Primary Adult Naïve CD4+ CD45RA+ Cells

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Goal: To obtain large numbers of highly pure primary CD4+ CD45RO- CD25- cells from adult peripheral blood, and prepare them as naïve, Th0, and Th1 cells. This protocol combines two separate magnetic bead-based purification strategies, a positive selection step with Dynal CD4 beads followed by a negative selection step with EasySep Naïve Human T CD4 T cell beads, to give desired purity but with higher yields than can be obtained with FACS sorting and cells are "untouched" with no antibodies bound to their surface.

Reagents:

1) Dynal CD4 Positive Isolation Kit (Invitrogen)
2) EasySep Negative Selection Human Naïve CD4 T Cell Enrichment Kit (Stemcell)
3) 1 buffy coat (Research Blood Components)
4) Anti-CD25-biotin
5) RPMI + 1% FCS
6) T Cell Media = RPMI 1640 + 10%FCS + Pen/Strep + 2mM L-glut + 10mM Hepes + 50μM 2-βME
7) 2X Freezing media = 8ml FCS + 2ml DMSO
8) Human recombinant IL-12 (R&D Systems)
9) PBE (PBS plus 2mM EDTA)

Step 1: Positive Selection of CD4 Cells with Dynal Beads

1) Buffy coat should contain 0.5-1 x 10^9 cells. Of these around 10% should be CD4 T cells, so there are about 0.5-1 x10^8 CD4 T cells in the pack.
2) Dilute Buffy coat 1 part cells to 2 parts PBE
3) Wash Dynal beads once with PBE. Add beads at ratio of 5 beads to 1 cell = 5 x 10^8 beads = 1.2ml of beads.
4) Incubate for 20 minutes at 4°C with gentle mixing.
5) Place tube on magnet for 2 minutes.
6) Discard supernatant.
7) Gently wash bead-bound cells 3 times with cold PBE.
8) Resuspend the bead-bound cells in 10 ml of RPMI/1% FCS.
9) Release cells by adding 400μl of Detach-a-bead solution and incubate for 45min at room temp with gentle rotation.
10) Place on magnet to remove beads.
11) Wash beads with RPMI/1% FCS x 2 to remove as many cells as possible.
12) Collect all cells and count. Cells should be highly pure CD4+ cells.

Step 2: CD45RO and CD25 depletion

1) Wash cells x 2 in PBE to remove Detachabead.
2) Per million purified CD4 T cells, add 0.5 anti-CD25-biotin.
3) Incubate on ice x 30min.
4) Wash once gently with PBE.

5) Resuspend in PBE at $5x10^7$ cells/ml
6) Ad 50µl CD45RO-biotin (from kit) per ml of cell suspension.
7) Incubate at RT for 15min
8) Add 50ul Enrichment Cocktail per ml of cells
9) Incubate at RT for 10min.
10) Add 100µl StemCell nanoparticles and incubate for 10min.
11) Place tube in magnet for 10 min.
12) Pour of unbound CD4 cells.
13) Wash beads and repeat.
14) Count final cells, spin down, and resuspend in T Cell media.

<u>Step 3: Stimulation for DNAse I experiments</u>

1) For Naïve Cells, take 10 million in T Cell Media. Add 2X Freezing media and freeze immediately.
2) For Naïve Cells with Short Stim, Take 10 million and stimulate in T cell media at 37° and 7% CO_2 with plate-bound anti-CD3 + anti-CD28 for 4 hours. Then freeze cells.
3) For Th0 Cells, take 10 million and culture with CD3/CD28 beads with no cytokines added (neutral conditions) in T cell media at 37° and 7% CO_2 for 3 days. Remove beads, collect cells, and freeze.
4) For Th1 Cells, take 10 million and culture with CD3/CD28 beads + 1ng/ml IL-12 (Th1 conditions) in T cell media at 37° degrees and 7% CO_2 for 3 days. Remove beads, collect cells, and freeze.

SOP: **Isolation of human primary naïve $CD4^+$ T 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.

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-Cy7 (BD, Cat# 557832)
anti-CD45RO-FITC (eBioscience, Cat# 11-0457)
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)
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)

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. In addition to the naïve CD4+ cells, three other T cell populations can be obtained as described below; all these populations are also $CD3^+$ $CD4^+$ $CD25^-$:
   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.