***In vitro* Differentiation of Mouse Th0, Th1, Th2, and Th17 from Naïve CD4 T Cells**

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[Abstract] *In vitro* differentiation of helper T cells of various lineages is frequently used in T helper cell study. Naïve CD4 T cells can differentiate into certain lineage of T help cells *in vitro* in the presence of specific stimulatory cytokines and inhibition of cytokines that are essential for the differentiation of other lineages.

Materials and Reagents

1. Fetal bovine serum (FBS) (Gemini Bio-Products, catalog number: 900-108)
2. Penicillin/streptomycin solution (Life Technologies, Gibco®, catalog number:15140-122)
3. 2-mercaptoethanol (Life Technologies, Invitrogen™, catalog number: 21985-023)
4. IL-2 (R&D systems, catalog number: 402-ML-020)
5. IL-4 (Biolegend, catalog number: 574302)
6. IL-6 (Biolegend, catalog number: 575702)
7. IL-12 (R&D systems, catalog number: 419-ML-010)
8. TGF-beta (R&D systems, catalog number: 240-B-002)
9. Anti-CD3 (Biolegend, catalog number: 100302)
10. Anti-CD28 (Biolegend, catalog number: 102102)
11. Anti-IL-4 (Biolegend, catalog number: 504102)
12. Anti-IL-12 (Biolegend, catalog number: 505303)
13. Anti-IFNg (Biolegend, catalog number: 505702)
14. Phorbol 12-myristate-13-acetate (PMA) (Sigma-Aldrich, catalog number: 79346)
15. Ionomycin (Sigma-Aldrich, catalog number: 19657)
16. Monensin (ebioscience, catalog number: 00-4505-51)
17. Easysep CD4+ T cell enrichment Kit (STEMCELL Technologies, catalog number: 19752)
18. RPMI-1640 medium (Life Technologies, Gibco®, catalog number: 11875-093) (see Recipes)

Equipment

1. 48, 96-Well plate

2. Centrifuges
3. Water bath
4. Fluorescence activated cell sortor (FACS)

Procedure

1. Enrich CD4⁺ T cells by Easysep CD4⁺ T cell enrichment kit following the manufacturer's instructions. Make sure the purity of CD4⁺ T cells is above 70% after enrichment.
2. Sort CD4⁺ CD44^{lo}CD62L^{hi} naïve T cells from enriched CD4⁺ T cells.
3. Count the sorted cells and plate them as follows:
 - a. For 96-well-plate, 1 x 10⁵ cells/well in 200 µl culture medium for each well.
 - b. For 48-well-plate, 2 x 10⁵ cells/well in 500 µl culture medium for each well.
4. Set up the differentiation culture (numbers are final concentration)

	Th0	Th1	Th2	Th17
anti-CD3 (plate coated)	5 µg ml ⁻¹	5 µg ml ⁻¹	5 µg ml ⁻¹	5 µg ml ⁻¹
anti-CD28	1 µg ml ⁻¹	1 µg ml ⁻¹	1 µg ml ⁻¹	1 µg ml ⁻¹
IL-2	20 ng ml ⁻¹	20 ng ml ⁻¹	20 ng ml ⁻¹	20 ng ml ⁻¹
IL-4			100 ng ml ⁻¹	
IL-12		20 ng ml ⁻¹		
TGF-beta				1 ng ml ⁻¹
IL-6				100 ng ml ⁻¹
anti-IL-4		10 µg ml ⁻¹		10 µg ml ⁻¹
anti-IFNγ			10 µg ml ⁻¹	10 µg ml ⁻¹
anti-IL-12			10 µg ml ⁻¹	

5. Transfer the culture to a new plate after 2 days without digesting or changing the medium.
6. Culture for 3 more days. If medium turns yellow, replace it with new medium with neutralizing antibodies (no cytokines).
7. Add 50 ng ml⁻¹ PMA and 1 µg ml⁻¹ ionomycin to the cells and incubate for 1 h.
8. Add 2 µM monensin and incubate cells for another 4 h.
9. Verify the lineage of T helper cells by determine the levels of intracellular IFN-gamma, IL-4, or IL-17 by FACS.

Note: Please see the following link for common FACS staining protocol (<http://www.bio-protocol.com/wenzhang.aspx?id=122&fl3=31>).

Recipes

1. RPMI-1640 culture medium
Supplemented with
5% FBS
1% Penicillin/streptomycin solution
50 μ M 2-mercaptoethanol