Intracellular cytokine staining on PBMCs using CyTOF™ Mass Cytometry

1. Principle

Production of cytokines plays an important role in the immune response. Cytokines are involved in many different pathways including the induction of many anti-viral proteins by IFN gamma, the induction of T cell proliferation by IL-2 and the inhibition of viral gene expression and replication by TNF alpha. Cytokines are not preformed factors but are rapidly produced and secreted in response to cellular activation.

Intracellular cytokine detection at the single-cell level has emerged as the premier technique for studying cytokine production. It detects the production and accumulation of cytokines within the endoplasmic reticulum after cell stimulation, allowing direct TH1 versus TH2 determination.

In this protocol, we use a CyTOF™ mass cytometer to acquire the ICP-MS data. The current mass window selected is approximately AW 103-203, which includes the lanthanides used for most antibody labeling, as well as iridium and rhodium for DNA intercalators. The principle steps of intracellular cytokine staining is as follows:

- Cells are activated for a few hours using either a specific peptide or a non-specific activation cocktail
- An inhibitor of protein transport (e.g. Brefeldin A) is added to to retain the cytokines within the cell
- Next, EDTA is added to remove adherent cells from the activation vessel

- After washing, antibodies to cell surface markers can be added to the cells
- The cells are then fixed in paraformaldehyde and permeabilized
- The anti-cytokine antibody is added and the cells can be analyzed by CyTOF.

2. Materials and Equipment

2.1. PBMC, fresh or thawed frozen
2.2. Complete RPMI (RPMI, 10% FBS, Pen-Strep, glutamine)
2.3. Benzonase (Sigma Catalog # B7651)
2.4. Brefeldin A (Sigma Catalog # B7651)
2.5. Monensin, 1000x (Biolegend Catalog # 420701)
2.6. 0.5M EDTA
2.7. 2% Para-formaldehyde (PFA), made in 1x CyPBS
2.8. CyPBS (1x PBS without heavy metal contaminants, such as 10X PBS from Rockland; made in MilliQ water)
2.9. CyFACS buffer (1x CyPBS with 0.1% BSA, 2 mM EDTA and 0.05% Na Azide; made in MilliQ water). Do NOT use FBS!
2.10. MilliQ water: no contact with beakers or bottles washed with soap (due to barium content of most commercial soaps).
2.11. Phenotyping antibodies, filtered with 0.1 um spin filters
2.12. Live-dead stain: 5 mg/mL maleimide-DOTA (eg, Macrocyclics B-272) loaded with 139-Lanthanum* or 115-Indium* (*: natural-abundance metal chloride salt used; >95% specified isotope; trace-metal pure 99.99%)
2.13. Ir-intercalator stock solution from DVS (Rh103-intercalator can be

used).

2.14. Saponin-based permeabilization buffer: 10x (eg, eBiosciences ca# 00 8333-56)

2.15. 96- well round-bottom plates

2.16. 37°C water bath

2.17. Biosafety cabinet

2.18. Centrifuge

2.19. CO_2 incubator at 37°C

2.20. Calibrated pipettes

3 Procedure

Thaw PBMC

3.1 Warm media to 37°C in water bath. Each sample will require 22ml of media with benzonase. Calculate the amount needed to thaw all samples, and prepare a separate aliquot of warm media with 1:10000 benzonase (25 U/ml). Thaw no more than 3 samples at a time. Run one control PBMC with each batch of samples.

3.2 Remove samples from liquid nitrogen and transport to lab on dry ice.

3.3 Place 10ml of warmed benzonase media into a 15ml tube, making a separate tube for each sample.

3.4 Thaw frozen vials in 37°C water bath.

3.5 When cells are nearly completely thawed, carry to hood.

3.6 Add 1ml of warm benzonase media from appropriately labeled centrifuge tube slowly to the cells, then transfer the cells to the centrifuge tube. Rinse vial with more media from centrifuge tube to retrieve all cells.

3.7 Continue with the rest of the samples as quickly as possible.

3.8 Centrifuge cells at 1550rpm (RCF=805) for 8 minutes at room temperature.

3.9 Remove supernatant from the cells and resuspend the pellet by tapping the tube.

3.10 Gently resuspend the pellet in 1ml warmed benzonase media. Filter cells through a 70 micron cell strainer if needed. Add 9 ml more warmed benzonase media to the tube.

3.11 Centrifuge cells at 1550 rpm for 8 minutes at room temperature. Remove supernatant from the cells and resuspend the pellet by tapping the tube.

3.12 Resuspend cells in 1ml warm media.

3.13 Count cells with Vicell (or hemocytometer if necessary). To count, take 20 ul cells and dilute with 480 ul PBS in vicell counting chamber. Load onto Vicell as PBMC with a 1:25 dilution factor.

3.14 Adjust the cell concentration to 5-10 * 10^6 cells/ml with warm media (no more benzonase at this point.)

3.15 Using a multichannel pipette, add 200 µl cells (1 * 10^6 cells) into each well of a 96-well deep well plate. Split each sample into two or more wells keeping one as an unstimulated control and the others for different types of stimulation.

3.16 Rest overnight (6-18h) at 37°C in CO_2 incubator.

Stimulate Cells

3.17 After overnight rest at 37°C, add the activation reagents and secretion inhibitor (Brefeldin A/ Monensin) to the well for stimulation. Add only the secretion inhibitor to the unstimulated control well.

Reagent	Stock Concentration	Intermediate Dilution	Final Concentration
phorbol12-myristate13acetate (PMA)	1 mg/mL in DMSO (store in aliquots at -20C)	1:1000 in PBS	10 ng/ml
Ionomycin	1 mg/ml in DMSO (store in aliquots at -20C)	1:10 in PBS	1 ug/ml
Phytohemagglutinin (PHA)	1 mg/mL in DMSO (store at 4C)	1:10 in PBS	1 ug/ ml
SEB	50 μg/mL in PBS	None	1 μg/mL (1:50)
Peptide mixes	0.5-1 mg/mL/pep in DMSO (store in aliquots at -20C)	1:10 in PBS	1 μg/mL/peptide (1:50 - 1:100)
Brefeldin A	5 mg/mL in DMSO (store in aliquots at -20C)	1:10 in PBS	10 μg/mL (1:50) or 5μg/mL (1:100) with monensin
Monensin	5 mg/mL in ethanol (store at -20C)	1:10 in PBS	10 μg/mL (1:50) or 5 μg/mL (1:100) with brefeldinA
Anti-CD3/ CD28	Follow manufacturer instruc	-	-

Note: [1] It is important to avoid solvent toxicity. Final DMSO+ethanol concentration from all sources (peptides, brefeldin A, monensin) should not exceed 0.5%.

[2] For most cytokines: use brefeldin A at 10 μg/mL final concentration (see stock preparation table). For CD107 and CD154: use monensin at 10 μg/mL final concentration (see stock preparation table). For assays combining cytokines and CD107 or CD154: use brefeldin A and monensin at 5 μg/mL final concentration each.

[3]Addition of costimulatory antibodies is optional. Add 1 μg/mL final concentration of CD28 and/or CD49d (labeled antibody can be used if analysis of the marker is desired).

3.18 Incubate the cells for 4 hours (PMA+Ionomycin stimulation, PHA+Ionomycin stimulation) or 6-8 hours (anti-CD3/CD28 stimulation, peptide stimulation) at 37°C, in a CO_2 incubator.

Note: For most cytokines 6-12 h incubation at 37°C is sufficient; For IL-10 and TGF incubate for 12-24 h

3.19 Add EDTA to a final concentration of 2 mM and incubate for 15 min at room temperature.

Staining

3.20 Wash 2x with 250 ul CyFACS buffer. 1550 rpm, 10 min at room temperature.

3.21 Make surface Ab cocktail in CyFACS buffer (Filter with 0.1 um spin filter). 70 ul per reaction. Incubate on ice for 45 min.

3.22 Wash 2X in CyFACS buffer.

3.23 Resuspend cells in 100ul of 1:3000 diluted 5 mg/ml 115-DOTA maleimide (titrated if new stock) in CyPBS, Incubate 30 min on ice.

3.24 Wash 3x in CyFACS buffer.

3.25 Resuspend in 100 ul of 2%PFA in CyPBS, Incubate 4 degrees overnight.

3.26 Wash 2x in 1x eBioscience perm buffer (1x in milliQ water). 2000rpm (RCF=805), 10 min at 4 degree.

3.27 Make intracellular staining cocktail in 1x perm buffer and filter with 0.1 um spin filter, 70 ul per reaction. Incubate on ice for 45 min.

3.28 Wash 3x in CyFACS buffer

3.29 Resuspend in 100 ul 1:2000 Ir-Interchelator diluted in 2% PFA (in CyPBS).

3.30 Incubate 20 min at room temp.

3.31 Wash 2x in CyFACS buffer

3.32 Wash 3x in MilliQ water

3.33 Resuspend in MilliQ water (1 to 1.5 ml) for running on CyTOF.