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A method to *in vitro* differentiate Th9 cells from mouse naïve CD4 T cells

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Abstract

CD4⁺ T helper cells with the ability to produce effector cytokines provide host protection by regulating immune responses against pathogens. In contrast, these cells are responsible for the development of various inflammatory disease. Previous studies using naïve CD4⁺ T cell activation *in vitro* have shown the requirement of various cytokine combinations in addition to TCR activation to differentiate naïve CD4⁺ T cells into various effector T helper lineages. The recently discovered CD4⁺ T helper subset is IL-9-producing Th9 cells. Since Th9 cell differentiation *in vitro* is essential in understanding the molecular mechanism in regulating Th9 cell development, it is critical to develop a basic protocol in polarizing naïve CD4⁺ T cells to Th9 cells *in vitro*. Here we describe a simple method for Th9 cell culture conditions *in vitro* that can be used for other molecular analyses.

Keywords

Naïve CD4⁺ T cells; Th9; IL-9; IL-4; TGF- β

1. INTRODUCTION

T helper type 9 (Th9) cells are a new T helper cell subset that has been characterized in recent years. Th9 cells secrete IL-9 that is instrumental in modulating allergic inflammation and immunity to intestinal parasites. IL-9 has broad effects on various cell types that play a pivotal role for the development of immunity and inflammation, including smooth muscle cells, stem cells, lymphocytes, mast cells, and epithelial cells [1,2]. Th9 cell differentiation requires both IL-4 and TGF- β signaling [3,4]. IL-4 activates STAT6 to induce the expression of transcription factors GATA3, IRF4 and BATF while TGF- β induces PU.1 expression. These transcription factors are required for optimal Th9 cell differentiation [5–9]. Other cytokines such as TSLP, IL-2, and IL-1 have been shown to modulate IL-9 production by CD4⁺ T cells, yet less is known about their downstream modulators [10,6]. Thus, proper Th9 cell differentiation protocol *in vitro* is essential in shedding light on the signaling pathways that control Th9 cell development.

2. MATERIALS

Prepare and store all reagents at 4°C (unless indicated otherwise).

2.1 Equipment

1. Centrifuges.
2. Fluorescence-activated cell sorter (FACS).
3. MultiStand for magnetic Separators.
4. MiniMACS and MidiMACs magnetic Separators.
5. MS and LS Columns.
6. 15 ml and 50 ml polypropylene conical tubes.
7. Polystyrene tissue culture plates.
8. 60 × 15 mm culture dish.
9. Frosted slides.
10. 0.22 µm and 0.45 µm filters.

2.2 Additional reagents

1. T cells re-stimulation cocktail: GolgiStop (Monensin solution), Phorbol 12-myristate 13-acetate (PMA), and Ionomycin.
2. Formaldehyde solution for molecular biology, 36.5-38% in H₂O.
3. Trypan blue.

2.3 Mice

We use spleen and lymph nodes from 6–12 weeks old C57BL/6 mice. All experiments are performed with the approval of the University Institutional Animal Care and Use Committee.

2.4 Buffers

1. Prepare RPMI complete medium for mouse under sterile conditions: 500 ml RPMI-1640 with 10% (v/v) Fetal bovine serum (FBS), 200 U/ml Penicillin/Streptomycin, 1 mM sodium pyruvate, 1 mM L-Glutamine, 50 µM MEM Non-Essential Amino Acids solution, 5 mM HEPES, and 50 µM 2-mercaptoethanol. Filter using 0.22 µm filter. Store at 4°C.
2. Prepare magnetic cell sorting (MACS) buffer under sterile conditions: phosphate-buffered saline (PBS), 0.5% (w/v) Bovine serum albumin (BSA), and 2 mM EDTA pH 8. Filter using 0.22 µm filter. Store at 4°C.
3. Red Blood cell lysis buffer: Add 8.3 g NH₄Cl and 10 mM Tris-HCl pH 7.4 to 900 ml water. Mix and adjust pH to 7.5 ± 0.2. Add water to make 1 liter, filter using 0.22 µm filter. Autoclave and store at 4°C.

4. Fluorescence-activated cell sorting (FACS) buffer: PBS, 0.02% (w/v) BSA, and 0.01% (w/v) sodium azide. Mix well and filter using 0.22 μ m filter. Store at 4°C.
5. Permeabilization buffer: PBS, 2% (w/v) BSA, 0.1% (w/v) saponin, and 0.01% (w/v) sodium azide. Mix well and filter using 0.22 μ m filter. Store at 4°C.

3.5 Antibodies and cytokines

See manufacture's datasheet for antibody and cytokine storage conditions. Small aliquots should be made to prevent multiple freeze thaw cycles.

1. CD4⁺ T cell biotin-antibody cocktail for depletion of non-CD4⁺ T cells: biotin-conjugated monoclonal anti-mouse CD8a, CD45R, CD11b, CD25, CD49b, TCR γ / δ , and Ter-119 antibodies.
2. Monoclonal (mouse IgG1) anti-biotin microbeads antibody.
3. Monoclonal anti-CD62L (L-selectin, rat IgG2a) microbeads antibody.
4. Th9-polarizing antibody and cytokine cocktail: purified anti-mouse CD3 (clone 145-2C11), purified anti-mouse CD28 (clone 37.51), and anti-mouse IFN γ (clone XMG) antibodies; recombinant human TGF- β and IL-2 cytokines, and recombinant mouse IL-4 cytokine.
5. Fluorochrome conjugated anti-mouse IL-9 and IL-4 antibodies.

3. METHODS

3.1 Naïve CD4⁺ T cell Isolation (see Note ¹, ², and ³)

3.1.1 Sample preparation

1. Process the spleen and/or lymph nodes by the frosted slide method. Place the spleen in the bottom half of a 60 $\times$ 15 mm culture dish with 5 ml of PBS.
2. Press the spleen and/or lymph nodes between the frosted ends of two microscope slides using a gentle circular motion until only the empty capsule remains.
3. Wash the frosted slide with 10 ml MACS buffer and transfer to a new 15ml tube through a 0.45 μ m filter.
4. Rinse the culture dish and filter with 5 ml MACS buffer.
5. Centrifuge samples at 300 $\times$ g for 5 minutes at 4°C.
6. Resuspend cell pellets in 3 ml red blood cell lysis buffer and incubate at room temperature for 3 minutes.
7. Add 20 ml of RPMI complete medium to stop the reaction.

¹Perform fast and keep reagents cold during naïve CD4⁺ T cell isolation.

²AutoMACS Separator can be used for negative and positive selection during the isolation of CD4⁺CD62L⁺ T cells. Using either magnetic separator or autoMACS Separator, one can achieve greater than 90% cell purity.

³Alternatively, total splenocytes can be labeled with fluorochrome antibodies and sorted by flow cytometry for naïve CD4⁺CD25⁻CD44^{low}CD62L^{high} T cells.

8. Centrifuge samples at 300×g for 5 minutes at 4°C.
9. Wash once with 10 ml MACS buffer by centrifuging at 300×g for 5 minutes at 4°C.
10. Perform live cell count using Trypan Blue.
11. Aspirate supernatant completely and resuspend cell pellet in 400 µl of MACS buffer per 10⁸ total cells.
6. Add 100 µl of CD4⁺ T cells biotin-antibody cocktail (including biotin-conjugated monoclonal anti-mouse CD8a, CD45R, CD11b, CD25, CD49b, TCRγ/δ, and Ter-119 antibodies per 10⁸ cells. (see **Note** ⁴)
12. Mix well and incubate at 4°C for 10 minutes.
13. Add 300 µl of MACS buffer and 200 µl of anti-biotin microbeads per 10⁸ cells.
14. Mix well and incubate at 4°C for 15 minutes.
15. Centrifuge samples at 300×g for 5 min at 4°C.
16. Aspirate supernatant and resuspend pellet in 500 µl of MACS buffer.

3.1.2 Magnetic separation-depletion of non-CD4⁺ T cells

1. Place LS column in the Midi MACS Separator.
2. Rinse column with 3 ml of MACS buffer.
3. Apply cell suspension onto the column.
4. Wash the column 3 times with 3 ml MACS buffer.
5. The flow-through contains the unlabeled pre-enriched CD4⁺ T cells.
6. Centrifuge samples at 300×g for 5 minutes at 4°C.
7. Aspirate supernatant completely and resuspend cell pellet in 800 µl of MACS buffer.
8. Add 200 µl of CD62L (L-selectin) microbeads to samples.
9. Mix well and incubate at 4°C for 15 minutes.
10. Centrifuge samples at 300×g for 5 minutes at 4°C.
11. Aspirate supernatant and resuspend pellet in 500 µl of MACS buffer.

3.1.3 Magnetic separation-positive selection of CD4⁺CD62L⁺ T Cells

1. Place MS column in the Mini MACS Separator.
2. Rinse column with 500 µl of MACS buffer.
3. Apply cell suspension onto the column.

⁴MS and LS columns can retain up to 1×10^7 and 1×10^8 labeled cells, respectively. The amount of antibodies should be optimized for up to 10^8 cells. Scale up buffer volume and antibodies for higher cell numbers.

4. Wash the column 3 times with 500 μ l MACS buffer.
5. Remove column and place it on a fresh collection tube.
6. Add 1 ml of MACS buffer onto the column. Push the plunger into the column to flush out the magnetically labeled CD4⁺CD62L⁺ T Cells.
7. Wash cells once with 10 ml MACS buffer by centrifuging samples at 300 $\times$ g for 5 minutes at 4°C.
8. Perform live cell count using Trypan Blue exclusion method.
9. Resuspend pellet in RPMI compete medium to 1×10^6 cells per ml.

4.2 Setup Th9 cell culture

1. Coat tissue culture plate with 2 μ g/ml anti-CD3 diluted in PBS for at least 2 h at 37°C or overnight at 4°C.
2. Aspirate PBS from tissue culture plate.
3. Seed cell suspension onto antibody-coated tissue culture plate at the concentration of 1 million cells per ml.
4. Prepare and add Th9 cytokine and antibody cocktail containing 1 μ g/ml anti-CD28, 20 ng/ml IL-4, 2 ng/ml human TGF- β , and 10 μ g/ml anti-IFN γ . (see **Note 5**)
5. Gently rock tissue culture plate back and forth to mix properly.
6. Incubate plate in the incubator at 37°C and 5% CO₂ for 72 h.
7. After 72 h, expand cell culture by gently pipetting up and down to remove cells and transfer to a new plate with fresh RPMI compete medium (3 times the original volume).
8. Add 20 ng/ml IL-4, 2 ng/ml human TGF- β , and 50 U/ml human IL-2 to the culture medium.
9. Incubate plate in the incubator at 37°C and 5% CO₂ for an additional 48 h.

3.3 Assess Th9 differentiation by flow cytometry (see **Note 6** and **7**)

3.3.1 Re-stimulation of Th9 cells

1. Harvest and count cells.
2. Wash cells once with 10 ml RPMI compete medium.
3. Centrifuge samples at 300 $\times$ g for 5 minutes at 4°C.

⁵The concentration of IL-4 and TGF- β can be adjusted to optimize Th9 cell culture conditions. We have observed that increasing the concentration of anti-CD3 and anti-CD28 antibodies up to 10 μ g/ml can enhance IL-9 production.

⁶Resting differentiated Th9 cells or other T helper cells (Th1 or Th2) can be used as negative controls for evaluating Th9 differentiation using flow cytometry.

⁷Th9 cell differentiation can be assessed using qPCR or ELISA. Day 5 differentiated cells are washed and restimulated for 6 h or 24 h with 2 μ g/ml anti-CD3 for qPCR or ELISA, respectively.

4. Aspirate supernatant and resuspend pellet in RPMI complete medium to 1×10^6 cells per ml.
5. Seed cell suspension onto tissue plate at 1×10^6 cells per ml.
6. Add 50 ng/ml PMA and 750 ng/ml Ionomycin to the medium.
7. Incubate cells in the incubator at 37°C and 5% CO₂ for 2 h.
8. Add GolgiStop solution at 1:1000 dilution and culture for an additional 4 h.

3.3.2 Cell staining for flow cytometry

1. Wash cells twice with 3 ml FACS Buffer. Centrifuge samples at 300×g for 5 minutes at 4°C.
2. Add surface antibodies if needed and incubate for 30 minutes at 4°C. (see **Note 8**)
3. Wash cells once with 3 ml FACS buffer. Centrifuge samples at 300×g for 5 minutes at 4°C.
4. Fix cells in 100 µl per 1×10^6 cells using 2% formaldehyde for 10 minutes at room temperature. Keep in the dark.
5. Wash cells once with 3ml FACS buffer and once with 1 ml permeabilization buffer by centrifuging samples at 300×g for 5 minutes at 4°C.
6. Add fluorochrome conjugated antibodies diluted in FACS buffer to the samples and incubate 30 minutes at 4°C in the dark. (see **Note 8**)
7. Wash cells once with 3 ml FACS buffer. Centrifuge samples at 300×g for 5 minutes at 4°C.
8. Resuspend cells in 500 µl FACS buffer.
9. Cells can be kept at 4°C in the dark for flow cytometry analysis (Fig. 1).

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⁸Dead cells can interfere with the interpretation of flow cytometry data. The Live/Dead cell viability kit that stains dead cells can be performed to differentiate between live and dead cells in flow cytometry. Live/Dead cell viability kit is comparable with common fixation and permeabilization methods for intracellular staining. Fluorochrome conjugated antibodies should be used at proper concentration according to manufacturer's protocol. Alternatively, antibodies can be titrated to determine optimal concentration.

- cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nature immunology*. 2008; 9(12):1347–1355. DOI: 10.1038/ni.1677 [PubMed: 18997793]
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Figure 1.

Flow cytometry obtained from day 5 unstimulated or PMA and Ionomycin (PI) re-stimulated Th9 cells that were stained using luorochrome conjugated anti-mouse IL-9 and IL-4 antibodies.