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Mass Cytometry Assays for Antigen-Specific T Cells Using CyTOF

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Abstract

T Cells specific for a single antigen tend to be rare, even after expansion of memory cells. They are commonly detected by in vitro stimulation with peptides or protein, followed by staining for intracellular cytokines. In this protocol, we use CyTOF® mass cytometry to collect single-cell data on a large number of cytokines/chemokines, as well as cell-surface proteins that characterize T cells and other immune cells. We also include a method for magnetic bead enrichment of antigen-stimulated T cells, based on their expression of CD154 and CD69. Coupling magnetic enrichment with highly multi-parameter mass cytometry, this method enables the ability to dissect the frequency, phenotype, and function of antigen-specific T cells in greater detail than previously possible. Rare cell subsets can be examined, while minimizing run times on the CyTOF.

Keywords

Antigen-specific; Intracellular staining; Cytokines; T Cells; CyTOF; Magnetic enrichment

1 Introduction

Mass cytometry, CyTOF (Fluidigm Corporation), is based on the concept of using heavy-metal isotopes to label antibodies for flow cytometry, rather than fluorescent tags [1–3]. The isotopes are attached to the antibodies via a metal-chelating polymer, and the labeled cells are introduced sequentially into a mass spectrometer for the quantitative detection of the metal labels associated with each cell. This novel technology affords the ability to combine many more specific antibodies in a single experiment without significant spillover between detector channels.

Like conventional flow cytometry, CyTOF can be used for intracellular as well as cell-surface staining. Intracellular cytokine staining (ICS) can be done to look at the intersection of cell phenotype and function, and is often used with multiparameter flow cytometry to dissect antigen-specific T-cell responses to infection or vaccination [4]. ICS was first adapted to the CyTOF platform by Newell et al. [5], who showed the diversity of CD8+ T cell functions and phenotypes, which nevertheless were biased based on pathogen specificity. A general protocol for CyTOF ICS has also been published [6].

As the throughput on the CyTOF is lower than conventional flow cytometry, magnetic enrichment of target population is helpful for the analysis of rare antigen-specific T cells. The principal steps of enrichment are as follows: first, cells are activated for 4 h using specific peptides in the presence of monensin, CD107a-metal conjugate (if analyzing for

CD107a in the assay [7, 8]), and either CD40 or CD154-biotin antibody [9–11]. Anti-CD28 and CD49d costimulatory monoclonal antibodies (mAbs) can be used to enhance the T-cell stimulation if desired [12]. During the first 4 h of stimulation, CD69 and CD154 accumulate on the surface of activated (antigen-responsive) cells. The addition of brefeldin A after 4 h further blocks the secretion of cytokines to allow their intracellular detection. After stimulation, EDTA is added to dislodge adherent cells from the culture plates. Next, cells are labeled with CD154-biotin (if not already in the stimulation cocktail) and CD69-biotin antibodies, followed by anti-biotin microbeads and magnetic enrichment on MS MACS columns (Miltenyi Biotec) [13]. After washing, the enriched cells are stained with antibodies to cell surface markers. The cells are then fixed in paraformaldehyde and permeabilized. We use a gentle detergent, saponin, as the permeabilization buffer because it is less destructive to surface and intracellular epitopes compared to harsh detergents or methanol. After permeabilization, the metal-conjugated anti-cytokine antibodies are added to the cell suspension. The stained cells are analyzed by the mass cytometer. A schematic of these steps is shown in Fig. 1.

2 Materials

2.1 Reagents (See Note 1)

1. Peripheral blood mononuclear cells (PBMCs).
2. Complete RPMI medium: RPMI-1640, 10% FBS, 1× Pen-strep-Glutamine.
3. ≥ 250 units/μL Benzonase® Nuclease.
4. 0.5 M EDTA.
5. PBS.
6. 5 mg/mL Brefeldin A: dissolved in dimethyl sulfoxide (DMSO) and frozen in small aliquots at −80 °C.
7. 5 mg/mL Monensin: dissolved in ethanol and stored at −20 °C (no need to aliquot since it will not freeze at −20 °C).
8. Anti-Human CD28/CD49d Costimulatory Reagent (BD Biosciences, San Jose, CA).
9. Anti-Human CD40 pure (Miltenyi Biotec, San Diego, CA).
10. Anti-Human CD154-Biotin (Miltenyi Biotec).
11. Anti-Human CD69-Biotin (Miltenyi Biotec).
12. Anti-Biotin Multisort kit (Miltenyi Biotec).
13. Cell-ID™ Cisplatin (Fluidigm Corporation, South San Francisco, CA).
14. Maxpar® Metal Conjugated Antibodies (Fluidigm Corporation) filtered with 0.1 μm spin filters (Millipore, Temecula, CA).
15. Maxpar® Cell Staining Buffer (Fluidigm Corporation).
16. 5× Maxpar® Fix I Buffer (Fluidigm Corporation).

17. Maxpar® Perm-S Buffer (Fluidigm Corporation).
18. 125 μM or 500 μM Cell-ID™ Intercalator–Ir (Fluidigm Corporation).
19. Maxpar® Fix and Perm Buffer (Fluidigm Corporation).
20. Maxpar® Water (Fluidigm Corporation).
21. (Optional) Dead Cell Removal Kit (Miltenyi Biotec).
22. (Optional) Human Fc-receptor Blocking Solution (BioLegend, San Diego, CA).

2.2 Equipment and Supplies

1. Multiwell plates: 24-well tissue culture plates, 2 mL deep well v-bottom 96-well plates (Corning Life Science, Corning, New York).
2. Pipettors: calibrated pipettors, multichannel pipettor.
3. Cell strainers: 70 μm cell strainers, tubes with cell strainer caps.
4. 30 μm Pre-separation filters (Miltenyi Biotec).
5. Biosafety cabinet.
6. CO_2 incubator at 37 °C.
7. Water bath at 37 °C.
8. Centrifuge.
9. Vi-CELL counter (Beckman Coulter, Brea, CA) or equivalent, or a microscope with hemocytometer.
10. MiniMACS separator (Miltenyi Biotec).
11. MS MACS columns (Miltenyi Biotec).
12. CyTOF® mass cytometer (Fluidigm Corporation).

3 Methods

3.1 Thawing of PBMC

1. Warm complete RPMI 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 dilution of benzonase (final concentration at 25 units/mL). Benzonase is added to prevent cell aggregation. Thaw not more than three samples at a time.
2. Remove samples from liquid nitrogen and transport to a lab on dry ice.
3. Place 10 mL of warm medium with benzonase 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 the biosafety cabinet.

6. Add 1 mL of warm medium with benzonase from appropriately labeled centrifuge tube slowly to the cells, and then transfer the cells to the centrifuge tube. Rinse vial with more medium from the centrifuge tube to retrieve all of the cells.
7. Continue with the rest of the samples as quickly as possible.
8. Centrifuge cells at 473 × *g* for 8 min at room temperature.
9. Remove the supernatant from the cells and resuspend the pellet by tapping the tube.
10. Gently resuspend the pellet in 1 mL of warm medium with benzonase. Filter cells through a 70 μm cell strainer if needed. Add 9 mL more warm medium with benzonase to the tube.
11. Centrifuge cells at 473 × *g* for 8 min at room temperature. Remove the supernatant from the cells and resuspend the pellet by tapping the tube.
12. Resuspend cells in 1 mL of warm medium.
13. Count cells with Vi-CELL (or hemocytometer). To count, take 20 μL of cells and dilute with 480 μL of PBS in a Vicell counting chamber. Load onto Vi-CELL as PBMC with a 1:25 dilution factor.
14. Adjust the cell concentration to 5–10 × 10^6 cells/mL with warm medium (no more benzonase at this point).
15. Using a multichannel pipettor, add 200 μL of cells (for at least 1 × 10^6 cells) into each well of a 2 mL deep-well v-bottom 96-well plate. If more cells are needed for enrichment, 24-well tissue culture plates are used for 10^7 cells in 1 mL of medium. Split each sample equally 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 a CO_2 incubator.

3.2 Cell Activation

For stimulation without cell enrichment, proceed to Subheading 3.2.1; for stimulation with antigen-specific T-cell enrichment, proceed to Subheading 3.2.2.

3.2.1 Without Enrichment

1. After overnight rest at 37 °C, add the activation reagents and secretion inhibitor (brefeldin A or monensin) to the well for stimulation (*see* Table 1) (*see* **Notes 2** and **3**). Add only the secretion inhibitor to the unstimulated control well. If doing CD107a staining, add CD107a antibody during the stimulation.
2. Add stimuli as directed in Table 2 (*see* **Note 4**). Incubate the cells for 4 h (PMA + ionomycin stimulation, PHA + ionomycin stimulation) or 6–8 h (SEB, anti-CD3/CD28, peptide stimulation) at 37 °C, in a CO_2 incubator (*see* **Note 5**).
3. Proceed to Subheading 3.3.

3.2.2 With Enrichment

1. After overnight rest of cells, spin down cells and remove the supernatant. Make a cocktail of the following: 1000 μL medium +1 μL monensin stock solution (final concentration at 5 μg/mL) + 10 μL CD28/CD49d co-stimulatory reagent + optimal concentration of CD107a-metal conjugate (if analyzing for CD107a) + either 20 μL CD40 Ab or optimal concentration of CD154-Biotin. Add 1 mL of this cocktail to resuspend each cell pellet. Add antigens (e.g., peptides from JPT at 1 μg/mL final concentration) or other stimuli to respective wells for stimulation (*see* **Note 6**).
2. 4 h later, add 1 μL of brefeldin A stock solution (final concentration at 5 μg/mL) to each well, mix well by pipetting, and continue to incubate for 4 h.
3. To enrich for antigen-specific cells, transfer cells from a 24-well plate to a 2 mL deep-well v-bottom 96-well plate. Spin down at 473 × *g* for 10 min at 4 °C. The same volume and centrifuge conditions are used in additional wash steps for enrichment. Flick or aspirate to remove the supernatant. Gently resuspend the pellet in 1 mL of cold Maxpar Cell Staining Buffer with pipette. Repeat wash step and centrifugation with 1 mL of cold Maxpar Cell Staining Buffer.
4. An optional dead cell removal step can be included at this step by using, for example, Dead Cell Removal Kit.
5. Make cocktail of the following: 20 μL CD69-biotin +20 μL CD154-biotin +60 μL Maxpar Cell Staining Buffer. Incubate cells with this cocktail at 4 °C for 20 min, and then wash with 1 mL of cold Maxpar Cell Staining Buffer. Spin and discard the supernatant.
6. Make cocktail of 20 μL anti-biotin microbeads +80 μL Maxpar Cell Staining Buffer. Incubate cells with this cocktail at 4 °C for 20 min, and then wash with 1 mL of cold Maxpar Cell Staining Buffer. Spin and discard the supernatant.
7. Set up magnetic columns: Use a cooled MiniMACS separator, and pre-wet pre-separation filters and MS MACS columns with 500 μL of cold Maxpar Cell Staining Buffer.
8. Resuspend cells in 1 mL of Maxpar Cell Staining Buffer, apply cell suspension through the pre-separation filters and columns at 500 μL twice; wash wells with 1 mL of Maxpar Cell Staining Buffer, and pass through the columns at 500 μL twice again.
9. Collect the flow-through containing unlabeled cells in a 15 mL tube and pass through a new column if desired. To check for carry over in the negative fraction, spin down the flow-through at 473 × *g* for 10 min at 4 °C, and proceed to later staining steps.
10. Remove the column from the magnet, put above the well of a 2 mL deep well v-bottom 96-well plate, add 500 μL of Maxpar Cell Staining Buffer into the column, and immediately flush out the magnetically labeled cells as the positive

fraction by firmly pushing the plunger into the column. Repeat with 500 μL of Maxpar Cell Staining Buffer into the same or another well.

11. Spin down the cells at 473 × *g* for 10 min at 4 °C. Combine the two wells into one well (if applicable) in 1 mL of Maxpar Cell Staining Buffer. Add 20 μL of Release Reagent to dissociate the beads from cells, and incubate for 10 min at 4 °C.

12. Pass the dissociated beads and cells over a second magnetic column, collecting the flow-through (cells without beads). For details, see: https://www.miltenyibiotec.com/~/media/Images/Products/Import/0001100/IM0001122.ashx?force=1 (*see* **Note 7**).

3.3 Viability Dye and Cell-Surface Staining

1. At the end of stimulation, add EDTA to a final concentration of 2 mM and incubate for 15 min at room temperature.

2. Wash cells 2× with 500 μL of PBS per well, spin at 473 × *g* for 10 min at room temperature, and discard the supernatant. The same volume and centrifuge conditions are used in additional wash steps before fixation with PFA.

3. Resuspend cells to 1×10^7 mL^{-1} in PBS and add Cell-ID Cisplatin to a final concentration of 5 μM (1000× dilution of 5 mM stock solution).

4. Incubate at room temperature for 5 min.

5. Wash with Maxpar Cell Staining Buffer using 5× the volume of the cell suspension. Spin and discard the supernatant.

6. An optional step of Fc-blocking can be done by using, for example, Fc-Receptor Blocking Solution. Incubate for 10 min at room temperature. Without washing off Fc-Receptor Blocking solution continue to the next steps.

7. Make surface antibody cocktail in Maxpar Cell Staining Buffer and filter with 0.1 μm spin filter, 100 μL per reaction. Incubate on ice for 45 min. Use Fluidigm recommended concentration (or optimal titer as determined for self-made conjugates) for each antibody.

8. Wash 3× in Maxpar Cell Staining Buffer. Spin and discard the supernatant.

3.4 Fixation and Permeabilization

1. Resuspend cells in 1 mL of 1× Maxpar Fix I buffer (diluted in PBS) to each well. Incubate at room temperature for 10–30 min or 4 °C overnight.

2. Wash 2× with 1 mL of Maxpar Perm-S Buffer. Spin at 787 × *g* for 10 min at 4 °C and discard the supernatant. The same volume and centrifuge conditions are used in the following wash steps.

3.5 Intracellular Staining

1. Make intracellular staining cocktail in Maxpar Perm-S Buffer and filter with 0.1 μm spin filter, 100 μL per reaction. Incubate on ice for 45 min.
2. Wash 3× in Maxpar Cell Staining Buffer. Spin and discard the supernatant.
3. Resuspend cells in 1 mL of 125 nM Cell-ID Intercalation-Ir (a 1000× dilution of the 125 μM stock solution in Fix and Perm Buffer). Incubate for 1 h at room temperature or leave overnight at 4 °C.
4. Wash 2× in Maxpar Cell Staining Buffer. Spin and discard the supernatant.
5. Wash 3× in Maxpar Water. Spin and discard the supernatant.
6. Resuspend cells in Maxpar Water to a concentration of 2.5–5 × 10^5 mL^{-1}. Filter cells into tubes with cell strainer caps.
7. Acquire data on CyTOF. *See* Fig. 2 for representative data and gating.

4 Notes

1. *See* ref. 6 for suppliers and catalogue numbers of reagents used in this chapter.
2. It is important to avoid solvent toxicity. Final DMSO + ethanol concentration from all sources (peptides, brefeldin A, monensin) should not exceed 0.5%.
3. For most cytokines: Use brefeldin A at 10 μg/mL final concentration (*see* the stock preparation table). For CD107 and CD154: Use monensin at 10 μg/mL final concentration. For assays combining cytokines and CD107 or CD154: Use brefeldin A and monensin at 5 μg/mL final concentration each.
4. Addition of costimulatory antibodies is optional. These antibodies can increase the frequency of cells responding to specific antigen. Add 1 μg/mL final concentration of CD28 and/or CD49d (labeled antibody can be used if analysis of the marker is desired).
5. For most cytokines, 6–12 h incubation at 37 °C is sufficient. For IL-10, optimal incubation time is 12–24 h, but detection in 6 h is possible.
6. The addition of staining antibodies for CD107a and CD154 allows these targets to be stained despite being only transiently expressed on the cell surface during activation. Metal-labeled antibodies are titrated in separate experiments for their optimal concentration. The amount of staining antibody should be scaled up to account for the larger (1 mL) incubation volume. Alternatively, CD40 antibody can be used during stimulation to block the interaction of CD154 with CD40, which triggers the re-uptake of CD154. The addition of monensin prevents the acidification of vesicles that might contain these stained complexes and that would otherwise lead to their degradation. *See* refs. 1–6 for details.
7. Milltenyi beads may contain barium and other heavy metals, which could interfere with detection of certain mass channels, and which could shorten

detector life. We therefore recommend cleaving and separating cells from beads as described in Subheading 3.2.2 **steps 11** and **12**.

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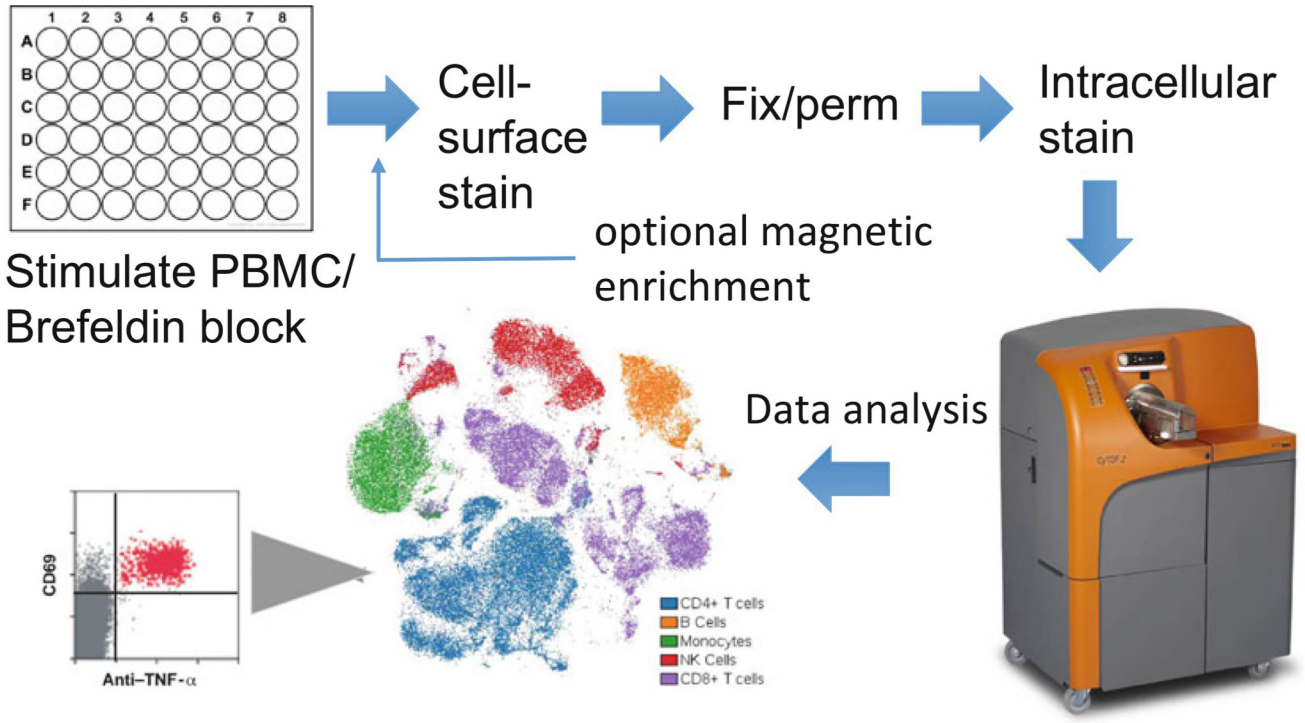

Fig. 1.
Workflow for CyTOF with intracellular cytokine staining (ICS)

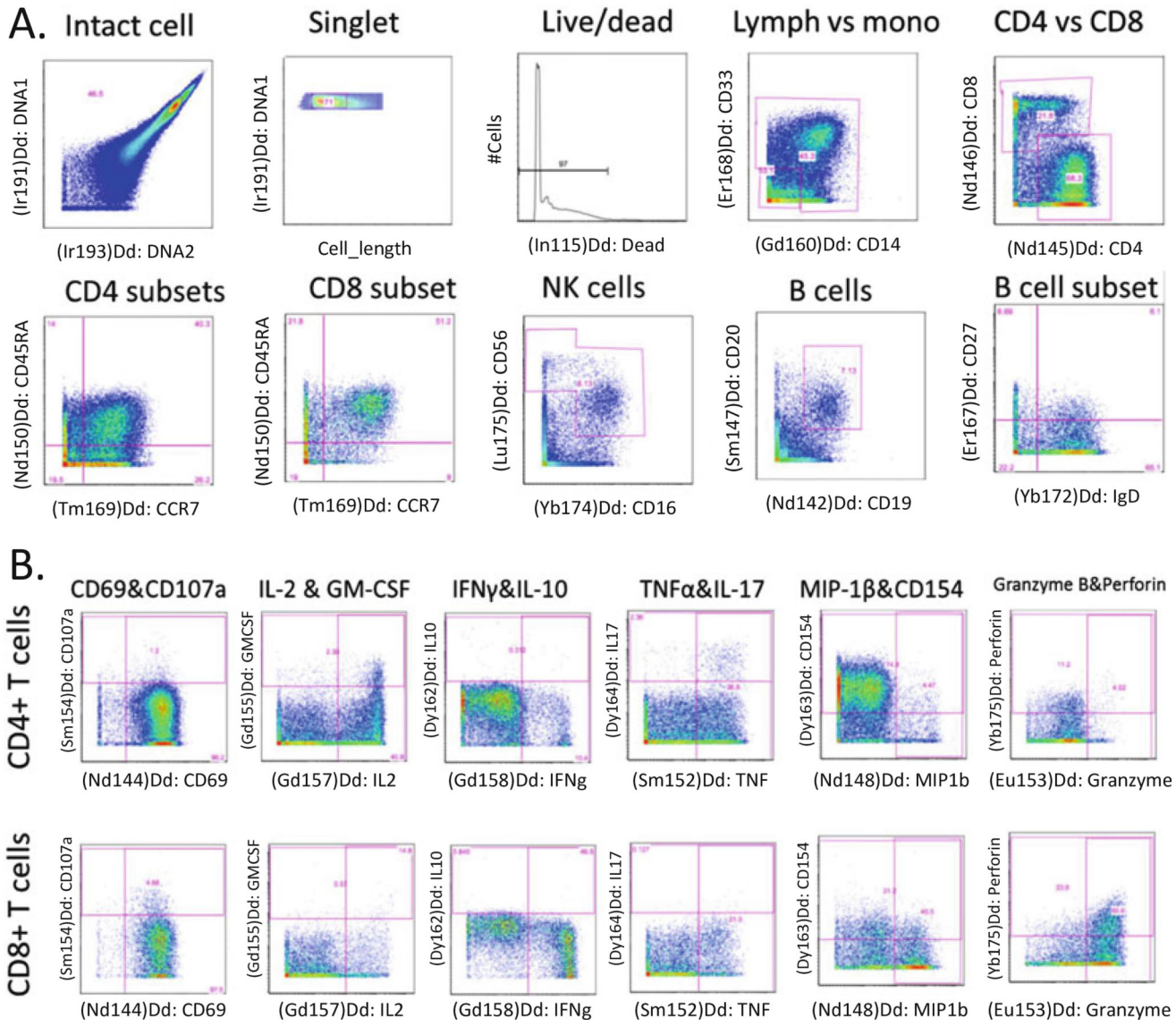

Fig. 2.

Representative CyTOF ICS data. (**a**) Initial gating on intact cells, singlets, and live cells, followed by gating of major cell subsets and T-cell differentiation markers. (**b**) Representative data from intracellular cytokines, after PMA + ionomycin stimulation

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 −20 °C)	1:10 in PBS	10 μg/mL (1:50) or 5 μg/mL (1:100) with brefeldinA

Table 2

Activators

Reagent	Stock concentration	Intermediate dilution	Final concentration
Phorbol 12-myristate 13-acetate (PMA)	1 mg/mL in DMSO (stored in aliquots at −20 °C)	1:1000 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 (1:50)
Anti-CD3/CD28	Follow manufacturer instruction	–	–
Peptide mixes (JPT, Acton, MA)	0.5–1 mg/mL/pep in DMSO (stored in aliquots at −20 °C)	1:10 in PBS	1 μg/mL/peptide (1:50–1:100)