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)

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 = 1.2 ml 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 400 ul of Detach-a-bead solution and incubate for 45 minutes 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 30 minutes.
4) Wash once gently with PBE.

5) Resuspend in PBE at 5x107 cells/ml
6) Ad 50ul CD45RO-biotin (from kit) per ml of cell suspension.
7) Incubate at RT for 15'
8) Add 50ul Enrichment Cocktail per ml of cells
9) Incubate at RT for 10min.
10) Add 100ul 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.

Step 3: Stimulation for DNAse I experiments

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 + 1 ng/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.