PROCEDURE:	Intracellular Staining of Peripheral Blood and Bone Marrow Cells for Detection of Cytokine Producing Cells

PRINCIPLE:

Multicolor immunofluorescent staining can detect and characterize intracellular cytokines as well as surface antigens on individual cells within a mixed population using multiparameter flow cytometry. Cells are activated in culture with an antigen to stimulate cytokine response then incubated with a protein transport inhibitor to prevent cytokines from secreting into the media. Cells are initially stained for surface antigens then fixed with paraformaldehyde. Cells can then be permeablilized to allow cytokine specific monoclonal antibodies to penetrate the cell membrane and fluorescently label cytokines of interest.

EQUIPMENT, MATERIAL and REAGENTS:

Equipment:
Centifuge with swinging bucket rotor (Sorvall RT 6000D)
Vortex mixer, variable speed
Pipettors, adjustable volume (Pipetman or Denville)
Repeating pipettor, adjustable volume (Eppendorf)
Sample tube racks
Timer
Fluorecent microscope

Materials:
50 mL polypropylene conical tubes
9 inch sterile glass pasteur pipets
sterile disposable transfer pipet
hemacytometer with glass cover slip
12 x 75 mm polystyrene round bottom tubes
Pipette tips, polypropylene, 1-10μl and 10-200μl (Denville)
Syringe pipet tips for repeating pipet (Eppendorf)

Reagents:

1x Phosphate Buffer Saline (PBS) (MediaTech #21-040-CV)

Lymphocyte Separation Media (MediaTech #25-072-CI)
Complete Media (see reagent procedure)
Brefeldin A (BFA) (Sigma #B-7651)
Phorbol 12-Myristate 13 Acetate PMA (Sigma #P8139)
Ionomycin (Sigma # I-0634)
Staining Media (1x PBS with 3% FCS and 2mM EDTA)
Fluorescent labeled monoclonal antibodies (Becton-Dickinson or Pharmingen)
Cytoperm/Cytofix Buffer (BD Pharmingen #2090KZ)
Perm/Wash Buffer (BD Pharmingen #2091KZ)

PROCEDURE:

Isolation of Mononuclear Cells by Ficoll Gradient

1. Transfer 20 ml of ACD-A or sodium heparinized blood to a 50 mL conical tube.

2. Add an equal amount of 1xPBS to each conical. Mix gently.

3. Add 10 mL of Lymphocyte Separation Medium to the bottom of each tube by underlayering the solution using a glass pasteur pipet.

4. Centrifuge 50 mL conical tubes at 1500 rpms with the brake off for 20-30 minutes.

5. Aspirate off the top layer that contains the plasma/platelet layer.

6. Remove the mononuclear cell layer gently with a sterile plastic disposable pipet.

7. Place mononuclear layer into a fresh 50 mL conical tube.

8. Wash 2x by adding 1xPBS to bring volume up to 40 mL , vortex gently, centrifuge samples for 5 minutes at 1500 rpms and remove supernatant

9. Add 1xPBS to desired volume to perform a cell count and viability of the isolated mononuclear cells using ethidium bromide/acridine orange DNA-binding dyes.

Stimulation of Cells:

1. Add 1 x 10^6 cells into 1 mL of complete media in a 12x75mm polystyrene round bottom tube.

2. Add stimulating antigen at the desired concentration.

3. Cap and vortex suspension gently. Loosen cap and place in a 37^0 C incubator with 5% CO_2 at a 5 degree slant for 5 hours.

4. In the last 4 hour of incubation, add 10 μg/mL of Golgi Plug (brefeldin A -secretion inhibitor) to all tubes.

5. In the last 4 hour of incubation also add PMA (25ng/mL) and Ionomycin (1μg/mL) to positive controls tubes.

6. At completion of culture stimulation, add 1 mL of staining media to each tube, vortex gently, and centrifuge at 1500 rpms for 5 minutes.

7. Aspirate supernatant and proceed with the following staining procedure.

Extracellular Staining Procedure:

1. Add 100 μl of staining media to each tube, vortex gently.

2. Pipet appropriate amount of each extracellular antibody into each tube, vortex gently.

3. Incubate samples in the dark at room temperature for 15 minutes.

4. Wash cells 2x by adding 1 mL of staining media, vortex gently, centrifuge samples for 5 minutes at 1500 rpms and remove supernatant.

Intracellular Staining Procedure

1. Fix and permeabilize cells by adding 250 μl of Cytofix/Cytoperm solution (BD Pharmingen) then incubate for 10-20 minutes at 4^0C.

2. Wash cells 2x in 1 mL of**1x Perm/Wash solution**** (BD Pharmingen), vortex gently, centrifuge at 1500 rpms for 5 minutes and remove supernatant.

   **** From here on out, intracellular staining must be in the presence of 1x Perm/Wash Solution for cells to remain permeabilized**

3. Add 100 μl of1x Perm/Wash solution (BD Pharmingen).

4. Pipet appropriate amount of intracellular cytokine monoclonal antibody into each respective tube, vortex gently.

5. Incubate at 4^0C for 30 minutes in the dark.

6. Wash cells 2x in 1 mL of1x Perm/Wash solution (BD Pharmingen), vortex gently, centrifuge at 1500 rpms for 5 minutes and remove supernatant.

7. Add 250 μl of staining media to each tube and store in the dark until ready for flow cytometric acquisition.

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