, W.W.; writing – review & editing, X.A.; funding acquisition, X.A.; supervision, X.A.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

- Aggarwal, S., Ghilardi, N., Xie, M.H., de Sauvage, F.J., and Gurney, A.L. (2003). Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* *278*, 1910–1914.
- Cote-Sierra, J., Foucras, G., Guo, L., Chiodetti, L., Young, H.A., Hu-Li, J., Zhu, J., and Paul, W.E. (2004). Interleukin 2 plays a central role in Th2 differentiation. *Proc. Natl. Acad. Sci. U S A* *101*, 3880–3885.
- DeGrendele, H.C., Kosfisz, M., Estess, P., and Siegelman, M.H. (1997). CD44 activation and associated primary adhesion is inducible via T cell receptor stimulation. *J. Immunol.* *159*, 2549–2553.
- Goldstein, L.A., Zhou, D.F., Picker, L.J., Minty, C.N., Bargatze, R.F., Ding, J.F., and Butcher, E.C. (1989). A human lymphocyte homing receptor, the hermes antigen, is related to cartilage proteoglycan core and link proteins. *Cell* *56*, 1063–1072.
- Killar, L., MacDonald, G., West, J., Woods, A., and Bottomly, K. (1987). Cloned, Ia-restricted T cells that do not produce interleukin 4(IL 4)/B cell stimulatory factor 1(BSF-1) fail to help antigen-specific B cells. *J. Immunol.* *138*, 1674–1679.
- Laurence, A., Tato, C.M., Davidson, T.S., Kanno, Y., Chen, Z., Yao, Z., Blank, R.B., Meylan, F., Siegel, R., Hennighausen, L., et al. (2007). Interleukin-2

signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26, 371–381.

Liao, W., Lin, J.X., Wang, L., Li, P., and Leonard, W.J. (2011). Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nat. Immunol.* 12, 551–559.

Ma, Q., Wang, Y., Lo, A.S., Gomes, E.M., and Junghans, R.P. (2010). Cell density plays a critical role in ex vivo expansion of T cells for adoptive immunotherapy. *J. Biomed. Biotechnol.* 2010, 386545.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and Coffman, R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136, 2348–2357.

Schlegel, P.G. (1997). The role of adhesion and costimulation molecules in graft-versus-host disease. *Acta Haematol.* 97, 105–117.

Stritesky, G.L., Yeh, N., and Kaplan, M.H. (2008). IL-23 promotes maintenance but not commitment to the Th17 lineage. *J. Immunol.* 181, 5948–5955.

Wang, W., Cohen, J.A., Wallrapp, A., Trieu, K.G., Barrios, J., Shao, F., Krishnamoorthy, N., Kuchroo, V.K., Jones, M.R., Fine, A., et al. (2019). Age-related dopaminergic innervation augments T helper 2-type allergic inflammation in the postnatal lung. *Immunity* 51, 1102–1118 e1107.

Zhu, J., Cote-Sierra, J., Guo, L., and Paul, W.E. (2003). Stat5 activation plays a critical role in Th2 differentiation. *Immunity* 19, 739–748.

Zhu, J., Yamane, H., and Paul, W.E. (2010). Differentiation of effector CD4 T cell populations (\*). *Annu. Rev. Immunol.* 28, 445–489.