Quantification of Th1 and Th17 Cells with Intracellular Staining Following PMA/Ionomycin Stimulation

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Cytokine-producing cells are at the center of the adaptive immune responses, and quantifying these cells is an important aspect to build understanding of the immune response. In particular, Th1 and Th17 cells have been implicated in the pathogenesis of such diseases as inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis. Quantification of Th1 and Th17 cells can provide important information in research of these diseases and other Th1- and Th17-mediated immune disorders. In vitro stimulation of cells followed by surface and intracellular staining, presented here, has the advantage of detecting the cytokines directly instead of relying exclusively on surrogate surface markers which, although showing enrichment for the effector T cells, are not specific markers for the cytokine-producing cells. © 2015 by John Wiley & Sons, Inc.

Keywords: Th1 cells • Th17 cells • intracellular staining • flow cytometry • adaptive immunity

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INTRODUCTION

Effector $CD4^+$ T cells, for a long time considered to be either Th1 or Th2 cells, include Th9, Th17, and Th22 cells (Cosmi et al., 2014). These cells are characterized by what cytokines they secrete. Th1 cells secrete IFN-γ, Th2 cells secrete IL-4, IL-5, or IL-13, Th9 cells secrete IL-9, Th17 cells secrete IL-17, and Th22 cells secrete IL-22. It should be noted that these are the cytokine signatures that best define the cell types, but are not intended to be a comprehensive list of all the cytokines that each subset can produce.

These cytokine-producing cells are at the center of the adaptive immune responses, and quantifying these cell types improves our understanding of the immune response. Th1 and/or Th17 cells have been implicated in the pathogenesis of diseases such as inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis. Quantification of Th1 and Th17 cells can provide important information in research of these diseases and other Th1 and/or Th17 mediated immune disorders (Petermann and Korn, 2011; Miossec and Kolls, 2012; Monteleone et al., 2012).

There are two main methods to detect Th1 and Th17 cells in human peripheral blood with flow cytometry. One relies exclusively on surface staining of cells, while the other relies on both surface and intracellular staining. The former method stains cell surface markers

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such as CXCR3, CCR6, or CD161 to detect Th1 and Th17 cells (Mahnke et al., 2013), and provides a decent alternative to the more time-consuming method of intracellular staining. Although this method enriches for effector T cells, the surface markers typically used are not specific to cytokine-producing cells. Thus, detection of cells stained only for these surface markers overpredicts the number/frequency of these cells compared to detection of cells stained for intracellular cytokines.

The latter method (Basic Protocol 1) utilizes in vitro stimulation of cells followed by surface staining to detect surface markers and intracellular staining to detect cytokines. The method of in vitro stimulation followed by surface and intracellular staining has the advantage of detecting cytokines directly instead of relying exclusively on surrogate surface markers.

BASIC PROTOCOL

QUANTIFICATION OF Th1 AND Th17 CELLS WITH INTRACELLULAR STAINING

In this protocol, peripheral blood mononuclear cells (PBMCs) are isolated, stained with propidium iodide and acridine orange, and counted. After a period of stimulation with PMA/ionomycin/brefeldin A, cells are stained for CD3, CD4, and CD8 surface markers and then for intracellular cytokines. Stained cells can then be analyzed on a flow cytometer (see *UNIT 6.2*).

PBMCs can be isolated from whole blood by a number of methods (*UNIT 5.1*), including Ficoll gradient centrifugation, but this protocol provides an alternative approach that takes approximately 30 min less time, which in some cases might be advantageous. Traditional ways of counting cells (e.g., trypan blue exclusion and hemacytometer; see *APPENDIX 3A*) can also be used, but the advantage of using propidium iodide (PI) and acridine orange (AO) with an automated cell counter is that red blood cells (RBCs) in the cell preparation will not affect the ability to quantify the PBMCs.

PMA/ionomycin is not the only way to activate cells, but it stimulates a robust response and bypasses TCR-specificity.

Although this protocol describes the basic staining of CD3, CD4, and CD8 surface markers and intracellular cytokine staining, it can be used to stain other surface proteins. However, the effect of PMA/ionomycin on the expression of each protein of interest should be tested empirically.

Materials

Ficoll-Paque Premium (GE Healthcare Life Sciences, cat. no. 17-5442-02)
SepMate-50 tubes (Stemcell Technologies, cat. no. 15450)
Human blood
PBS (*APPENDIX 2A*)
PBS with 2% FBS (P2F)
AO/PI staining solution (Nexcelom, cat. no. CS2-0106-5ML)
Activation medium (see recipe)
Fixable viability dye eFluor 506 (eBioscience, cat. no. 65-0866)
FcR blocking reagent (Miltenyi Biotec, cat. no. 130-059-901)
Anti-human CD3, Brilliant Violet 785 (Biolegend, cat. no. 317329)
Anti-human CD4, Brilliant Violet 421 (BD, cat. no. 562424)
Anti-human CD8, Qdot 705 (Life Technologies, cat. no. Q10059)
Fixation/permeabilization solution (eBioscience, cat. no. 00-5521-00)
Permeabilization buffer (eBioscience, cat. no. 00-8333)
Anti-human IL-17A, Alexa Fluor 488 (eBioscience, cat. no. 53-7179-41)
Anti-human IFN-γ, PE-CF594 (BD Horizon, cat. no. 562392)

15-ml and 50-ml conical tubes
0.5-ml microcentrifuge tubes
Cellometer counting chamber (Nexcelom, cat. no. CHT4-PD100)
Cellometer Auto 2000 cell viability counter (Nexcelom)
96-well round bottom plates
Incubator

Isolate PBMCs

1. Add 15 ml Ficoll-Paque Premium to the bottom of a SepMate-50 tube, according to the manufacturer's instructions.
2. Dilute 10 to 20 ml of blood with an equal volume of P2F, and carefully add the diluted blood to the SepMate tube by releasing it down the side of the tube.
3. Centrifuge 10 min at 1200 × *g*, room temperature, with the brake on.
4. Pour the cells into a new 50-ml conical tube.

   Do not to invert the tube for more than 2 sec.

5. Fill the tube with P2F, and centrifuge 10 min at 400 × *g*, room temperature.
6. Aspirate the supernatant, and suspend the cells with 8 ml P2F.

Count PBMCs

7. Add 20 µl AO/PI staining solution to a 0.5-ml microcentrifuge tube.
8. Add 20 µl of cell suspension to the tube with AO/PI staining solution, and mix by pipetting up and down.
9. Add 20 µl of the cell mixture to a Nexcelom counting chamber.
10. Count the cells with Cellometer Auto 2000.
11. Wash the cells from step 6 by filling the tube with P2F and centrifuging 10 min at 400 × *g*, room temperature.
12. Suspend the cells in P2F to a concentration of 5×10^6 cells/ml.

Stimulate cells with PMA/ionomycin/brefeldin A

13. Pipet 200 µl of cell suspension (1×10^6 cells) into each well of a 96-well round bottom plate.

    The number of wells to be used will depend on the experimental setup.

14. Centrifuge the plate for 5 min at 470 × *g*, 4°C.
15. Aspirate the supernatant with a needle attached to a vacuum source.
16. Suspend the cells in 200 µl activation medium, containing PMA, ionomycin, and brefeldin A.
17. Incubate 4 hr at 37°C, 5% CO_2.

Stain cells for surface markers and intracellular cytokines

18. Remove the 96-well plates from the incubator and centrifuge 5 min at 470 × *g*, room temperature, and aspirate the supernatant.
19. Wash the cells by suspending each cell pellet with 200 µl of PBS, pellet the cells by centrifuging 5 min at 470 × *g*, room temperature, and aspirate the supernatant.
20. Repeat step 19.

21. Suspend cells in appropriate wells with 200 µl Fixable Viability Dye eFluor 506 diluted 1:1000 in PBS. Suspend cells in the blank (control) well with 200 µl PBS.
22. Incubate 30 min at 4°C.
23. Pellet the cells by centrifuging 5 min at 470 × *g*, room temperature, and aspirate the supernatant.
24. Suspend the cells in each well with 200 µl P2F, centrifuge 5 min at 470 × *g*, room temperature, and aspirate the supernatant.
25. Suspend the cells with 100 µl of FcR blocking reagent.
26. Incubate 15 min at 4°C.
27. Add 100 µl P2F to each well. Pellet cells by centrifuging 5 min at 470 × *g*, room temperature, and aspirate the supernatant.
28. Suspend cells in appropriate wells with 100 µl of surface-antibody mix (CD3/CD4/CD8) or single color antibody. Suspend cells in blank control wells with P2F.

    Antibodies should be titrated to determine the optimal concentration.
29. Incubate 15 min at 4°C.
30. Add 100 µl P2F to each well. Pellet the cells by centrifuging 5 min at 470 × *g*, room temperature, and aspirate the supernatant.
31. Suspend with 200 µl Fixation/Permeabilization solution.
32. Incubate 30 min at 4°C.
33. Pellet the cells by centrifuging 5 min at 470 × *g*, room temperature, and aspirate the supernatant.
34. Wash the cells by suspending with 200 µl permeabilization buffer, pellet the cells by centrifuging 5 min at 470 × *g*, room temperature, and aspirate the supernatant.
35. Repeat step 34.
36. Suspend cells in appropriate wells with 100 µl of intracellular-antibody mix (IL-17A/IFN-γ) or single color antibody. Suspend cells in blank control wells with permeabilization buffer.

    Antibodies should be titrated to determine the optimal concentration.
37. Incubate 30 min at room temperature.
38. Add 100 µl permeabilization buffer to each well. Pellet the cells by centrifuging 5 min at 470 × *g*, room temperature, and aspirate.
39. Wash the cells by suspending with 200 µl permeabilization buffer, pellet the cells by centrifuging 5 min at 470 × *g*, room temperature, and aspirate the supernatant.
40. Suspend the cells with 200 µl P2F.
41. Analyze on flow cytometer.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Activation medium

Thaw aliquots of PMA, ionomycin, and brefeldin A just before use. Dilute PMA 1:10 with X-VIVO 15 medium (Lonza, cat. no.) by adding 180 µl of medium to a 20 µl aliquot of 0.1 mg/ml PMA. Add 4 ml X-VIVO 15 medium to a 15-ml conical tube, and then add 4 µl of the 1:10 diluted PMA, 8 µl of 0.5 mg/ml ionomycin solution, and 8 µl of 5 mg/ml brefeldin A solution. Final concentrations in the activation medium are 10 ng/ml PMA, 1 µg/ml ionomycin, and 10 µg/ml brefeldin A.

Brefeldin A, 5 mg/ml

Prepare 5 mg/ml brefeldin A (Sigma) in DMSO. Store 20-µl aliquots up to 6 months at –20°C.

Ionomycin, 0.5 mg/ml

Prepare 0.5 mg/ml ionomycin (Sigma) in absolute ethanol. Store 20-µl aliquots up to 6 months at –20°C.

Phorbol 12-myristate 13-acetate (PMA), 0.1 mg/ml

Prepare 0.1 mg/ml PMA (Sigma) in DMSO. Store 20-µl aliquots up to 6 months at –20°C.

COMMENTARY

Background Information

A well known problem for detecting cytokine-expressing T cells following PMA-stimulation is that CD4 gets down-regulated (Weyand et al., 1987), making it challenging or impossible to gate on the $CD4^+$ T cells in flow cytometric analysis. Down-regulation of CD4 occurs in three sequential steps. First, PMA stimulates dissociation of CD4 and $p56^{kk}$, which allows CD4 to interact with clathrin-coated pits and vesicles. Second, PMA increases the clustering of CD4 in the clathrin-coated pits, thus increasing the rate of CD4 endocytosis. Third, PMA induces redistribution of the internalized CD4 to degradation pathways instead of the recycling pathways (Pelchen-Matthews et al., 1993). Approaches that have been utilized to improve detection of $CD4^+$ cells include: choosing a CD4 antibody clone that can be added with the intracellular antibodies following fixation/permeabilization (Kemp and Bruunsgaard, 2001); utilizing surrogate markers, such as $CD3^+$ (Eastaff-Leung et al., 2010) or $CD3^+CD8^-$ (Kallas et al., 1999), to identify the cell-type of interest; or using alternative methods of stimulation, such as SEB, which does not stimulate every cell (Roederer et al., 2004).

Critical Parameters and Troubleshooting

It is important to use fluorescent probes to exclude dead cells from analysis, as dead cells can bind fluorescent antibodies nonspecifically. This nonspecific binding makes it difficult to draw the gates correctly for the cytokine analysis and usually leads to an overestimated number of cells of interest. Another critical element is to utilize a bright fluorophore for CD4 due to the aforementioned CD4 downregulation during PMA stimulation. Using CD4-antibody conjugated with Brilliant Violet 421, it is possible to detect CD4 on the cells following PMA stimulation. To improve gating, it is highly recommended to use CD8 antibody as well.

Anticipated Results

In our experience with healthy individuals, 5 to 40% of $CD4^+$ T cells produce IFN-γ, but there is substantial individual variability, with the mean and median close to 20%. IL-17A production in healthy individuals can range from 0.3 to 2.0% of $CD4^+$ T cells, with the mean and median approximately 1%.

A gating strategy is shown in Figure 6.35.1. The first gate is drawn around the lymphocyte

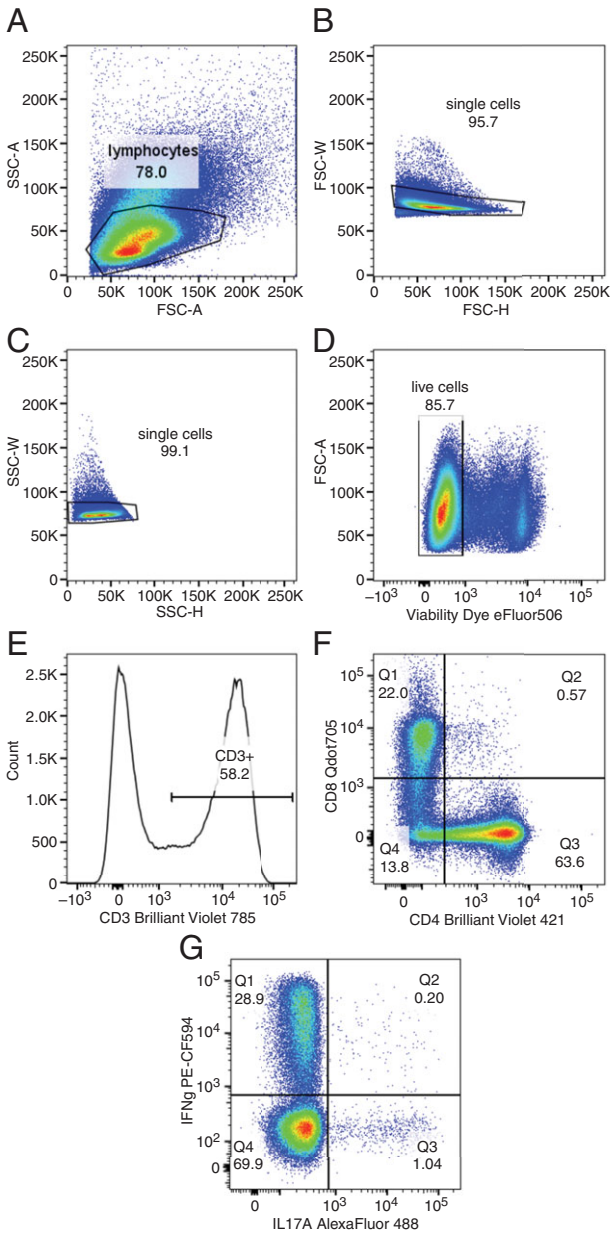

Figure 6.35.1 Gating strategies for identifying Th1 and Th17 cells following intracellular staining. See Anticipated Results.

population (Fig. 6.35.1A), and subsequent gates drawn to exclude any doublets (Fig. 6.35.1B,C). Dead cells are excluded from the analysis by gating only on the cells that are eFluor 506-negative (Fig. 6.35.1D). $CD3^+$ T cells are identified with a histogram (Fig. 6.35.1E), while the $CD4^+$ T cells are identified by gating on the $CD4^+CD8^-$ cells on a CD4/CD8 dot plot (Fig. 6.35.1F). Finally, the frequency of the IL-17A- and IFN-γ-producing cells can be quantified (Fig. 6.35.1G).

Further examples of the gating-strategy are provided in the supplementary data files provided with this protocol (go to *http://www.currentprotocols.com/protocol/cy635*).

FlowJo Workspace:

- Analysis.wsp

Data files:

- Cells_PFE88.fcs
- Cells_PFE160.fcs
- Cells_PFE184.fcs
- Cells_PFE210.fcs

Time Considerations

The entire protocol takes ~12 hr to complete. Following the fixation after the surface staining, cells can be washed twice with P2F and stored in P2F at 4°C overnight, if necessary.

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