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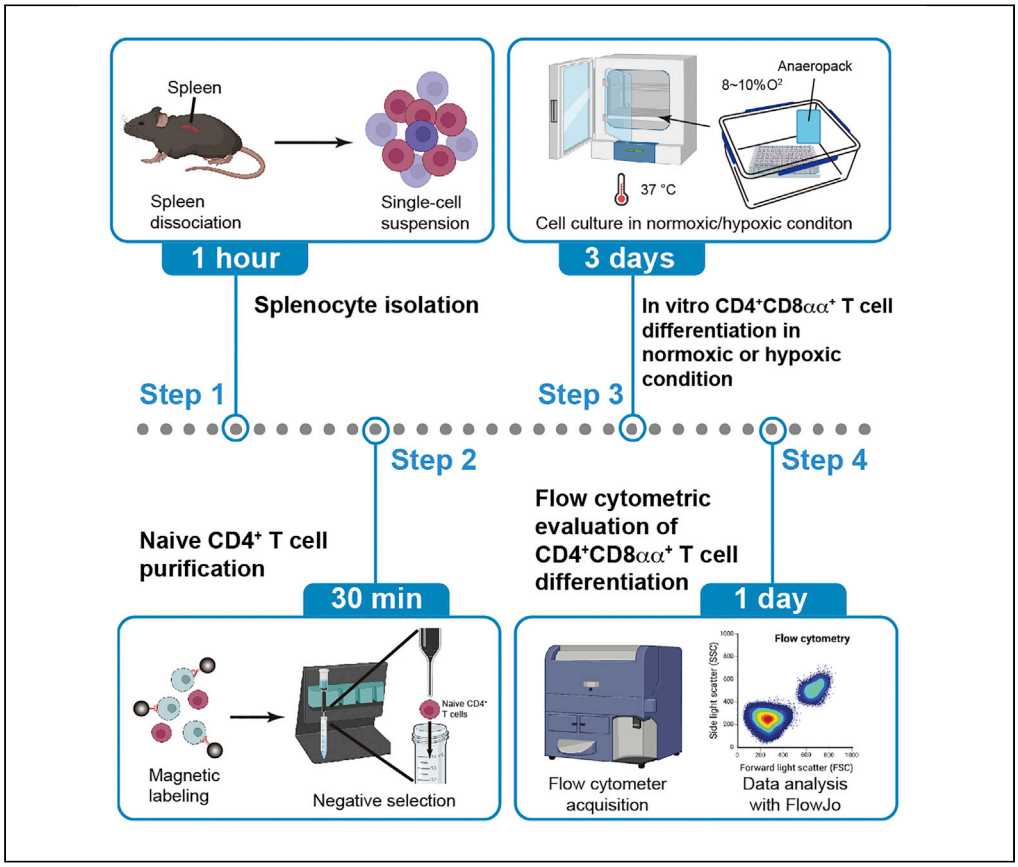
Protocol to isolate and enrich mouse splenic naive CD4$^+$ T cells for *in vitro* CD4$^+$CD8αα$^+$ cell induction



Recent studies have shown that CD4$^+$CD8αα$^+$ T cells are induced in the hypoxic environment of the small intestinal epithelium. Herein, we describe a protocol for CD4$^+$CD8αα$^+$ T cell induction from freshly isolated naive CD4$^+$ T cells, including procedures for the isolation and enrichment of mouse splenic T cells. In addition, we present an approach that can induce more CD4$^+$CD8αα$^+$ T cells by artificially creating a hypoxic environment *in vitro*.

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

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Highlights

Protocol for CD4$^+$CD8αα$^+$ T cell induction from freshly isolated naive CD4$^+$ mouse T cells

Increased CD4$^+$CD8αα$^+$ T cells induction via an artificial hypoxic environment *in vitro*

FACS analysis, purity check for the isolation, and result of CD4$^+$CD8αα$^+$ T cell induction

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Protocol to isolate and enrich mouse splenic naive CD4$^+$ T cells for *in vitro* CD4$^+$CD8αα$^+$ cell induction

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SUMMARY

Recent studies have shown that CD4$^+$CD8αα$^+$ T cells are induced in the hypoxic environment of the small intestinal epithelium. Herein, we describe a protocol for CD4$^+$CD8αα$^+$ T cell induction from freshly isolated naive CD4$^+$ T cells, including procedures for the isolation and enrichment of mouse splenic T cells. In addition, we present an approach that can induce more CD4$^+$CD8αα$^+$ T cells by artificially creating a hypoxic environment *in vitro*.

For complete details on the use and execution of this protocol, please refer to Harada et al. (2022).

BEFORE YOU BEGIN

Preparation of mice

All animal experiments described herein were approved by the Institutional Review Board for Animal Experiments of Keio University and were performed according to the institutional guidelines and home office regulations.

C57BL6/J mice were used for spleen collection. Both female and male mice were used for this study and were aged between 6 and 12 weeks at the time of experiments.

Preparation of cell culture plates and surgical tools

Timing: 20 min; antibody coating: 16 h

1. 96-well plate preparation for naive CD4$^+$ T cell culture.
   a. Dilute anti-CD3ε antibody (3 mg/mL) at 1: 3,000 with sterile DPBS (1 ×, pH 7.4) and pipet 50 μL in each well of 96-well flat-bottom plate.
   b. Place the plate in the refrigerator (2°C–8°C) for 16 h.

   Note: DPBS should be kept ice-cold, and the coating solution should be prepared in polypropylene tube.

   Alternatives: The coating can also be done by incubating at 37°C in a cell incubator for 2–3 h.

2. Surgical tool for harvesting spleen.

Prepare a pair of fine scissors and 2 pair of forceps for harvesting spleen.

Preparation of hypoxic culture condition using Anaeropack

Timing: 20 min; equilibration: 16 h

3. Preparation of hypoxic culture condition with airtight container and Anaeropack.

Place an Anaeropack and culture medium in 50 mL tube in a Rectangular jar (see key resources table) for 16 h at 37°C until the day of T cell culture.

Note: Anaeropack is a disposable O_2-absorbing and CO_2-generating agent to reduce the O_2 concentration to 8%–10% and to keep the CO_2 concentration at approximately 5% (hypoxic condition). Rectangular jar for Anaeropack is a sealed airtight container and is also available from same company.

Preparation of cell culture media and solutions

Timing: 1 h

Prepare the buffers and antibodies cocktails as described in the "materials and equipment" section.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD4 (clone: RM4-5; BV421) (dilution 1:200)	BioLegend	Cat# 100544; RRID: AB_11219790
CD8α (clone: 53-6.7; PE-Cy7) (dilution 1:200)	BD Biosciences	Cat# 552877; RRID: AB_394506
CD8β (clone: eBioH35-17.2; APC) (dilution 1:200)	eBioscience	Cat# 17-0083-81; RRID: AB_657760
CD44 (clone: IM7; APC) (dilution 1:200)	BioLegend	Cat# 103012; RRID: AB_312963
CD45 (clone: 30-F11; BV510) (dilution 1:200)	BioLegend	Cat# 103138; RRID: AB_2563061
CD62L (clone: MEL-14; FITC) (dilution 1:200)	BioLegend	Cat# 104406; RRID: AB_313093
TCRβ (clone: H57-597; APC-Cy7) (dilution 1:200)	BioLegend	Cat# 109220; RRID: AB_893624
Foxp3 (clone: FJK-16s; PE) (dilution 1:200)	eBioscience	Cat# 12-5773-82; RRID: AB_465936
CD16/32 (Fc blocker, clone: 2.4G2) (dilution 1:200)	BD Biosciences	Cat# 553142; RRID: AB_394657
CD3ε (clone: 145-2C11) (dilution 1:3000)	BioLegend	Cat# 100359; RRID: AB_2616673
CD28 (clone: 37.51) (dilution 1:1000)	BioLegend	Cat# 102116; RRID: AB_11147170
Chemicals, peptides, and recombinant proteins		
EDTA	Nacalai Tesque	Cat# 06894-85
Ammonium chloride	Nacalai Tesque	Cat# 02424-55
Sodium azide	Wako	Cat# 195-11092
Mouse Naive $CD4^+$ T cell isolation kit	Miltenyi Biotec	Cat# 130-104-453
TGF-β	R&D Systems	Cat# 7666-MB-005
Retinoic acid (RA)	Tokyo Chemical Industry	Cat# 0064-1G
IFN-γ	PeproTech	Cat# 315-05
Fetal bovine serum (FBS)	Thermo Fisher Scientific	Cat# 10270-106
HBSS	Nacalai Tesque	Cat# 17460-15
RPMI1640	Nacalai Tesque	Cat# 30264-85
DPBS	Nacalai Tesque	Cat# 14249-24
Penicillin/streptomycin	Nacalai Tesque	Cat# 09367-34
Sodium pyruvate	Thermo Fisher Scientific	Cat# 11360070
MEM/NEAA	Thermo Fisher Scientific	Cat# 11140050
HEPES	Thermo Fisher Scientific	Cat# 15630080
β-mercaptoethanol	Thermo Fisher Scientific	Cat# 21985023

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Foxp3/transcription factor staining buffer set	eBioscience	Cat# 00-5523-00
AnaeroPack-MicroAero-7L	Mitsubishi Gas Company	Cat# A-29
LS Columns	Miltenyi Biotec	130-042-401
MidiMACS Separator	Miltenyi Biotec	130-042-302
Experimental models: Organisms/strains		
Mouse: C57BL/6J (6–12-week-old male)	The Jackson Laboratory	JAX:000664
Software and algorithms		
GraphPad Prism 8	GraphPad Software	https://www.graphpad.com/
FlowJo Vx software	TreeStar	https://www.flowjo.com/
Other		
96-well Flat-Bottom Microplate	Thermo Scientific	Cat# 167008
96 well Round Bottom Microplate with lid	IWAKI	Cat# 3870-096
15 mL High Clarity polypropylene Centrifuge Tube	Falcon	Cat# 352196
50 mL High Clarity polypropylene Centrifuge Tube	Falcon	Cat# 352070
60-mm plastic dish	IWAKI	Cat# 1010-060
100 μM cell strainer	Greiner	Cat# 542000
1 mL syringe	Terumo	Cat# SS-01T
Rectangular jar (Large)	Mitsubishi Gas Company	Cat# A-112
Oxygen Monitor	JIKCO	Cat# OXY-2
Flow Cytometry System	BD Biosciences	BD FACSCanto II
CO2 incubator	Sanyo	Cat# MCO-18AIC

MATERIALS AND EQUIPMENT

HBSS wash solution		
Reagent	Final concentration	Amount
Penicillin-Streptomycin (100 ×)	1 ×	5 mL
Fetal Bovine Serum	1.5%	7.5 mL
HBSS	N/A	Up to 500 mL
Total	**N/A**	**500 mL**
Store at 4°C for up to 2 months.		

T cell culture medium		
Reagent	Final concentration	Amount
Penicillin-Streptomycin (100 ×)	1 ×	5 mL
Fetal Bovine Serum	10%	50 mL
β-mercaptoethanol	50 μM	500 μL
Sodium pyruvate (100 ×)	1 ×	5 mL
MEM NEAA (100 ×)	1 ×	5 mL
RPMI 1640	N/A	Up to 500 mL
Total	**N/A**	**500 mL**
Store at 4°C for up to 2 months.		

FACS buffer		
Reagent	Final concentration	Amount
Fetal Bovine Serum	2%	10 mL
Sodium azide	0.01%	50 mg
DPBS	N/A	Up to 500 mL
Total	**N/A**	**500 mL**
Store at 4°C for up to 2 months.		

MACS buffer		
Reagent	Final concentration	Amount
Bovine Serum Albumin	0.5%	2.5 g
EDTA (0.5 M)	2 mM	2 mL
DPBS	N/A	Up to 500 mL
Total	**N/A**	**500 mL**
Store at 4°C for up to 2 months.		

RBC lysis buffer		
Reagent	Final concentration	Amount
Ammonium chloride	0.84%	1.68 g
ddH_2O	N/A	Up to 200 mL
Total	**N/A**	**200 mL**
Store at 4°C for up to 2 months.		

Mixed cytokine solution (for 1 well, 4 × concentrated)		
Reagent	Final concentration	Amount
TGF-β (20 ng/mL)	2 ng/mL	20 μL
IFN-γ (200 ng/mL)	20 ng/mL	20 μL
Retinoic acid (10 μM)	10 nM	2 μL
T cell culture medium	N/A	Up to 50 μL
Total	**N/A**	**50 μL**
Prepare on demand. Store at 4°C before use.		

⚠ CRITICAL: β-mercaptoethanol and sodium azide are toxic reagents, so suitable protective equipment are needed and handle with caution.

STEP-BY-STEP METHOD DETAILS

Naive $CD4^+$ T cell isolation from mouse spleen

Timing: 120 min

The following steps provide the procedure of collection of spleen and isolation of naive $CD4^+$ T cells using commercially available kit.

1. Euthanize C57BL/6J mice by cervical dislocation, disinfect with 70% ethanol to prevent contamination.
2. Cut open the abdomen and expose the spleen in the abdominal cavity (Figure 1A).
3. Collect the spleen and place in a petri dish containing 5 mL of HBSS solution on ice (Figure 1B).
4. Put a 100 μm cell strainer on top of a 50 mL conical tube.
5. Place the spleen on the cell strainer and crush with a syringe plunger (Figures 1C and 1D) (add ice-cold HBSS several times to rinse off the cells).
6. Adjust the volume to 30 mL for each tube with ice-cold HBSS solution.
7. Centrifuge (600 × *g*, 5 min, at 4°C). Discard supernatant.
8. Add 2 mL of 0.84% (vol/wt) ammonium chloride on the cell pellet to hemolyze and resuspend by pipetting. Incubate 5 min at RT.
9. Add 20 mL of ice-cold HBSS solution and centrifuge (600 × *g*, 5 min, at 4°C).
10. Remove supernatant and resuspend cell pellet with 10 mL of HBSS solution.
11. Count cell number and transfer 5 × 10^7 cells into new 15 mL conical tube.
12. Fill up volume to 10 mL with ice-cold MACS buffer and centrifuge (600 × *g*, 5 min, at 4°C).

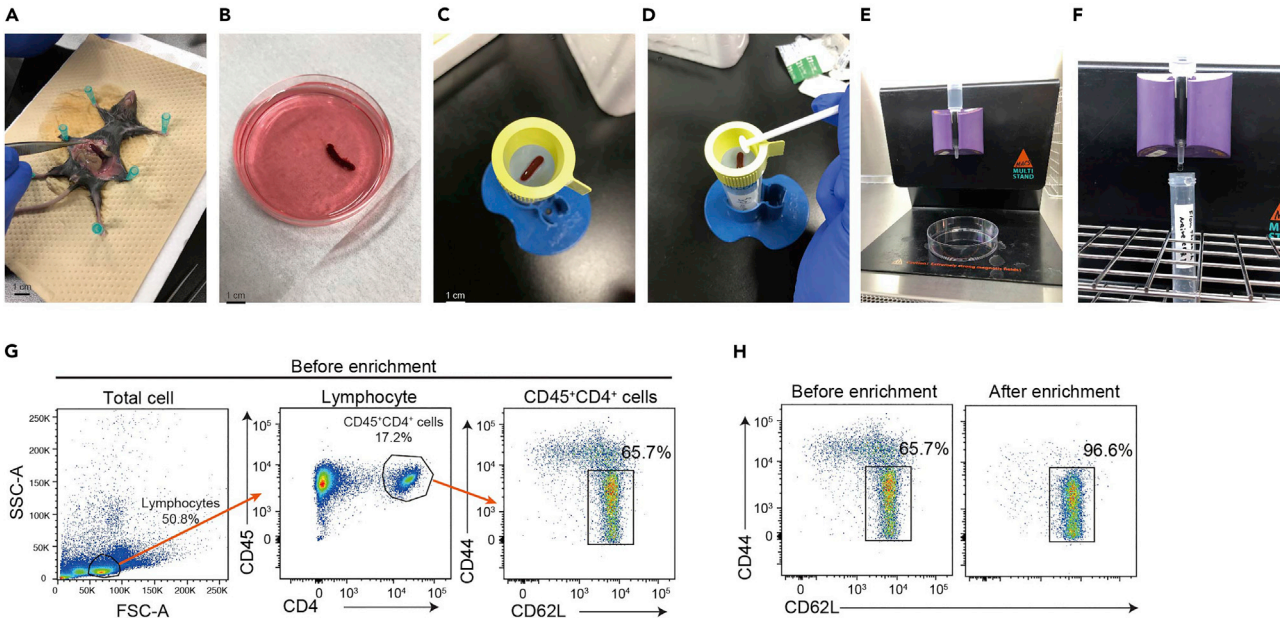

Figure 1. Spleen harvesting and naive CD4$^+$ T cell enrichment

(A) Euthanize C57BL/6J mice according to the institutional guidelines. Collect spleen using scissors and forceps.

(B) Place the spleen in a petri dish containing 5 mL of HBSS solution.

(C) Place the spleen on a 100 μm cell strainer.

(D) Crush the spleen with a syringe plunger.

(E) Place LS column in the magnetic field and rinse the column with 3 mL of MACS buffer.

(F) Collect flow-through containing unlabeled cells.

(G) Representative gating strategy for analysis of CD45$^+$ CD4$^+$ cells before enrichment.

(H) FACS plot showing the expression of CD62L and CD44 before or after enrichment.

13. Resuspend cell pellet in 200 μL of ice-cold MACS buffer.
14. Add 50 μL of Biotin-Antibody Cocktail (included in the isolation kit) and mix well. Incubate for 5 min in the refrigerator (2°C–8°C).

    Note: This cocktail contains biotin-conjugated monoclonal antibodies against CD8α, CD11b, CD11c, CD19, CD25, CD45R (B220), CD49b (DX5), CD105, Anti-MHC class II, Ter-119, and TCRγ/δ to remove unwanted immune cells.

15. Add 100 μL of MACS buffer, 100 μL of Anti-Biotin Microbeads and 50 μL of CD44 Microbeads (both are included the isolation kit).

    Note: By excluding CD44-positive cells in addition to non-CD4 cells at this point, only naive CD4$^+$ cells can be obtained in a single column operation.

16. Mix well and incubate for 10 min in the refrigerator (2°C–8°C).
17. To remove unbound antibodies and microbeads, add 5 mL of ice-cold MACS buffer to cell suspension and centrifuge (600 × *g*, 5 min, at 4°C).
18. Place LS column in the MidiMACS separator and rinse the column with 3 mL of MACS buffer (Figure 1E).
19. Resuspend cell pellet with 1 mL of MACS buffer and apply onto the column. Collect flow-through containing unlabeled cells, representing the enriched naive CD4$^+$ T cells (Figure 1F).
20. Centrifuge (600 × *g*, 5 min, at 4°C). Discard supernatant.
21. Wash with 10 mL of T cell culture medium and centrifuge (600 × *g*, 5 min, at 4°C).

22. Remove supernatant and resuspend cell pellet with 1 mL of T cell medium.
23. Count cell number and adjust 1 × 10^5 cells per 50 μL of T cell medium.
24. To check the quality of the purification, take 50 μL of aliquot and transfer to 5 mL tubes.
25. Wash with 200 μL of FACS buffer and Centrifuge (800 × *g*, 3 min, at 4°C).
26. Discard supernatant and resuspend cells in 50 μL of Fc blocker solution (concentration per antibody: 1:200).
27. Incubate cells for 10 min at 4°C.
28. Add antibody cocktail (anti-CD45, CD4, CD44 and CD62L, concentration per antibody: 1:200) is added to the cells (see key resources table).
29. Incubate cells for 30 min in the dark at 4°C.
30. After incubation, fill up volume to 200 μL with FACS buffer and centrifuge (800 × *g*, 3 min, at 4°C).
31. Remove supernatant and resuspend cell pellet with 400 μL of FACS buffer.
32. Analyze cells on a flow cytometer. Representative gating strategy and an example of FACS analysis of purification is shown in Figures 1G and 1H.

In vitro culture for $CD4^+CD8\alpha\alpha^+$ T cell induction in normoxic or hypoxic condition

Timing: 4 days

This step describes seeding naive $CD4^+$ T cells and stimulation by pre-coated anti-CD3 and soluble anti-CD28 with cytokines to initiate differentiation.

33. Wash pre-coated 96-well flat-bottom plate (see before you begin for details) 3 times with DPBS.
    a. Remove coating solution with a suction (Figure 2A).
    b. Add 100 μL of DPBS to each well using multichannel pipette (Figure 2B).
    c. Remove DPBS with a suction.
    d. Repeat b and c two more times.
    e. In the end, add 100 μL DPBS to keep the pre-coated bottom wet.
34. Remove DPBS from washed 96-well plate right before use.
35. Add 50 μL of enriched naive $CD4^+$ T cell suspension (1 × 10^5 cells) per well onto pre-coated 96-well plate.
36. Add 50 μL of anti-CD28 solution (final concentration: 1 μg/mL diluted with culture medium) to each well.
37. Add 50 μL of mixed cytokine solution to each well.
38. Add 50 μL of T cell culture medium to fill up volume to 200 μL.

Alternatives: The medium added at the end can be a free slot for any compounds or neutralizing antibodies you wish to use. In that case, you can prepare four times the concentration and add 50 μL to the culture.

39. Place the cells into an 5% CO_2 incubator at 37°C for 4 days.
40. For the culture in hypoxic condition, use the medium stored in hypoxic condition to prepare enriched cells, anti-CD28 solution and mixed cytokine solution (Figures 2C and 2D).
41. Put the cells in the airtight container with a new Anaeropack and close immediately.

⚠ CRITICAL: Ensure to use newly opened Anaeropack at the beginning of the incubation. Use hypoxic medium as soon as it is opened.

42. Place the airtight container into an incubator at 37°C for 4 days (Figure 2E).

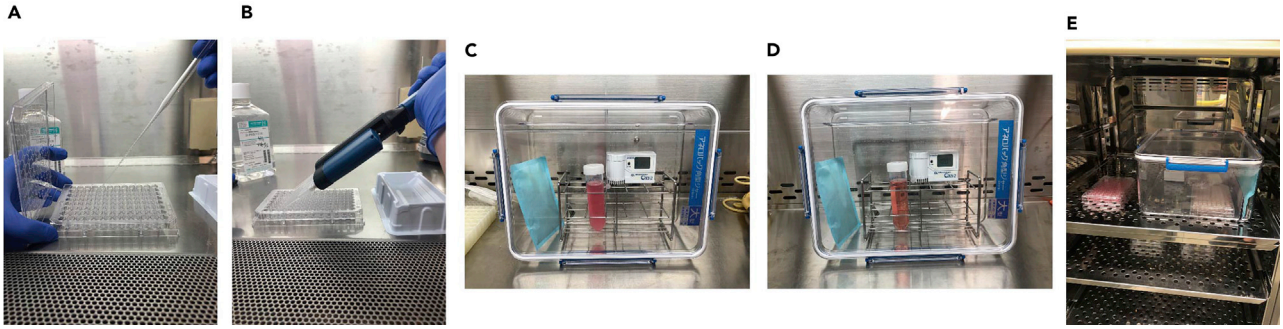

Figure 2. *In vitro* culture for CD4$^+$CD8αα$^+$ T cell induction in normoxic or hypoxic condition

(A) Remove coating solution with a suction.

(B) Add 100 μL of DPBS to each well using multichannel pipette.

(C) Place an Anaeropack and culture medium in 50 mL tube in Rectangular jar.

(D) The medium after 16 h incubation with Anaeropack resulting in hypoxic atmosphere (8%–10% O_2).

(E) Place the airtight container into an incubator at 37°C.

Flow cytometric analysis for CD4$^+$CD8αα$^+$ T cell differentiation

43. Harvest all cells (per well) 4 days after the initiation of culture.
44. Transfer cells into new 96-well U-bottom plate and centrifuge (800 × *g*, 3 min, at 4°C).
45. Discard supernatant and resuspend the cells in 50 μL of Fc blocker solution (see key resources table).
46. Incubate cells for 10 min at 4°C.
47. Add antibody cocktail (anti-CD45, TCRβ, CD4, CD8α and CD8β, concentration per antibody: 1:200 diluted with FACS buffer) to the cells.

    Alternatives: Dead cell contamination can be adequately excluded by gating during the analysis process. Although we did not use Live/Dead cell staining in this protocol, dead cells can be more reliably excluded if used.

48. Incubate cells for 30 min in the dark at 4°C.
49. After incubation, fill up volume to 200 μL with FACS buffer and centrifuge (800 × *g*, 3 min, at 4°C).
50. Discard supernatant and resuspend the cells in 100 μL of fixation buffer (included in Foxp3/transcription factor staining buffer set, see key resources table).
51. Incubate cells for 30 min in the dark at 4°C.
52. Centrifuge the cells (800 × *g*, 3 min, at 4°C).
53. Discard supernatant and resuspend the cells in 200 μL of permeabilization buffer (included in Foxp3/transcription factor staining buffer set, see key resources table).
54. Centrifuge the cells (800 × *g*, 3 min, at 4°C).
55. Discard supernatant and resuspend the cells in 50 μL of anti-Foxp3 solution (concentration: 1:100) diluted with permeabilization buffer.
56. Incubate cells for 1 h in the dark at 4°C.
57. Centrifuge the cells (800 × *g*, 3 min, at 4°C).
58. Discard supernatant and resuspend the cells in 200 μL of FACS buffer.
59. Transfer cells into 5 mL tubes and fill up volume to 400 μL with FACS buffer.
60. Acquire cells on a flow cytometer and analyze data using FlowJo software (Representative gating strategy and results in Figures 3A–3C).

EXPECTED OUTCOMES

Here, we provide a reproducible procedure for the induction of CD4$^+$CD8αα$^+$ T cells *in vitro*.

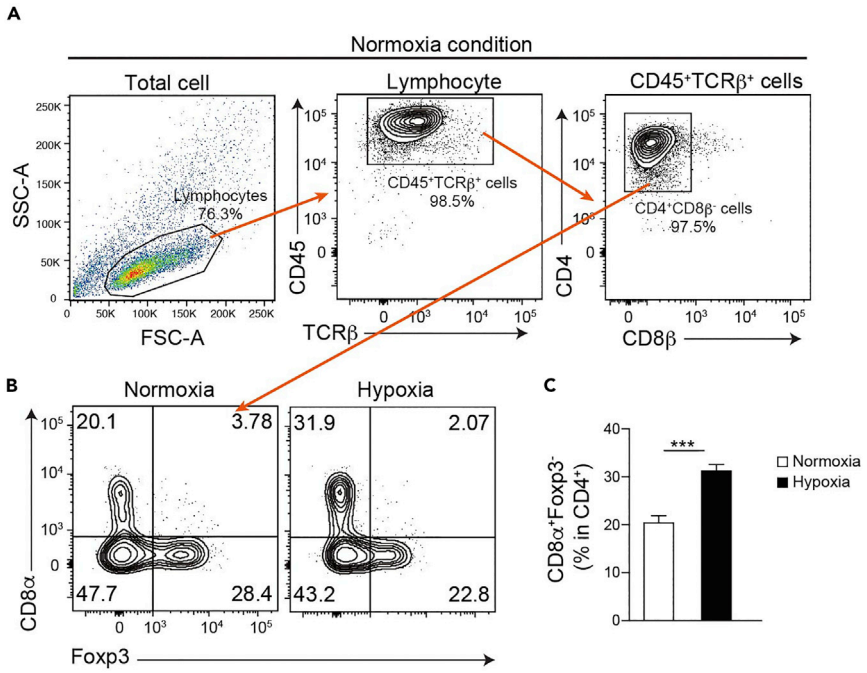

Figure 3. Representative result of CD4$^+$CD8αα$^+$ T cell induction

(A) Representative gating strategy for analysis of CD45$^+$TCRβ$^+$CD4$^+$CD8β$^-$ cells after *in vitro* CD4$^+$CD8αα$^+$cell induction in normoxic condition.

(B and C) Representative flow cytometric figures of surface CD8α and intracellular Foxp3 (A) and frequencies of CD4$^+$CD8αα$^+$ cells (B) among CD45$^+$TCRβ$^+$CD4$^+$CD8β$^-$ cells after *in vitro* CD4$^+$CD8αα$^+$cell induction in normoxic and hypoxic condition. Data adapted from Harada et al. (2022).

Naive T cells express a cell adhesion molecule CD62L on their surface (Goldstein et al., 1989). Using the method presented here, over 95% of the cells should be CD62L$^+$ naive CD4$^+$ T cells after purification (Figure 1G).

CD4$^+$CD8αα$^+$ T cells are induced by aryl hydrocarbon receptor ligands from food or intestinal bacteria and the surrounding environment, such as IL-15, food antigens, and MHC class ll molecules in the intestinal epithelium (Bilate et al., 2016, 2020; Cervantes-Barragan et al., 2017; Mucida et al., 2013). It also has been reported that TGF-β, RA and IFN-γ are required for the differentiation of CD4$^+$CD8αα$^+$ T cells *in vitro* (Konkel et al., 2011; Reis et al., 2014). While it was previously known that induction of the same cells using OT-II cells yields about 15% of CD4$^+$CD8αα$^+$ T cells within CD4$^+$ cells, this protocol succeeds in achieving the same level of differentiation from polyclonal naive CD4$^+$ T cells.

We have previously identified that hypoxic condition induces more CD4$^+$CD8αα$^+$ T cells *in vitro* (Harada et al., 2022). Around 15% of total CD4$^+$ T cells are developed into CD4$^+$CD8αα$^+$ T cells in normoxia condition whereas around 30% of total CD4$^+$ T cells are developed into CD4$^+$CD8αα$^+$ T cells in hypoxia condition (Figures 3A and 3B). More CD4$^+$CD8αα$^+$ T cell differentiation may be obtained by extending the incubation period in either normoxic or hypoxic condition.

LIMITATIONS

This protocol provides the basics of the culture and differentiation of CD4$^+$CD8αα$^+$ T cells from mouse splenic naive CD4$^+$ T cells. The percentage of CD4$^+$CD8αα$^+$ T cells in total CD4 T cells at the end of culture *in vitro* can be changed due to the culture condition, incubation period, reagents. *In vitro* cultured CD4$^+$CD8αα$^+$ T cells do not fully represent the CD4$^+$CD8αα$^+$ T cells in vivo.

Although polyclonal T cells were used in this protocol, it may be more appropriate to use OT-II T cells and antigen-presenting cells carrying suitable antigens in order to reflect the physiological environment.

TROUBLESHOOTING

Problem 1

The purity of splenic naive $CD4^+$ T cells after isolation is low (step 32).

Potential solution

Incomplete hemolysis of red blood cells could be the cause. Add another 2 mL of 0.84% (vol/wt) ammonium chloride and incubate another 5 min at RT for efficient lysis of red blood cells. Furthermore, low purity may be due to poor washing of unbound antibodies and magnetic beads before LS column step. Wash cells twice after antibody and microbeads labeling. Increase by 10% the recommended amount of antibody cocktail and magnetic beads can also improve the purity.

Problem 2

The number of naive $CD4^+$ T cells obtained was very low (step 32).

Potential solution

The process of crushing organs is insufficient. Make sure that the organ is adequately homogenized. Frequent rinsing during the homogenizing process will increase the recovery rate. To obtain more splenic naive $CD4^+$ T cells, use younger mice (6–10 weeks old). Do not use a spleen that is black in color which occasionally can occur and may be an indication of organ necrosis.

Problem 3

No $CD4^+CD8\alpha\alpha^+$ T cells differentiation was observed (step 42).

Potential solution

Prepare new and fresh antibodies for coating, cytokines and media. The conditions shown are similar to Treg differentiation conditions, so if you see a lot of Tregs in culture, refreshing the IFNγ cytokine and trying a concentration gradient should solve the problem.

Problem 4

Proliferation of cultured cell is not good.

Potential solution

The culture should be started as soon as possible after the purification process. Plate wash should be done immediately before culture, and preparation of cytokine mix, etc. should be done during the waiting time for each process.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tomohisa Sujino (tsujino1224@keio.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not involve generation/analysis of datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Y.H., K.M., and T.S. conceived the study, designed experiments, interpreted results, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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