Intracellular Cytokine Staining (ICS) on Human Lymphocytes or Peripheral Blood Mononuclear Cells (PBMCs)

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[Abstract] 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 by flow cytometry has emerged as the premier technique for studying cytokine production at the single-cell level. It detects the production and accumulation of cytokines within the endoplasmic reticulum after cell stimulation, allowing direct TH1 versus TH2 determination. It can also be used in combination with other flow cytometry protocols for immunophenotyping using cell surface markers or with MHC multimers to detect an antigen specific response, making it an extremely flexible and versatile method. This capability, combined with the high throughput nature of the instrumentation, gives intracellular cytokine staining an enormous advantage over existing single-cell techniques such as ELISPOT, limiting dilution, and T cell cloning. The principle steps of intracellular cytokine staining is as follows:

1. Cells are activated for a few hours using either a specific peptide or a non-specific activation cocktail;
2. An inhibitor of protein transport (*e.g.* Brefeldin A) is added to retain the cytokines within the cell;
3. Next, EDTA is added to remove adherent cells from the activation vessel;
4. After washing, antibodies to cell surface markers can be added to the cells;
5. The cells are then fixed in paraformaldehyde and permeabilized;
6. The anti-cytokine antibody is added and the cells can be analyzed by flow cytometer.

Materials and Reagents

1. PBMC (fresh or thawed frozen)
2. RPMI-1640 (Hyclone, catalog number: SH30027.01)
3. FBS (Atlanta Biologicals, catalog number: S11150)

4. 100x Pen-strep-Glutamine (Hyclone, catalog number: SV30082.01)
1. Benzonase (Sigma-Aldrich, catalog number: B7651)
2. PBS (10x stock) (Rockland, catalog number: MB-008)
3. Sodium azide (10% w/v solution) (Teknova, catalog number: S0209)
4. PMA (Sigma-Aldrich, catalog number: P8139)
5. Ionomycin (Calbiochem®, catalog number: 407952)
6. Dynabeads Human T Activator CD3/CD28 (Life Technologies, Invitrogen™, catalog number: 111.32D)
7. Brefeldin A (Sigma-Aldrich, catalog number: B7651)
8. 1,000x monensin (BioLegend, catalog number: 420701)
9. 0.5 M EDTA (Sigma-Aldrich, catalog number: E-5134)
10. LIVE/DEAD® fixable red dead cell stain (Life Technologies, Invitrogen™, catalog number: L23102)
11. 10x FACS lysing solution (BD Biosciences, catalog number: 349202)
12. 10x FACS permeabilizing solution 2 (BD Biosciences, catalog number: 347692)
13. Fluorochrome-linked surface markers [*e.g.* CD3-V500 (BD Biosciences, catalog number: 561416); CD8-V450 (BD Biosciences, catalog number: 560348); CD4-PerCP-Cy5.5 (BD Biosciences, catalog number: 341654)]
14. Fluorochrome-linked cytokine antibodies [*e.g.* IFN gamma-FITC (BD Biosciences, catalog number: 340449); IL-17 PE (BD Biosciences, catalog number: 560438); IL-2 PE-Cy7 (BD Biosciences, catalog number: 560707); IL-22-APC (R&D systems, catalog number: IC7821A); TNF- Alexa fluor 700 (BD Biosciences, catalog number: 557996)]
15. BD CompBeads [(anti-mouse Igκ, anti-rat Igκ, or anti-rat/hamster Igκ; BD Biosciences), for creating single-color compensation controls (BD Biosciences, catalog number: 560707)]
16. Immunoglobulin capture beads for single-color compensation (*e.g.*, BD Biosciences, catalog number: 560497)
17. Complete RPMI (see Recipes)
18. FACS buffer (see Recipes)

Equipment

1. 96- deep well V-bottom plates (Corning, catalog number: 3960) (1 ml washes in a 2 ml well volume)
2. Falcon round-bottom FACS tubes
3. Magnet for Dynabead separation (BD Biosciences, IMag, catalog number: 552311)
4. 37 °C water bath

5. Biosafety cabinet
6. Centrifuge
7. CO_2 incubator at 37 °C
8. Calibrated pipettes
9. ViCell (Beckman Coulter) or Hemocytometer cell counter

Procedure

A. Thaw PBMC

1. Warm complete medium to 37 °C in water bath. Each sample will require 22 ml of medium with benzonase. Calculate the amount needed to thaw all samples, and prepare a separate aliquot of warm medium with 1:10,000 benzonase (final concentration: 25 U/ml). Thaw no more than 3 samples at a time.
   Note: Run one control PBMC with each batch of samples if doing a large longitudnal study split over sevral batches.
2. Remove samples from liquid nitrogen and transport to lab on dry ice.
3. Place 10 ml of warmed benzonase medium into a 15 ml tube, making a separate tube for each sample.
4. Thaw frozen vials in 37 °C water bath.
5. When cells are nearly completely thawed, carry to hood.
6. Add 1 ml of warm benzonase medium from appropriately labeled centrifuge tube slowly to the cells, then transfer the cells to the centrifuge tube. Rinse vial with more medium from centrifuge tube to retrieve all cells.
7. Continue with the rest of the samples as quickly as possible.
8. Centrifuge cells at 1,550 rpm (RCF = 473), 10 min at room temperature.
9. Decant or aspirate supernatant from the cells and resuspend the pellet by tapping the tube.
10. Gently resuspend the pellet in 1 ml warmed benzonase medium. Filter cells through a 70 µm cell strainer if needed. Add 9 ml more warmed benzonase medium to the tube.
11. Centrifuge cells at 1,550 rpm (RCF = 473), 10 min at room temperature. Decant or aspirate supernatant from the cells and resuspend the pellet by tapping the tube.
12. Resuspend cells in 1 ml warm medium.
13. Count cells with Vicell (or hemocytometer if necessary). To count, take 20 µl cells and dilute with 480 µl PBS in vicell counting chamber. Load onto Vicell as PBMC with a 1:25 dilution factor.
14. Adjust the cell concentration to 5-10 x 10^6 cells/ml with warm medium (no more benzonase at this point).

15. Using a multichannel pipette, add 200 µl cells (1 x 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.
16. Rest overnight (6-18 h) at 37 °C in CO_2 incubator.
    Note: The percentage of both CD^{4+} and CD^{8+} cytokine producing T cells is increased after an overnight resting prior to stimulation (Horton et al., 2007).

B. Stimulate cells

1. 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.

Table 1. Protein secretion inhibitors

Reagent	Stock concentration	Intermediate dilution	Final concentration
Brefeldin A	5 mg/ml in DMSO (stored in aliquots at -20 °C)	1:10 in PBS	10 µg/ml (1:50) or 5 µg/ml (1:100) with monensin
Monensin	5 mg/ml in ethanol (stored at 4 °C)	1:10 in PBS	10 µg/ml (1:50) or 5 µg/ml (1:100) with brefeldin A

Table 2. Activators

Reagent	Stock concentration	Intermediate dilution	Final concentration
Phorbol12-myristate13 acetate (PMA)	1 mg/ml in DMSO (stored in aliquots at -20 °C)	1:1,000 in PBS	10 ng/ml
Ionomycin	1 mg/ml in DMSO (stored in aliquots at -20 °C)	1:10 in PBS	1 µg/ ml
Phytohemagglutinin (PHA)	1 mg/ml in DMSO (stored at 4 °C)	1:10 in PBS	1 µg/ ml
SEB	50 µg/ml in PBS	None	1 µg/ml
Peptide mixes	0.5-1 mg/ml/pep in DMSO (stored in aliquots at -20 °C)	1:10 in PBS	1 µg/ml/peptide
Anti-CD3/CD28	Follow manufacturer instructions	-	-

Notes:

a. *It is important to avoid solvent toxicity. Final DMSO + ethanol concentration from all sources (peptides, brefeldin A, monensin) should not exceed 0.5%.*
b. *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.*
c. *Fluorochrome-labeled CD107 and CD154 can be added into the culture during the stimulation at a concentration of 2 µg/ml. This allows for staining of target molecules that are re-internalized by cells during the activation process.*
d. *Addition of costimulatory antibodies is optional. These can increase the cytokine response to protein antigens, peptides, and SEB by amplifying the signal for low-affinity T cells (Waldrop et al., 1998). Add 1 µg/ml final concentration of CD28 and/or CD49d (labeled antibody can be used if analysis of the marker is desired).*

2. Incubate the cells for 4 h (PMA + Ionomycin stimulation, PHA + Ionomycin stimulation) or 6-8 h (SEB, 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 optimal incubation time is 12-24 h.
3. Add EDTA to a final concentration of 2 mM and incubate for 15 min at room temperature.
   Note: If Dynabeads have been used for stimulation, use the I Mag for removal of beads during this step.
4. Wash the cells with PBS at 1,550 rpm (RCF = 473), 8 min at room temperature.

C. Staining

1. Repeat PBS wash at 1,550 rpm (RCF = 473), 8 min, room temperature and resuspend the cells in 500 µl PBS.
2. Stain with Red LIVE/DEAD cell viability dye according to the manufacturer's instructions.
3. Incubate at room temperature for 30 min in dark.
4. Centrifuge the cells at 1,550 rpm (RCF = 473), 10 min, room temperature.
5. Flick or aspirate to remove supernatant and wash cells with PBS at 1,550 rpm (RCF = 473), 10 min, room temperature.
6. Repeat another wash in FACS buffer, at 1,550 rpm (RCF = 473), 10 min, room temperature.
7. Flick or aspirate to remove supernatant and resuspend cells in residual volume.
8. Prepare the surface staining cocktail according to titre per test for each antibody (pre-determined/provided by manufacture). See an example below for staining ten tubes/wells.

Note: The manufacturer's recommended dose is a good starting point, but antibody titrations on representative cells can improve results on unsatisfactory stainings.

Table 3. Surface staining cocktail

Antibody panel	Stain	Titre (µl/sample)	X # of samples	Total µl
CD3	V 500	5	10	50
CD4	PerCPCy 5.5	10	10	200
CD8	V 450	5	10	50

Note: If doing simultaneous tetramer analysis, cells should be stained with the pMHC multimer prior to staining with the anti-coreceptor antibodies, in a separate step, washed and then stained with the surface staining cocktail.

9. Add calculated volume of staining cocktail for each sample to (20 µl in the example above). Add appropriate amount of single antibodies to beads for compensation controls.
10. Incubate cells for 30 min at room temperature in the dark.
11. Add 2 ml FACS buffer to each well/tube.
12. Centrifuge the cells at 1,550 rpm (RCF = 473), 10 min, room temperature.
13. Flick or aspirate to remove supernatant and wash cells again with FACS buffer at 1,550 rpm (RCF = 473), 10 min, room temperature.
14. Prepare 1x BD FACS lysing solution (10x diluted to 1x in water).
15. Add 2 ml per tube/well, mix well and incubate the cells for 10 min at room temperature, in the dark.
16. Centrifuge cells at 2,000 rpm (RCF = 787) for 10 min at 4 °C and flick or aspirate to remove supernatant.
17. Wash cells twice in FACS buffer.
18. Prepare 1x BD FACS Perm-2 buffer (10x diluted to 1x in water).
19. Resuspend the cell pellet in 500 µl of 1x FACS permeabilizing solution (prepared above) and incubate for 15 min in dark.
20. Centrifuge cells at 2,000 rpm (RCF = 787) for 10 min and flick or aspirate to remove supernatant.
21. Wash cells twice in FACS buffer.
22. Flick or aspirate to remove supernatant and resuspend cells in residual volume.
23. Prepare the intracellular staining cocktail according to titre per test for each antibody (pre-determined/provided by manufacture). See example below for staining ten tubes/wells.

Table 4. Intracellular staining cocktail

Antibody panel	Stain	Titre(µl/ sample)	X # of samples	Total µl
IFN-γ	FITC	20	10	200
IL-17	PE	20	10	200
IL-4	APC	5	10	50
TNFα	Alexa700	1.2	10	12
IL-2	PE-Cy7	5	10	50

24. Calculate and add required volume of staining cocktail to each sample (51.2 µl in the example above) and incubate cells for 30 min, in the dark.
25. Add 2 ml FACS buffer to each well/tube.
26. Centrifuge cells at 2,000 rpm for 10 min and flick or aspirate to remove supernatant.
27. Wash cells twice in FACS buffer.
28. Flick or aspirate to remove supernatant and resuspend in a final volume of 150 µl FACS buffer for Flow.

Representative data

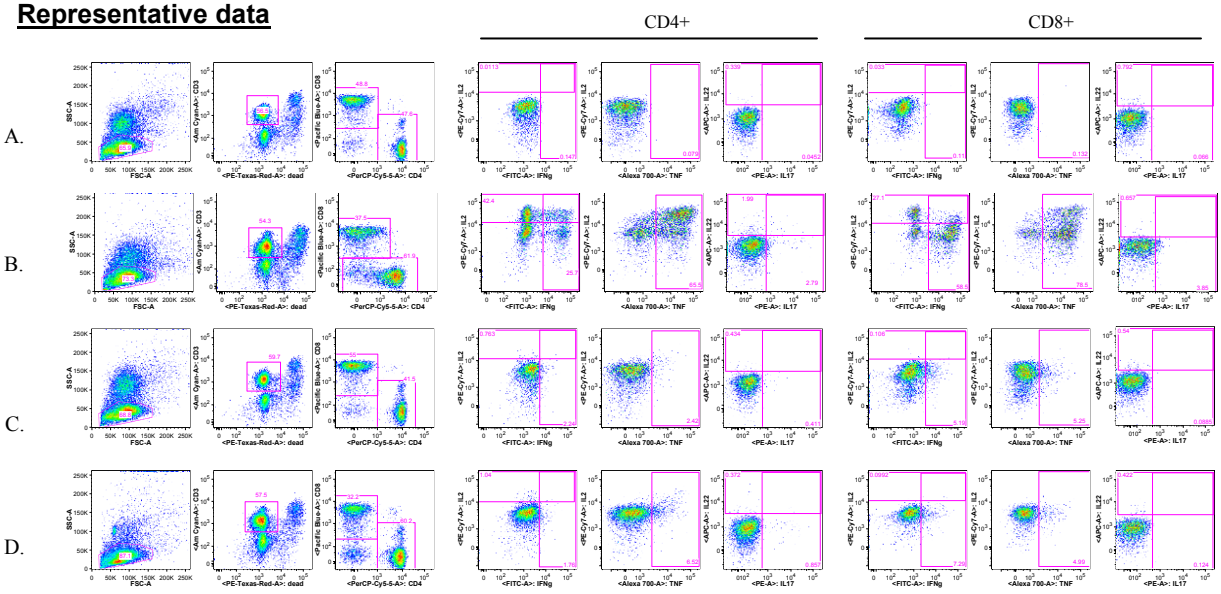

Figure 1. Intracellular cytokine production by T cells (IFNg, TNF, IL-2, IL-17, IL-22). The first three plots in each row show gating hierarchy for $CD4^+$ and $CD8^+$ T cells. The next panel of subsequent three plots shows cytokine production by $CD4^+$ T cells and the final panel shows the same for $CD8^+$ T cells. (A) unstimulated (B) PMA and Ionomycin stimulation (C) PHA and Ionomycin stimulation (D) anti CD3/CD28 stimulation.

Notes

1. Doing the adequate number of washes after staining, fixing and permeabilization of cells is very important for reducing the background level for cytokines.
2. If doing the experiment in a 96-well plate, leave a few wells empty between the unstimulated and stimulated conditions to minimize the chances of spillover and cross-contamination while washing.

Recipes

1. Complete RPMI
   RPMI
   10% serum (*e.g.* FBS)
   Pen-strep
   Glutamine
2. FACS buffer
   PBS with 2% serum (*e.g.* FBS) and 0.1% Na azide

References

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