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A Simple and Robust Protocol for *in vitro* Differentiation of Mouse Non-pathogenic T Helper 17 Cells from CD4$^+$ T Cells

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[Abstract] Functional and mechanistic studies of CD4$^+$ T cell lineages rely on robust methods of *in vitro* T cell polarization. Here, we report an optimized protocol for *in vitro* differentiation of a mouse non-pathogenic T helper 17 (T_H17) cell lineage. Most of the previously established protocols require irradiated splenocytes as artificial antigen presenting cells (APC) for TCR activation. The protocol described here employs plate-bound antibodies and a T_H17-polarizing cytokine cocktail to activate and differentiate naïve CD4$^+$ T (T_{nai}) cells, reflecting a simple and robust protocol for *in vitro* T_H17n differentiation. Using T cells that are genetically engineered with an IL-17 reporter, this protocol may enable the rapid production of a pure population of IL17-expressing CD4$^+$ T cells for system biology studies and high-throughput functional screening.
Keywords: T_H17, IL-17, Plate-bound, FACS, T cell polarization

[Background] Naive CD4$^+$ T cells circulate through secondary lymphoid tissues after emerging from the thymus. The activation of naïve CD4$^+$ T cells, by antigen-presenting cells that offer cognate antigens, initiates various differentiation programs that lead to the development of highly specialized T helper (T_H) cell lineages (Bhaumik and Basu 2017). T_H17 cells are a subset of pro-inflammatory T_H cells defined by the production of interleukin 17 (IL-17) (Hartigan-O'Connor *et al.*, 2011). To date, at least two different types of T_H17 have been described. Type 1, pathogenic or disease-inducing T_H17 (T_H17p) cells, are induced by the combined IL-6, IL-1β, and IL-23 cytokine stimulation, and produce IL-17 together with IFN-γ, which results in tissue inflammation and autoimmunity (Cua *et al.,* 2003; Awasthi *et al.,* 2009; Jager *et al.,* 2009; Lee *et al.,* 2012; Lee *et al.,* 2014). Type 2, non-pathogenic T_H17 (T_H17n) cells, are induced by TGF-β and IL-6 *in vitro*, and produce IL-17 but are not able to induce effective tissue inflammation or autoimmunity. T_H17n cells play a protective role in mucosal tissues, promoting tissue homeostasis as well as maintaining barrier function (Bettelli *et al.,* 2008; Ouyang *et al.,* 2008; Korn *et al.,* 2009; Guglani and Khader 2010; Gaffen *et al.,* 2011). Here, we report a simple and robust protocol for generating non-pathogenic T_H17 (T_H17n) cells *in vitro*.

Materials and Reagents

1. 48-well cell culture plates (ASi Alkali Scientific Inc., catalog number: TP9048)
2. CellPro™ 70 μm cell strainers (ASi, Falcon®, catalog number: MT4070)

3. 1 ml syringe (BD Tuberculin Slip Tip, catalog number: 9189520)
4. 50 ml centrifuge tubes (ASi, catalog number: C5602)
5. 15 ml centrifuge tubes (ASi, catalog number: C5600)
6. C57BL/6J mice (The Jackson Laboratory, catalog number: 000664)
7. C57BL/6-*IL17a*^{tm1Bcgen}/J mice (The Jackson Laboratory, catalog number: 018472)
8. RBC lysis buffer 10× (Biolegend®, catalog number: 420302)
9. Cell culture grade water (HyPure™, catalog number: SH30529.02)
10. MojoSort™ Mouse CD4 Naïve T Cell Isolation Kit (Biolegend®, catalog number: 480040)
11. Coating antibodies:
    a. Anti-mouse CD3ε (clone 145-2C11, Bio X Cell, catalog number: BE0001-1)
    b. Anti-mouse CD28 (clone 37.51, Bio X Cell, catalog number: BE0015-1)
12. Staining antibodies:
    a. APC anti-mouse CD4 (clone RM4-5, Biolegend, catalog number: 100516)
    b. PE/Cy7 anti-mouse IL-17A (clone TC11-18H10.1, Biolegend, catalog number: 506922)
13. Cell Stimulation Cocktail Plus Protein Transport Inhibitors (Invitrogen eBioscience™, catalog number: 00-4975-93)
14. FoxP3/Transcription Factor Staining Buffer Set (Fixation Diluent, Fixation Reagent, Permeabilization kit, eBioscience™, catalog number: 00-5523-00)
15. RPMI 1640 1× with L-glutamine (Corning, catalog number: 10-040-CV)
16. Fetal bovine serum (FBS) (ATLANTA biologicals™, R&D SYSTEMS, catalog number: S12450)
17. L-glutamine 200 mM (Corning, catalog number: 25-005-CI)
18. Penicillin-streptomycin solution (PS) 100× (Corning, catalog number: 30-002-CI)
19. Sodium pyruvate 100 mM (Corning, catalog number: 25-000-CI)
20. 2-mercaptoethanol (ME) 1000× (Life Technologies, Gibco®, catalog number: 21985-023)
21. T_H17 polarization cytokines and antibodies:
    a. Recombinant Human TGF-β1 (PeproTech, catalog number: 100-21C)
    b. Recombinant Murine IL-6 (PeproTech, catalog number: 216-16)
    c. Anti-mouse IFN-γ (clone XMG1.2, Bio X Cell, catalog number: BE0055)
    d. Anti-mouse IL-2 (clone JES6-1A12, Bio X Cell, catalog number: BE0043)
    e. Anti-mouse IL-4 (clone 11B11, Bio X Cell, catalog number: BE0045)
22. Phosphate-buffered saline (PBS) 1× (Corning, catalog number: 21-040-CV)
23. Bovine serum albumin (BSA) (Fisher BioReagents™, catalog number: BP1600-100)
24. Ethylenediamine tetraacetate acid (EDTA) 0.5 M (Fisher BioReagents™, catalog number: BP2482-500)
25. Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen™, catalog number: C1157)
26. Culture medium (see Recipes)
27. TCR-stimulation antibody mixture (see Recipes)
28. RBC lysis buffer (1×) (see Recipes)
29. MACS buffer (see Recipes)

30. T_H17 polarization medium (see Recipes)
31. Staining buffer (see Recipes)

Equipment

1. NovoCyte (ACEA Biosciences)

Software

1. FlowJo software (BD Biosciences)

Procedure

A. Naïve CD4$^+$ T cell isolation and seeding.
   1. Day -2, pre-coat 200 µl TCR-stimulating antibody mixture/well in a 48 well-plate at 4°C.
   2. Day 0, euthanize a C57BL/6J mouse (or IL17A-IRES-GFP-KI mouse), harvest the spleen and lymph nodes (LNs), and isolate naïve CD4$^+$ T cells using the MojoSort™ Isolation Kit according to the manufacturer's instructions.
      Note: Basic information regarding the anatomy of a laboratory mouse can be found at http://www.informatics.jax.org/cookbook/.
   3. Count the cells and seed 2.5 × 10^5 cells/well in the pre-coated 48-well plate with 500 µl T_H17 polarization medium at 37°C/5% CO_2.
      Note: To follow T cell proliferation, it is optional to stain naive CD4$^+$ T cells with CFSE according to the manufacturer's instructions.

B. FACS analysis of T_H17 cell differentiation (**Figure 1**)
   Day 3 (or anytime between day 2 and day 5; we routinely observed over 40% IL17$^+$ cells starting at day 2 and remaining for 3 additional days until the media's nutrients are depleted), treat the cells for 4 h with Cell Stimulation Cocktail Plus Protein Transport Inhibitors according to the manufacturer's for 4 h, and then stain the cells with the anti-mouse CD4 antibody (1:100) for 30 min at 4°C. Next, fix and permeabilize the cells using the FoxP3 Fixation/Permeabilization Kit according to the manufacturer's instructions. Finally, stain the cells with the anti-IL-17A antibody (1:100) at RT for 15 min before FACS analysis (if cultured cells are from an IL17A-IRES-GFP-KI mouse, FACS analysis can be performed directly without the procedures described above in B).
   Note: Basic principles and applications of flow cytometry can be found in the literature (McKinnon, 2018).

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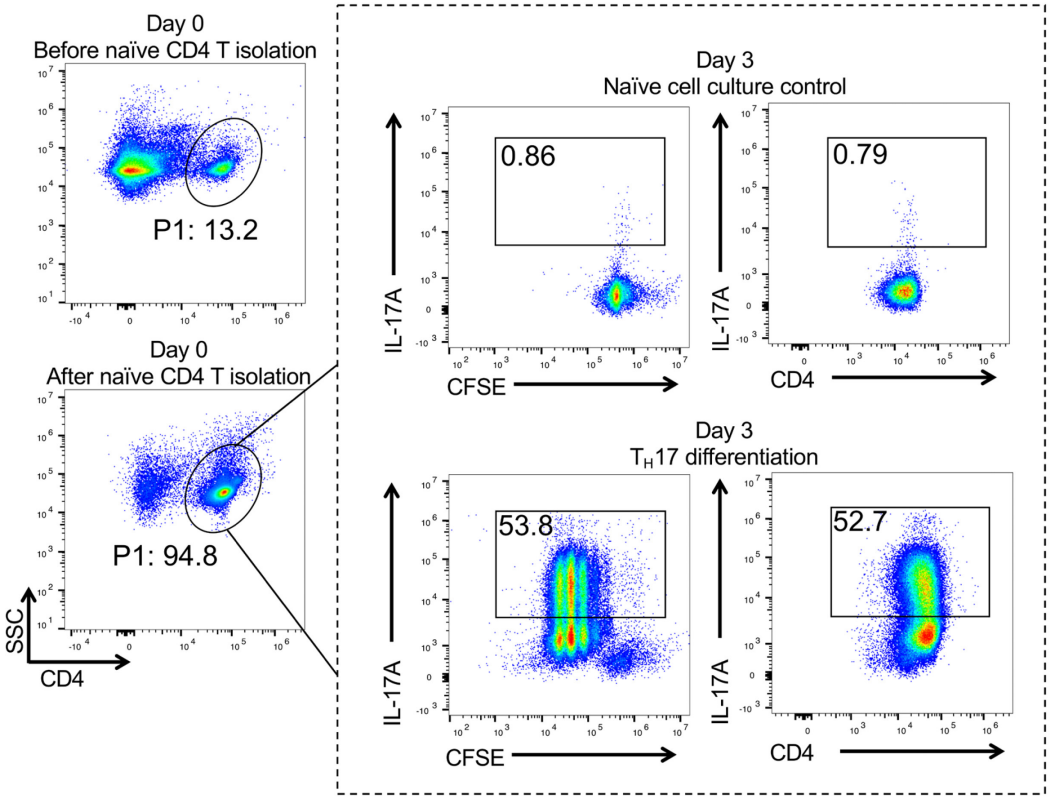

Figure 1. Naïve CD4⁺ T cell isolation and T_H17 differentiation. Flow cytometry data showing that more than 90% naïve CD4⁺ T cells can be obtained after isolation on day 0 (left panel). Day 3, CFSE labeling data showing that more than 50% T_H17 cells can be observed post-polarization (dotted line frame).

Data analysis

FlowJo software: Main gate P1; day 0 data, choose log SSC versus CD4 for gating CD4⁺ T cells. Day 3 data; within P1-CD4 gate (same gating scheme as day 0), choose log CFSE or CD4 versus IL-17A to analyze intracellular IL-17.

Recipes

1. Culture medium
   RPMI 1640 1×
   10% fetal bovine serum (FBS)
   2 mM L-glutamine
   1% sodium pyruvate
   1% PS
   1:1,000 2-ME
   Store at 4°C

2. TCR-stimulation antibody mixture
   10 μg/ml anti-mouse CD3 and 10 μg/ml anti-mouse CD28 antibodies in PBS
3. RBC lysis buffer (1×)
   1 ml 10× buffer with 9 ml cell culture grade water
   Store at 4°C
4. MACS buffer
   500 ml PBS containing 2.5 g bovine serum albumin (BSA) and 2 ml 0.5 M EDTA
   Store at 4°C, fliter using a 0.2-μm filter
5. T_H17 polarization medium
   Culture medium containing mIL-6 (50 ng/ml), hTGF-β1 (20 ng/ml), anti-mIL-2 (8 μg/ml), anti-mIL-4 (8 μg/ml), and anti-mIFN-γ (8 μg/ml)
   Prepare immediately before use
6. Staining buffer
   PBS containing 2% (w/v) BSA
   Store at 4°C

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Competing interests

The authors declare no conflicts of interest.

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