Title: FoxP3

PRINCIPLES OF THE PROCEDURE:

Expression of FOXP3 on CD3+, CD4+, and CD25+ cells is associated with T regulatory (Tregs) and some activated T cells.

Regulatory T cells/Tregs (also known as suppressor T cells) are a specialized subpopulation of T cells that act to suppress immune responses thereby maintaining homeostasis and self tolerance. Intense investigation has tried to determine exclusive protein markers for Tregs. This has been complicated by the identification of four regulatory T cells subsets; natural, anergic, Tr1 and CD8+CD28- suppressor T cells. There are still no cell surface molecules that uniquely distinguish functional regulatory cells from conventional T cells. It has been proposed that the "natural" regulatory T cells originally recognized by their constitutive expression of CD4 and CD25 are further defined by expression of the transcription factor Foxp3. Additionally Foxp3 has also been characterized in CD8+CD28- suppressor T cells.

T regulatory (Treg) cells are a subset of T lymphocytes which is characterized by $CD4^+/CD25^+/FOXP3^+$. These naturally occurring Treg cells originate in the thymus, and comprise 2-10% of peripheral $CD4^+$ T cells. It has been shown that Treg cells are able to inhibit T cells proliferation and cytokine production and play critical roles in preventing autoimmunity as well as in controlling tumor immunity and transplantation tolerance. Impaired Treg function or Treg cell deficiency will develop variety of autoimmune diseases, while higher frequency of Treg cells will cause hypo-immune response to pathogens.

SPECIMEN REQUIREMENTS:

Ficolled cells or whole blood

REAGENTS:

1. PBS
2. FCM Buffer
   Phosphate buffered saline
   Bovine serum albumin (Sigma A4503)
   Sodium azide (Fisher 5227)
   Na_2EDTA (Sigma)

Dissolve in 1 liter of 1X PBS, 10 g BSA, 0.04 g Na_2EDTA and 1 g sodium azide.
Stored at 4-8˚C, this solution is stable for 6 months.

3. Antibody Panels V606/V607 (ICATA) and V608/V609 (UREKA) with corresponding PECy7.
4. eBioscience Fix/Perm Concentrate: (Cat. #00-5123-43) at a 1:4 dilution with the Diluent.
5. EBioscience Fix/Perm Diluent: (Cat#00-5223-56).
6. EBioscience 10X Permeabilization Buffer: (cat# 00-8333-56) at a 1:10 dilution in dH_2O.
7. Biolegend Alexa Fluor 488 Mouse IgG1 Isotype: (Cat. #400134) at the appropriate titer.

appropriate titer.

9. Human IgG Block at 12mg/ml.
   Block IgG:
   12 mg/ml human IgG Cohn fraction II and III globulins (Sigma G-4386).
   Dilute in PBS.
   Store frozen. Thaw when needed and store in the refrigerator. Keep this sterile.
10. Methanol Free Formalin
   2% EM grade 10% buffered Formalin (Polysciences 08379)
   dilute in PBS

EQUIPMENT & INSTRUMENTATION

Rack
Ice
Pipettors and tips
Timer
12x75mm Flow Tubes
Waste container and Blotting cloth
Centrifuge
15ml and 50ml Centrifuge tubes for Buffers
BD Canto or BD LSRII
Winlist Flow Cytometry Analysis Software

PROCEDURE:

A. Ficoll Cells:

1. Centrifuge the cells at 1400 rpm for 5 minutes.
2. Aspirate off as much of the media without disturbing the pellet.
3. Measure the remaining volume.
4. Add FCM Buffer so that each tube will have a total volume of 100ul.
5. Add 10µl per $1x10^6$ cells of Human IgG to block the FC receptors on the cell surface. Vortex tube.
6. Place tube on ice for 10 minutes.

B. Whole Blood:

1. Place 2 - 3 mls of whole blood in a 15ml centrifuge tube.
2. Add 10 - 12 mls of cold PBS to the tube to wash and vortex.
3. Centrifuge at 3200 rpm for 3 minutes.
4. Aspirate off the PBS as close to the Buffy Coat as possible without disturbing it.
5. Add 10-12 mls of cold FCM Buffer to the tube to wash and vortex.
6. Aspirate off the FCM Buffer so to have 2 mls left.
7. Add 100 µl of Human IgG per ml of blood. i.e. 2 mls left add 200 µl of Human IgG. Vortex the tube.
8. Place on ice for 10 minutes.

C. The following part of the procedure is the same for either FICOLL Cells or Whole Blood

1. Aliquot 100 µl of cells to each flow tube.
2. Add the mab panels to each tube and vortex. Place on ice in the dark for a

3. After the 1 hour incubation vortex the tube and add 3.5 mls of Ammonium Chloride Lyse Solution. Cover the tubes and invert 3 times to mix completely and let sit at room temperature for 5 minutes.
4. Centrifuge at 3200 rpm for 3 minutes. Decant off the liquid and blot tubes 4 times and vortex.
5. Wash the tubes 2 times in 3 mls of FCM Buffer. Centrifuge at 1400 rpm for 5 minutes each time. Decant off the liquid and blot 4 times and vortex after each wash.
6. Resuspend in the 1:4 dilution of the Fix/Perm Buffer, 1 ml per tube. Incubate at room temperature in the dark for 30 minutes.
7. After the 30 minute incubation of the Fix/Perm, centrifuge at 1400rpm for 5 minutes. Decant and blot and vortex the tubes. Wash 2 times all tubes with 2 mls of the 1:10 dilution of Perm Buffer. Centrifuge at 1400 rpm for 5 minutes, decant, blot, (do not vortex until after the last wash). Make sure after the second wash and centrifuge that the tube is blotted very well; you want less than 50 µl of residual Perm Buffer left in the tube before adding the Human IgG.
8. Add 10 µl per tube of Human IgG and let sit on ice for 10 minutes.
9. After the 10 minute incubation on ice add the appropriate volume of the Isotype Control and FOXP3 antibody to each tube. Vortex and let incubate at room temperature for 1 hour in the dark.
10. Add 1ml of the 1:10 dilution of Perm Buffer to each tube to wash. Centrifuge at 1400 rpm for 5 minutes. Decant and blot the tubes. No vortex at this time.
11. Add 3 mls of FCM Buffer to wash each tube. Centrifuge at 1400 rpm for 5 minutes. Decant, blot and vortex the tubes.
12. Add between 300 µl and 500 µl of 2% Formalin to fix the cells.
13. Place the tubes in the refrigerator overnight and run samples on the BD Canto or BD LSRII the next day.

PROCEDURAL NOTES:

Ficolled cells stored in RPMI are stable for several days check viability.
Whole blood collected in EDTA is stable for 24 hours.
Whole blood collected in Heparin is stable for 48 hours.

QUALITY CONTROL GUIDELINES

Run an FMO (fluorescence minus one) control to set FOXP3 positive/negative regions

EXPECTED VALUES:

Not Determined

REPORTING RESULTS & CALCULATIONS

Fix/Perm Buffer (1:4 dilution): add 1ml of the concentrated Fix/Perm to 3 mls of the Fix/Perm diluent. Going to need 1 ml per tube overall so calculate accordingly.

Perm Buffer 1:10 dilution: add 1ml of the Perm Buffer to 9 mls of dH2O. Going to need 5mls of the Perm buffer per tube overall so calculate accordingly.

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