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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 µm 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)

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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

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Procedure

A. Lymphocyte isolation

1. Coat desired number of wells in a 96-well plate with 50 µl of anti-mouse CD3 (2.5 µg/ml) and anti-mouse CD28 (2.0 µg/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 x 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 (~1 x 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 x 10^5 cells/well in 100 µl complete IMDM medium (see Recipes).

B. Th17 cell differentiation

1. Prepare 2x Th17 differentiation condition medium (see Recipes)
2. Add 100 µl 2x Th17 differentiation condition medium to each well.
3. Culture for 3 additional days at 37 °C and 5% CO_2 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 µl complete IMDM medium (see Recipes).

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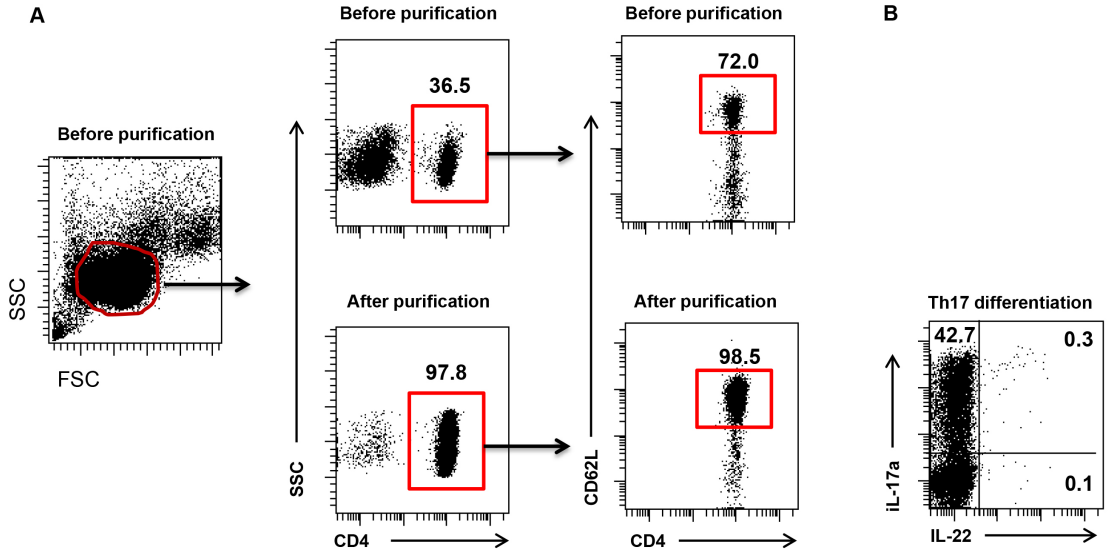

Figure 1. Naïve CD4 T cells purification and Th17 differentiation. A. Flow cytometry dot plot gate shows spleen lymphocytes (left), percentage of $CD4^+$ cells (middle) and frequency of purified naïve $CD4^+CD62L^+$ T cells. B. Data show IL-17 producing Th17 cells on day 3 post differentiation.

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% CO_2 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.

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8. Add 100 µl 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 µl 1x BD Perm/Wash™ buffer (300 *x g*, 5 min, 4 °C). Re-suspend pellet in 200 µl 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-β1
   80 ng/ml recombinant IL-6
   20 ng/ml recombinant IL-23
   10 µg/ml anti-IFNγ
   10 µg/ml anti-IL-4
3. FACS buffer
   PBS with
   0.5 % bovine serum albumin (BSA)
   0.05% sodium azide

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