

Lymphocyte Isolation, Th17 Cell Differentiation, Activation, and Staining

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[Abstract] *In vitro* Th17 (α , β T helper cell which produce IL-17A, IL-17F and IL-22) differentiation has been routinely used for functional T cells studies. Here we describe a method for Th17 cell differentiation.

Keywords: Th17, IL-17, FACS

[Background] T cells are critical to mediate host defense against bacteria, viruses and fungi as well as commensal (Kumar *et al.*, 2016). T cells can be further subdivided into T helper (Th1), Th2 and Th17 subsets based on their ability to generate specific cytokines. Naive T cells can be differentiated into specific T cell subsets in *in vitro* culture in response to specific cytokine stimulation. *In vitro* generated Th1, Th2 and Th17 cells have helped us to understand the molecular mechanism of their differentiation and their effector functions. Here, we have described a basic protocol for Th17 cell generation.

Materials and Reagents

1. 96-well tissue culture plate (CELLTREAT Scientific Products, catalog number: 229196)
2. Falcon® 70 μ m cell strainers (Corning, Falcon®, catalog number: 352350)
3. 50 ml conical tube (Denville Scientific, catalog number: C1056)
4. 1 ml syringe with cap (BD, catalog number: 305217)
5. 15 ml conical tube (Denville Scientific, catalog number: C1018-P)
6. 96-well round (U) bottom plate (CELLTREAT Scientific Products, catalog number: 229190)
7. C57BL/6 mice (Taconic Biosciences, catalog number: B6-F)
8. Coating antibodies: anti-mouse CD28 (Affymetrix, eBioscience, catalog number:14-0281), anti-mouse CD3e (Affymetrix, eBioscience, catalog number: 14-0033)
9. ELISA coating buffer (Biolegend, catalog number: 421701)
10. EasySep™ buffer (STEMCELL Technologies, catalog number: 20144) or PBS containing 2% fetal bovine serum (FBS) with 1 mM EDTA
11. EasySep™ Mouse Naïve CD4⁺ T Cell Isolation Kit (STEMCELL Technologies, catalog number: 19765)
12. Staining antibodies:
 - a. anti-mouse CD3-eFlour 450 (Affymetrix, eBioscience, catalog number: 48-0032)
 - b. anti-mouse CD4-Alexa Flour 700 (Affymetrix, eBioscience, catalog number: 56-0041)

- c. anti-mouse IL-22-APC (Affymetrix, eBioscience, catalog number: 17-7222)
- d. anti-mouse IL-17A-PE (Affymetrix, eBioscience, catalog number: 12-7177)
- e. anti-mouse CD62L-FITC (Affymetrix, eBioscience, catalog number: 11-0621)
13. Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, catalog number: 79346)
14. Ionomycin (Sigma-Aldrich, catalog number: I0634)
15. Fixation/Permeabilization Solution Kit with BD GolgiStop™ (BD, catalog number: 554715)
16. Cytofix/Cytoperm Plus Kit (with BD GolgiStop™) (BD, catalog number: 554715)
17. Iscove's modified Dulbecco's medium (IMDM) cell culture medium + glutamax (Thermo Fisher Scientific, Gibco™, catalog number: 31980-030)
18. HyClone™ fetal bovine serum (U.S.), characterized FBS (GE Healthcare, catalog number: SH30071.03)
19. HyClone™ penicillin-streptomycin 100x solution (GE Healthcare, catalog number: SV30010)
20. Recombinant porcine TGF-β1 (R&D Systems, catalog number: 101-B1)
21. Recombinant mouse IL-6 (R&D Systems, catalog number: 406-ML)
22. Recombinant mouse IL-23 (R&D Systems, catalog number: 1887-ML)
23. Anti-mouse IFN_γ (R&D Systems, catalog number: MAB485)
24. Anti-mouse IL-4 (R&D Systems, catalog number: MAB404)
25. Phosphate buffer saline (PBS) (Boston BioProduct, catalog number: BM220S)
26. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A3059)
27. Sodium azide (Sigma-Aldrich, catalog number: S2002)
28. Complete IMDM medium (see Recipes)
29. 2x Th17 differentiation condition medium (see Recipes)
30. FACS buffer (see Recipes)

Equipment

1. Tissue culture incubator (NuAire, model: LabGard Class II type A2)
2. Centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Sorvall Legend XFR)
3. BD LSRII Flow cytometer - BD
4. Scientific Industries Vortex Genie2 (Stellar Scientific, catalog number: SI-236)
5. EasyEight™ EasySep™ magnet (STEMCELL Technologies, catalog number: 18103)

Software

1. FACS Diva or Flow Jo software

Procedure

A. Lymphocyte isolation

1. Coat desired number of wells in a 96-well plate with 50 μ l of anti-mouse CD3 (2.5 μ g/ml) and anti-mouse CD28 (2.0 μ g/ml) for overnight at 4 °C. We used 1x ELISA coating buffer for antibodies dilution and coating. We washed plate once with 1x PBS before adding the cells.
2. Next day, euthanize a C57BL/6 mice and harvest spleen.
3. Place 70 micron cell strainer on 50 ml conical tube.

Place spleen on the cell strainer and homogenize/disrupt using 1 ml syringe cap. Syringe cap is placed on top of the spleen and rotate handle with hand to crush/homogenize the tissue. Add 1 ml EasySep buffer in cell strainer to facilitate homogenization. Collect flow through (contains cells) and add additional 9 ml EasySep buffer directly into the centrifuge tube. Centrifuge 300 x g for 10 min at 4 °C. Discard supernatant and re-suspend cells pellet with 1 ml EasySep buffer. Count cells (RBC lysis step is not required) and add 1 ml EasySep buffer/ 1×10^8 cells. Transfer cells into a 15 ml centrifuge tube.

Follow EasySep mouse CD4⁺CD62L⁺ naïve T cells isolation protocol.

Steps for naïve CD4⁺CD62L⁺ T cell isolation as per the [EasySep™ kit](#)

Notes:

- a. *The total number of cells in a naïve mouse spleen is ($\sim 1 \times 10^8$) without RBC lyses. We usually get 5-7 million naïve CD4 T cells after the purification steps.*
 - b. *Make sure the purity of isolated CD4⁺CD62L⁺ T cells is above 95% after enrichment (see Figure 1). Take small aliquots of purified cells (5,000-10,000 cells) and stain with anti-CD4-Alexa Flour 700 (1:100) and anti-CD62L-FITC (1:100) in ice cold FACS buffer for 30 min at 4 °C. After washing with FACS buffer check purity using flow cytometer by gating for CD4⁺CD62L⁺ T cells.*
4. Count the enriched cells and plate them as follows:
For a 96-well-plate, add 2×10^5 cells/well in 100 μ l complete IMDM medium (see Recipes).

B. Th17 cell differentiation

1. Prepare 2x Th17 differentiation condition medium (see Recipes)
2. Add 100 μ l 2x Th17 differentiation condition medium to each well.
3. Culture for 3 additional days at 37 °C and 5% CO₂ in a tissue culture incubator. You will see several T cell clusters on day 2 post differentiation and medium will turn slight yellowish. If medium turned very yellowish then add additional 50 μ l complete IMDM medium (see Recipes).

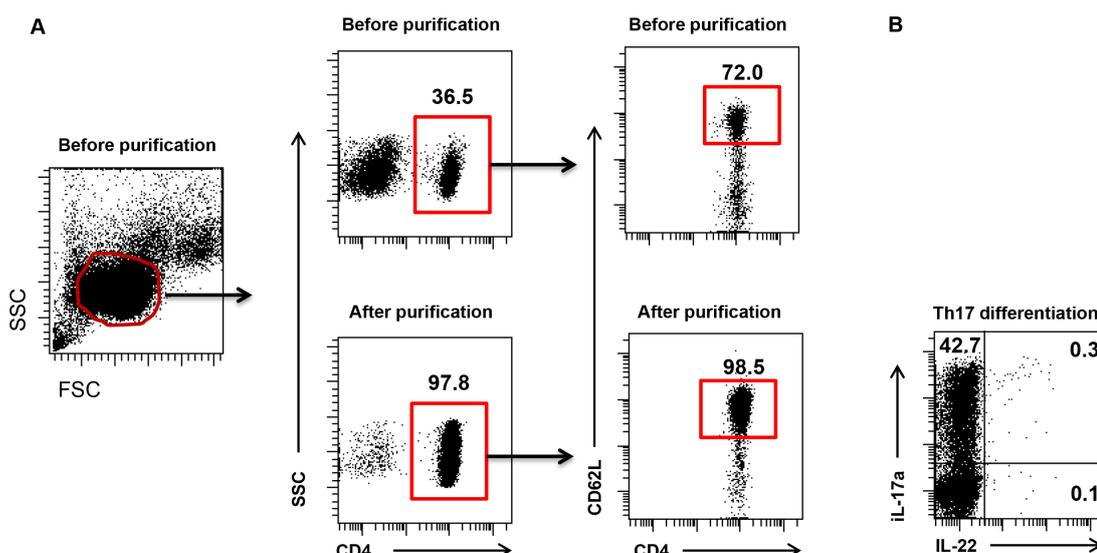


Figure 1. Naïve CD4 T cells purification and Th17 differentiation. A. Flow cytometry dot plot gate shows spleen lymphocytes (left), percentage of CD4⁺ cells (middle) and frequency of purified naïve CD4⁺CD62L⁺ T cells. B. Data show IL-17 producing Th17 cells on day 3 post differentiation.

C. Activation and staining

1. After day 3 post differentiation, transfer cells into a U-bottom 96-well plate using a multi-channel pipette (gently pipetting up and down to resuspend the cells) and centrifuge at 300 x g for 5 min at room temperature.
Discard supernatant by flicking plate in a quick single motion. To flick off the supernatant, move the plate upward and bring it straight down. Gently touch plate with the tissue paper to remove drops. Vortex plates using a benchtop Vortex Genie2 or any mini vortex mixer. Follow these steps for washing and decanting the supernatant.
2. Add 200 µl of complete IMDM medium containing 50 ng/ml PMA, 750 ng/ml ionomycin and GolgiStop (1:1,000) to the cells and incubate for 4 h at 37 °C and 5% CO₂ in a tissue culture incubator.
3. Wash twice with 200 µl ice cold FACS buffer (300 x g, 5 min, 4 °C). Discard supernatant by flicking plate in a quick single motion.
4. Re-suspend cells in 100 µl ice cold FACS buffer containing anti-mouse CD3-eFluor 450 (1:100), anti-mouse CD4-Alexa Fluor 700 (1:100) and incubate for 30 min at 4 °C (refrigerator).
5. Wash cells with FACS buffer (300 G, 2 min, 4 °C).
6. Fix cells with 100 µl BD Cytofix/Cytoperm for 20 min at 4 °C. Discard supernatant by flicking plate in a quick single motion.
7. Wash twice with 200 µl 1x BD Perm/Wash™ buffer (300 x g, 2 min, 4 °C). Discard supernatant by flicking plate in a quick single motion.

8. Add 100 μ l 1x BD Perm/Wash™ buffer containing anti-IL-17A-PE (1:100) and anti-IL-22-APC (1:100) antibodies and incubate for 45 min/overnight at 4 °C.
 Instead of surface staining steps, all antibodies including surface (anti-mouse CD3-eFluor 450, anti-mouse CD4-Alexa Fluor 700) and intracellular (anti-mouse IL-17A-PE and anti-mouse IL-22-APC) can be used together in a single step after fixation and Perm/Wash. We have stained cells with intracellular cytokines along with surface antibodies against CD3 and CD4.
9. Wash cells twice with 200 μ l 1x BD Perm/Wash™ buffer (300 x g, 5 min, 4 °C). Re-suspend pellet in 200 μ l FACS buffer.
10. Confirm the lineage of Th17 cell by determining the levels of intracellular IL-17A and IL-22 by FACS. A good Th17 differentiation will result into 20-40% IL-17A producing CD4 T cells.

Data analysis

FACS Diva or Flow Jo software can be used to analyze data. Plot a linear FSC versus SSC dot plot and create a gate (P1) to select all cells. Using P1 population plot a linear CD3 versus CD4 dot plot. Majority of differentiated cells will be positive for both CD3 and CD4. CD3⁺CD4⁺ double positive population (gate P2) will be analyzed for intracellular IL-17 and IL-22 staining.

Recipes

1. Complete IMDM medium (Pre-warmed at 37 °C before use)
 IMDM medium + glutamax
 10% FBS
 1% penicillin/streptomycin solution
2. 2x Th17 differentiation condition medium
 Complete IMDM medium (Pre-warmed at 37 °C) containing:
 10 ng/ml TGF- β 1
 80 ng/ml recombinant IL-6
 20 ng/ml recombinant IL-23
 10 μ g/ml anti-IFN γ
 10 μ g/ml anti-IL-4
3. FACS buffer
 PBS with
 0.5 % bovine serum albumin (BSA)
 0.05% sodium azide

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