Staining Mononuclear Cells for CTLA4 in Conjunction with Cell Surface Phenotyping

Obtaining a Mononuclear Cell Preparation

- Two 10ml Vacutainers (Sodium Heparin) of blood are collected from a normal, healthy donor by veinipuncture.
- The blood is pooled and QS to 35ml with room temperature Hanks Balanced Salt Solution (HBSS) into a 50ml conical.
- The blood / HBSS suspension is mixed to homogeneity with multiple inversions and underlaid with 15ml of RT ficoll using a variable speed automatic pipettor (with dispense set to slow) fitted with a 10ml serological pipette.
    - Dispense the ficoll slowly (<1ml / 30 seconds) for the first 30 seconds and then increase the speed of dispensing to an appropriate rate that will elevate the blood suspension without disturbing the blood - ficoll interface.
- Centrifuge balanced ficoll apparatus for 30', 25°C at 400g (1500rpm) without the brake.
- Harvest the mononuclear cells from betwixt the plasma – ficoll interface with a sterile 1ml pipet tip affixed to a clean P1000. Pool the MNCs into a sterile 50ml conical tube.
- QS the cells to 50ml with HBSS to wash away the platelets. Centrifuge for 5', 25°C at 400g (1500rpm) with the brake applied. Do not exceed 400g, lest platelets sediment into the pellet.
- Decant the supernatant and resuspend the cells in the residual buffer. QS the cells to 50ml with RT complete media for the second wash (cell counts using trypan blue have been erratic when cells are suspended in HBSS). Centrifuge for 5', 25°C at 400g (1500rpm) with the brake applied. Do not exceed 400g, lest platelets sediment into the pellet.
- Decant the supernatant and resuspend the cells in the residual buffer. The cells have now been washed twice and are resuspended in complete media. QS the cells to 10ml (if using two vacutainers of blood) with RT complete media and perform a cell count at a 1:5 dilution (10µl cells + 40µl trypan blue [or PI, if using the fluorescence microscope]). Alternatively, the clinical coulter counter can be used to determine the cell concentration.

Culturing Cells

- the concentrations are 0.5µg/ml _CD28 [final] and 0.25µg/ml _CD3 [final].
- Adjust the concentration of sample to 1 x 10^6 cells/ml with complete media.
- Divide the mixed cell suspension into two equal whole-number volumes (ml).
- Label one aliquot "Unstimulated" and the second "CD3/CD28"
- To the 'CD3/CD28' aliquot, add the appropriate volume of the following UNCONJUGATED, sterile antibodies to yield the final concentrations indicated below:
    - _CD3: [Stock] = 480µg / ml; [final] = 0.25µg / ml
      (the protocol calls for 50µl of a 1µg/ml stock per 200µl of cultured cells)
    - _CD28: [Stock] = 1000µg / ml; [final] = 0.5µg / ml
      (the protocol calls for 50µl of a 2µg/ml stock per 200µl of cultured cells)
- Mix the cell aliquots to homogeneity and aliquot 1ml fractions into each appropriate well of a 24 well tissue culture plate.
- Incubate the plate for the desired period of time (i.e. 4 hours or overnight).

Harvesting Cells for Flow Cytometry

- After the incubation period has elapsed, harvest the cell suspensions with consistent and gentle trituration with a sterile 1ml pipet tip affixed to a clean P1000 (set to 750µl, to minimize 'frothing').
- Pool two cultured wells of test article for every tube that will be stained, into individually labeled 15ml conical tubes (two wells are combined to account for cell loss during the harvesting and washing steps).
- QS the pooled cell suspensions to 15ml with PBS to wash away the cell culture media. Centrifuge the cells for 3', 4°C, 3200rpm. Decant the supernatant and resuspend cells in residual buffer.

Staining Cells for Surface Antigen Markers, Part A

- Transfer the cell suspensions to appropriately labeled 12 x 75mm falcon tubes. Add 6.7µl of mouse IgG to each tube to block FcR binding to antibodies and Incubate 20' on ice.
- Add the required volume of antibody (20µl) from ITN panel 33A (CD62L / CD25 / CD4 / CD8) to each appropriate tube and incubate in the dark, on ice for 20'.
- Wash the labeled cells with 3ml PBS and centrifuge the suspension for 3', 4°C, 3200rpm. Decant the supernatant and resuspend cells in residual buffer.
- Repeat the previous step such that a total of two washes are achieved.
- Add 200µl methanol-free formaldehyde to each tube to fix the cells. Incubate tubes overnight at 4°C or for 30' at room temperature.

Staining Cells for Intracellular CTLA4, Part B

- Wash away the formaldehyde from Step A above, with 3ml of PBS and centrifuge the suspension for 3', 4°C, 3200rpm. Decant the supernatant and resuspend cells in residual buffer.
- Add 50µl of Caltag permeablization buffer and mix *gently*.
- Add the required volume of intracellular antibody (20µl) from ITN panel 33B (CTLA4) to each appropriate tube and incubate at room temperature for 30' in the dark.
- Add 3ml of PBS and incubate for an additional 10' at room temperature in the dark.
- Centrifuge the suspension for 3', 4°C, 3200rpm. Decant the supernatant and resuspend cells in residual buffer.
- Add 200µl methanol-free formaldehyde to each tube to fix the cells. Incubate tubes at 4°C in the dark for at least 20' prior to acquisition.