SOP: **Isolation of human primary T cells and expansion of memory T cell subsets: naïve $CD4^+$, Th1, Th2, and Th17 cells**
Date modified: **6/25/2012**
Modified by: **A. Raubitschek/R.S. Hansen/T. Canfield (UW)**

Source Information

Cells are isolated from peripheral blood procured from a normal healthy donor.
Notes:
T-cell subsets are purified and then expanded in primary culture.

Materials List

anti-CCR6-PE (BD, Cat# 559562)
anti-CCR4-PE-Cy7 (BD, Cat# 557864)
anti-CXCR3-PE-Cy5 (BD, Cat# 558047)
anti-CD4-Qdot 655 (Invitrogen/Life Technologies, Cat# Q10007)
anti-CD3-APC-AF750 (eBioscience, Cat# 27-0078-13)
anti-CD45RO-FITC (eBioscience, Cat# 11-0477-73)
anti-CD25-APC (BD, Cat# 555434)
PBS (Sigma-Aldrich, Cat# D8537)
Ficoll-Paque (GE Healthcare Life Science, Cat# 17-1440-03)
BSA (Sigma-Aldrich, Cat# A2153)
EDTA (Sigma-Aldrich, Cat# EDS)
Trypan Blue (Sigma-Aldrich, Cat# T6146)
MACS CD4 T Cell Isolation Kit II (Miltenyi Biotec, Cat# 130-091-155)
MACS LS Columns (Miltenyi Biotec, Cat# 130-042-401)
AIM-V medium (Invitrogen/Life Technologies, Cat# 12055-083)
Human AB Serum (Cellgro, Cat# 35-060-CI)
Penicillin-Streptomycin Solution (Invitrogen/Life Technologies, Cat# 15070-063)
IL-4 (Cell Sciences, Cat# CRI104-20)
IL-12 (Cell Sciences, Cat# CRI111B)
IL-1b (Cell Sciences, Cat# CRI133B)
IL-2 (Prometheus, Cat# Proleukin)
anti-CD3/CD28 Dynabeads (Invitrogen/Life Technologies, Cat# 111.311)
PMA (Sigma-Aldrich, Cat# P1585)
Ionomycin (Sigma-Aldrich, Cat# I0634)
Brefeldin A (BFA; Sigma-Aldrich, Cat# B7651)
Perm/Wash (BD, Cat# 51-2091KZ)
Cytofix/Cytoperm (BD, Cat# 51-2090KZ)
anti-IL-4-FITC (eBioscience, Cat# 53-7049-42)
anti-IL-13-APC (BD, Cat# 501907)
anti-IL-17-PE (Cap17) (eBioscience, Cat# 12-7178-42)
anti-IFNg-PerCP-Cy5.5 (Biolegend, Cat# 502526)
anti-IL-17-biotin (Dec17) (eBioscience, Cat# 13-7179-85)
anti-CD45-biotin (Biolegend, Cat# 304004)
Streptavidin (Biosource, (Cat# SNN1001)

500mL Corning 0.2µm Filter System (Cat# 430758)
15mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430766)
50mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430828)
Graduated serological pipets (5, 10, 25, 50mL)
Hemocytometer
Micropipet with P20 tips
Micropipet with P200 tips
Micropipet with P1000 tips
70µm filter
Microscope (preferably phase contrast)
Eppendorf Refrigerated Centrifuge 5810R
MACS Separator
FACS AriaII Cell Sorter
FACS LSRII Flow Cytometer
Tissue culture plates (96-well; 48-well; 24-well; 6-well)
Tissue culture flasks (T75)

Procedure

A. Reagent Preparation

MACS buffer
0.5% BSA and 2mM EDTA in PBS, sterile filtered, degassed

FACS buffer
1% BSA in PBS, sterile filtered

Growth Medium
AIM-V medium supplemented with 2% AB serum and 1% Penicillin/Streptomycin, sterile filtered

B. Isolation of PBMCs from peripheral blood

1) Collect 100-400mL of whole blood from donor.
2) Aliquot blood into 50mL conical tubes.
3) Centrifuge at 1300 rpm 10 min (brake OFF).
4) Remove white middle band (buffy coat) along with small portion of plasma and RBC portions.
5) Dilute each collected buffy coat (from 50mL of whole blood) to 50mL in sterile PBS.
6) Aliquot 20mL of Ficoll-Paque in 50mL conical tubes. One conical tube of Ficoll-Paque will be needed for each 30mL of whole blood processed. Overlay 30mL of PBS-diluted buffy coat over each of the Ficoll-Paque aliquots.
7) Centrifuge at 1800 rpm 20 min (brake OFF).
8) Collect 15-20mL of PBMC layer and dilute to 50mL in sterile PBS.
9) Centrifuge at 1300 rpm 10 min.
10) Resuspend cell pellets in 5mL MACS Buffer and filter through 70µm filter, collecting all PBMCs in 50mL conical. Rinse filter with 5-10mL MACS buffer.

11) Take a 10µL volume and dilute to 100µL in 1:10 Trypan Blue:PBS. Count cells with hemocytometer.

C. MACS enrichment of $CD4^+$ T cells

1) Centrifuge at 1300 rpm 10 min.
2) Resuspend cell pellet in 40µL MACS buffer/10^7 cells. Add 10µL of Biotin-Antibody Cocktail (T cell Isolation Kit II) per 10^7 cells. Incubate 10 min at 4°C.
3) Add 30µL MACS buffer/10^7 cells. Add 20µL of anti-Biotin-Microbeads (T cell Isolation Kit II) per 10^7 cells. Incubate 15 min at 4°C.
4) Wash cells by adding MACS buffer to 50mL and centrifuge at 1300 rpm for 10 min. Resuspend pellet in 500µL MACS buffer/10^8 cells.
5) Place LS columns (1 per 10^8 cells) in magnetic field of MACS Separator.
6) Prepare column with 3mL MACS buffer.
7) Discard effluent and change collection tube (15mL conical).
8) Apply 500µL of PBMCs in MACS buffer to column.
9) Wash column with 3 times 3mL MACS buffer. Collect effluent (contains $CD4^+$ T cells).
10) Combine collected $CD4^+$ T cells and count on a hemocytometer as described above.

D. FACS separation of T cell subsets

1) Aliquot 10^6 cells into each of 8 wells in 96-well plate and use cells as single stain compensation controls.
2) Add FACs buffer to $CD4^+$ T cells to 50mL and centrifuge 1300 rpm 10 min.
3) Resuspend cells in 200µL of FACs buffer. Add:
   50µL anti-CCR6-PE
   100µL anti-CCR4-PE-Cy7
   100µL anti-CXCR3-PE-Cy5
   5µL anti-CD4-Qdot 655
   100µL anti-CD3-APC-Cy7
   100µL anti-CD45RO-FITC
   100µL anti-CD25-APC
4) Stain 30 min on ice.
5) Wash cells with 50mL of FACS buffer and centrifuge 1300 rpm 10 min (2 times).
6) Resuspend cells at approximately $20x10^6$ cells/ml in growth medium and filter through a 70µm filter.
7) Sort cells on FACS Aria II (4-way sort) into 6mL tubes containing 1mL of growth medium. Isolate four T cell populations that are all $CD3^+$ $CD4^+$ $CD25^-$:
   a. Naïve: $CD45RO^-$ $CCR6^-$ $CCR4^-$ $CXCR3^-$
   b. Th1: $CD45RO^+$ $CCR6^-$ $CCR4^-$ $CXCR3^+$
   c. Th17: $CD45RO^+$ $CCR6^+$
   d. Th2: $CD45RO^+$ $CCR6^-$ $CCR4^+$ $CXCR3^-$

E. Process naïve $CD4^+$ cells directly for either nuclear DNaseI treatment and/or RNA isolation

See relevant SOPs.

F. Stimulation and expansion of Th1, Th2, and Th17 cells

1) Wash cells with growth medium, centrifuge 1300 rpm 10 min, and resuspend at 10^5 cells/mL.
2) To cells, add medium, IL-2, anti-CD3/28 microbeads, and relevant cytokine (IL-12 for Th1, IL-4 for Th2, IL-1b for Th17) at final concentrations of: $5x10^5$ cells/mL, 50U/mL IL-2, 10µL/mL anti-CD3/CD28 microbeads (vortex beads before pipeting), and 10ng/mL IL-12, IL-4, or IL-1b.
3) Place cells in culture container of appropriate size and incubate at 37°C, 5% CO_2 for 72 hours.
4) After 72 hours add equal volume of culture medium and fresh cytokines to final concentrations of 50U/mL IL-2 and 10µg/mL IL-4, IL-12, or IL-1b.
5) Repeat splitting of culture every 48-72 hours while adding fresh cytokines.

G. Confirmation of cytokine profile by intracellular cytokine staining

1) On day 7, take 10^5 cells from each effector cell type and restimulate with 0.7µM Ionomycin and 10ng/mL PMA in presence of BFA (10µg/mL) in growth medium. Add 2µL/mL anti-CD4-Qdot 655.
2) Stain cells with the following using BD cytofix/cytoperm kit and protocol:
   a. anti-IL17-PE
   b. anti-IFNg-PerCP-Cy5.5
   c. anti-IL4-FITC
   d. anti-IL13-APC
3) Analyze culture and verify purity of effector populations. All populations should be >99% $CD4^+$. For Th1, >50% cells producing IFNg; <2% producing IL-17 or IL-4. For Th2, >50% of cells producing IL-4 or IL-13; <2% producing IL-17 or IFNg.

H. On day 8 process Th1 and Th2 cells for either nuclear DNaseI treatment and/or RNA isolation

See relevant SOPs.

I. Cytokine capture for enrichment of IL-17 producing cells

1) On day 9, count Th17 cells as described above.
2) Adjust Th17 cells to 10^6 cells/mL in growth medium. Add 5ng/mL PMA and 0.5µM Ionomycin.
3) Incubate 1.5 hours at 37°C, 5% CO_2.
4) Transfer cells to 50mL conical tube and wash cells with FACS buffer, and centrifuge 1300 rpm 10 min.
5) Stain cells sequentially in FACS buffer at 10^8 cells/mL on ice with:
   a. 20µL/mL anti-CD45-biotin (0.5mg/mL) for 30 min
   b. 5µL/mL Streptavidin (1mg/mL) for 15 min
   c. 40µL/mL anti-IL-17-biotin for 15 min

   Wash with 50mL FACS buffer between each stain. *Note: no wash step after final stain with anti-IL-17.

6) Dilute to 10^6 cells/mL in growth medium and incubate on rotator at 37°C for 1 hour.
7) Spin down at 1300 rpm 10 min, wash once with FACS buffer (50mL), and stain at 10^8 cells/mL with 200µL/mL anti-IL-17-PE and 200µL/mL anti-CD4-APC in FACS buffer for 30 min on ice.
8) Wash with 50mL FACS buffer, spin down, and resuspend at $2x10^7$ cells/mL in AIM-V medium with 2% AB serum and filter through 70µm filter.
9) Sort PE^+ APC^+ cells by FACS (Aria II cell sorter; PE^- APC^+ as controls).

J. Expansion of IL-17 producing cells

1) Resuspend sorted cells at 10^6 cells/mL in growth medium + IL-2 (50U/mL) and incubate for 48 hours.
2) After 48 hours, stimulate cells by adding medium, IL-2, anti-CD3/28 microbeads, IL-1b to final concentrations of $5x10^5$ cells/mL, 50U/mL IL-2, 10µL/mL anti-CD3/CD28 microbeads (vortex beads before pipeting), and 10ng/mL IL-1b.
3) After 72 hours, add equal volume of culture medium and fresh cytokines to final concentrations of 50U/mL IL-2 and 10ng/mL IL-1b.
4) Repeat splitting of culture every 48-72 hours while adding fresh cytokines.

K. Seven days post stimulation, verify IL-17 production of cells by intracellular cytokine staining as before

Verify purity of Th17 as >99% CD4+, >50% cells producing IL-17; <2% producing IFNg or IL-4.

L. Process Th17 cells for either nuclear DNaseI treatment and/or RNA isolation

See relevant SOPs.