

Intracellular Detection of T-Cell Cytokines

Differentiation of TH1 and TH2 Cells

Ursula Banning and Dieter Körholz

1. Introduction

The use of cytometry has become very important in different fields of modern biology and medicine (1–4). In addition to the simple measurement of cell surface marker expression to define certain cellular subsets in basic research as well as in clinical settings, more complex assays have been developed in recent years to analyze, for example, physiological responses, apoptosis, or cell cycling (5–9). The expression of intracellular proteins is another important feature for characterizing cellular function. The development of multicolor cytometers as well as the growing number of fluorochromes and dyes makes it possible to perform more complex analyses.

Cytokines play an important role in the interaction of different cells (10). Evaluation of cytokine content together with cell surface marker expression makes it possible to understand the relationship between different cell types and elucidate their special role in the hematopoietic system. The use of a cytometer makes it possible to analyze cells on a single-cell level in a comparatively short time. Staining of cell surface markers is currently a routinely used method in many laboratories. However, the detection of intracellular proteins is more complicated. Staining methods depend on the permeabilization of the membrane of the cell. Different solutions, such as formaldehyde or alcohols, are used to fixate the cells before incubation with detergents, which permeabilize the membrane reversible (11,12). To date, ready-to-use solutions are available that have been optimized for the use in flow cytometry. Primarily, cells have to be stimulated to express a detectable amount of susceptible protein. In addition,

From: *Methods in Molecular Biology*, vol. 215:
Cytokines and Colony Stimulating Factors: Methods and Protocols
Edited by: D. Körholz and W. Kiess © Humana Press Inc., Totowa, NJ

stimulated cells have to be treated with Monensin or Brefeldin A to prevent the secretion of the produced proteins (**13–15**).

T-cells play an important role in the immune system (**16**). T-cell-derived cytokines control pro-inflammatory and anti-inflammatory processes. The differentiation of T-cells according to the types of cytokines produced by these cells lead to the concept of TH-1 and TH-2 cells, which might be important for the evaluation of several diseases, such as autoimmune diseases, human immunodeficiency virus (HIV)-associated pathology, or graft-versus-host disease, one of the severest side effects of allogeneic transplantation (**17–21**). The TH-1 and TH-2 cell subset can be distinguished by analysis of cytoplasmic interferon (IFN)- γ (TH-1) or interleukin (IL)-4 (TH-2).

2. Materials

2.1. Isolation of Mononuclear Cells from Whole Blood

1. Blood, drawn in 9 mL EDTA or lithium–heparin tubes (Sarstedt, Nuremberg, Germany).
2. Phosphate-buffered saline (PBS): 8 g NaCl, 1.42 g $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 0.2 g KH_2PO_4 , 0.2 g KCl, add 1 L distilled water, pH 7.3, sterilize, and store at room temperature.
3. BicolI (Biochrome, Berlin, Germany), sterile, should be stored at 4–25°C and protected from light (storage in cold will increase the shelf life).
4. RPMI 1640 medium supplemented with the following:
10% heat-inactivated fetal calf serum (FCS)
10 U/mL penicillin
10 $\mu\text{g/mL}$ streptomycin
2 mM L-glutamine
This can be stored at 4°C up to 3 wk.
5. 0.83% Ammonium chloride: 8.29 g NH_4Cl , 1 g KHCO_3 , 0.0371 g EDTA; add 1 L with distilled water, sterilize, and store at room temperature.

2.2. Stimulation

1. 96 Flat-bottom 96-well cell culture plates.
2. Phorbol 12-myristate 13-acetate (PMA).
3. Phytohemagglutinine.
4. Ionomycin (Sigma, St. Louis, MO); dissolved in 96% ethanol, aliquoted, and stored at –80°C until use.
5. GolgiStop (BD Pharmingen, Heidelberg, Germany); should be stored at 4°C.
Caution: contains Monensin (toxic!) and is highly flammable.

2.3. Staining of Cells

1. 6-mL Polypropylene tubes.
2. Fix/Perm solution (BD Pharmingen, Heidelberg, Germany); stored in cold.
Caution: contains formaldehyde and saponin.

Table 1
Staining Panel

Tube	FITC	PE	ECD	PC5
1	IgG1 surface	IgG1 cytoplasmic	IgG1 surface	IgG1 surface IgG2a surface
2	IgG1 cytoplasmic	IgG1 surface	IgG1 surface	IgG1 surface IgG2a surface
3	CD4 surface	IL-4 cytoplasmic	CD3 surface	CD14 surface
4	IFN cytoplasmic	CD4 surface	CD3 surface	CD14 surface
5	CD8 surface	IL-4 cytoplasmic	CD3 surface	CD56 surface
6	IFN cytoplasmic	CD8 surface	CD3 surface	CD56 surface

3. Wash buffer (BD Pharmingen, Heidelberg, Germany); stored in cold. **Caution:** contains sodium azide and saponin.
4. Fluorescence-labeled antibodies (staining panel is described in **Table 1**):
 Anti-CD3-ECD (clone: UCHT1; isotype: mouse IgG1)
 Anti-CD4-FITC (clone: 13B8.2; isotype: mouse IgG1)
 Anti-CD4-PE (clone: 13B8.2; isotype: mouse IgG1)
 Anti-CD8 FITC (clone: B9.11; isotype: mouse IgG1)
 Anti-CD8 PE (clone: B9.11; isotype: mouse IgG1)
 Anti-CD14 PC5 (clone: RMO52; isotype: mouse IgG2a)
 Anti-CD56 PC5 (clone: N901-NKH1; isotype: mouse IgG1)
 Anti-IFN- γ FITC (clone: B27; isotype: mouse IgG1)
 Anti-IL-4 PE (clone: 8D4-8; isotype: mouse IgG1)

2.4. Flow Cytometric Analysis

The analysis was done on a Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Krefeld, Germany). For measurement and analysis, EXPO 32 software was used. The daily routine involves use of FlowCheck Fluorespheres (No. 6605359, Beckman Coulter, Krefeld, Germany) for check up of the cytometer, use of Coulter Isoton II (no. 8448011) for running, and Coulter Clenz Solution (no. 8456930) for cleaning.

3. Methods

3.1. Isolation of Mononuclear Cells from Whole Blood

1. Heparinized whole-blood samples are 1:2 diluted with PBS, pH 7.3 (*see Note 1*).
2. 5 mL of Ficoll are overlayed by up to 10 mL of the whole blood–PBS solution.
3. Centrifugate for 15 min at 800g without break.
4. Transfer mononuclear cell fraction to a new tube and wash with PBS to remove residual Ficoll solution (600g, 5 min).

5. Lysis of remaining erythrocytes may be done by incubation of the cell pellet with 1–2 mL 0.83% ammonium chloride for 5 min.
6. Incubation should be followed by intensive washing to remove ammonium chloride solution completely (at minimum, three times with 20 mL PBS).
7. After washing steps, the cell number is determined by counting.
8. Cells are suspended in RPMI 1640 supplemented with 10% heat-inactivated FCS and penicillin/streptomycin at a final density of 10^6 /mL (*see Note 2*).

3.2. Stimulation

1. Transfer 200 μ L of cell suspension ($=2 \times 10^5$ cells) per well to a 96-well flat-bottom cell culture plate.
2. Stimulate the cells with PMA (10 ng/mL) and Ionomycin (1 μ g/mL) for 4 h.
3. Add GolgiStop (0.6 μ L of stock solution 1:5 diluted with PBS) to the cultures to inhibit secretion of the produced cytokines.
4. Incubate the cells for 4 h at 37°C, 5% CO₂ (*see Note 3*).

3.3. Staining of Cells

1. After stimulation, transfer cells from two wells (approx 4×10^5 cells) into a 6-mL polypropylene-tube suitable for cytometer use.
2. To guarantee transfer of possibly all cells, wash the wells with PBS twice.
3. After the addition of 1 mL PBS, centrifugate the cells at 600g for 5 min.
4. Discard the supernatant and resuspend the cells in 100 μ L PBS.
5. For cell surface analysis, stain the cells with 10 μ L of each assigned fluorescence-labeled antibody and incubate cells for 10 min at room temperature in the dark. For analysis of different lymphocyte subsets, staining may be carried out according to the panel listed in **Table 1**.
6. Wash with 1 mL PBS and discard supernatant.
7. Add 250 μ L fixing and permeabilizing solution (Fix/Perm, BD Pharmingen).
8. Vortex cells and incubate for 25 min at 4°C.
9. After fixation and permeabilization, wash cells two times with 1 mL Wash-Buffer (BD Pharmingen, diluted 1:10 with distilled water).
10. Resuspend cells in 100 μ L PBS.
11. After the addition of 10 μ L cytokine-specific fluorescence-labeled antibody, vortex cells very gently and incubate for 30 min at 4°C (*see Note 4*).
12. Wash cells twice with 1 mL wash buffer.
13. Repeat washing with 1 mL PBS.
14. Resuspend cells in 500 μ L PBS and measure with the cytometer (*see Note 5*).

A typical experiment is shown in **Fig. 1**.

4. Notes

1. To combine intracellular staining with immunophenotyping and detection of plasma cytokine concentration, portions of whole blood and plasma should be taken before dilution with PBS!

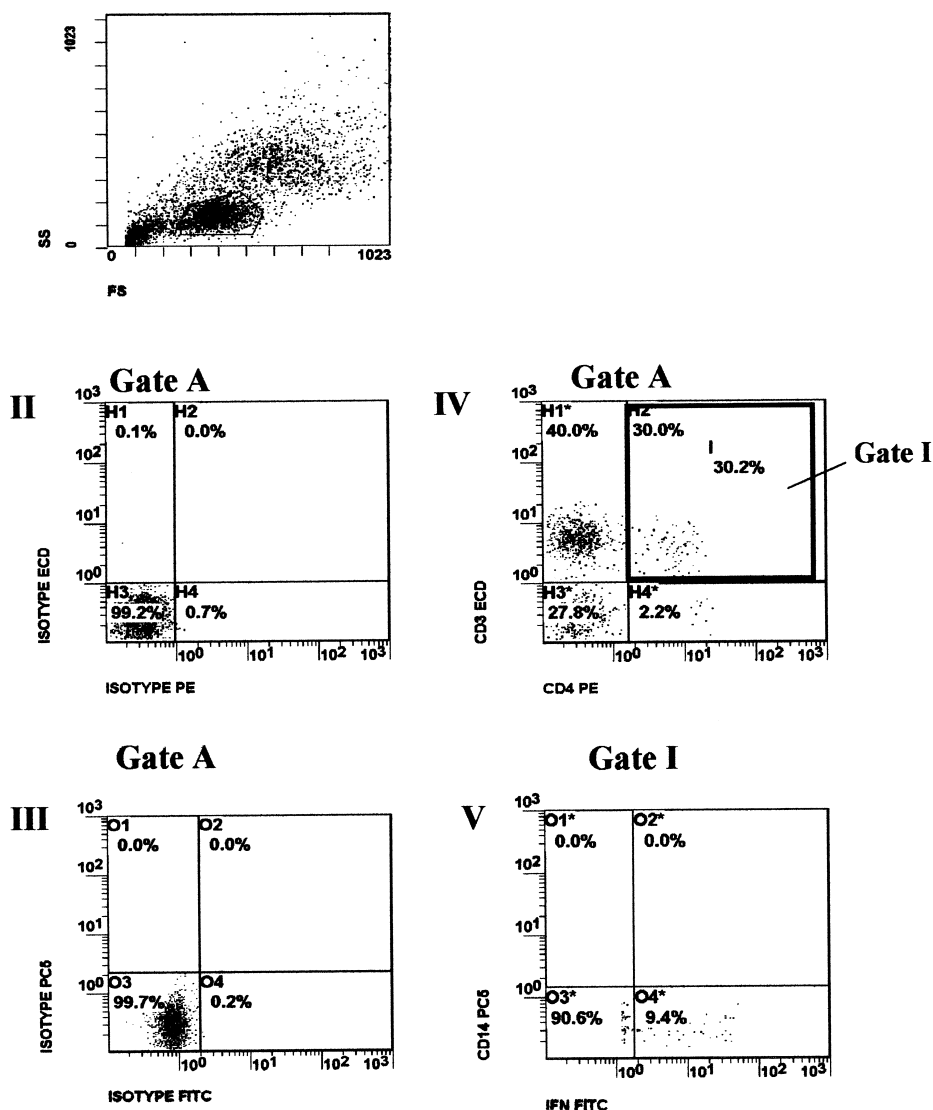


Fig. 1. IFN- γ content of CD3-positive lymphocytes. Shown is a cytoplasmic staining of cells from a patient after allogeneic transplantation. Lymphocytes are gated in gate A after FSC/SSC dot plot. (I). Left panel shows isotype control (II–III); right panel shows specific staining: (IV) CD4 PE vs CD3 ECD. (V) shows the IFN- γ positive portion of CD3–CD4 double positive cells (gate I in IV). No CD14-positive monocytes can be found in the gate.

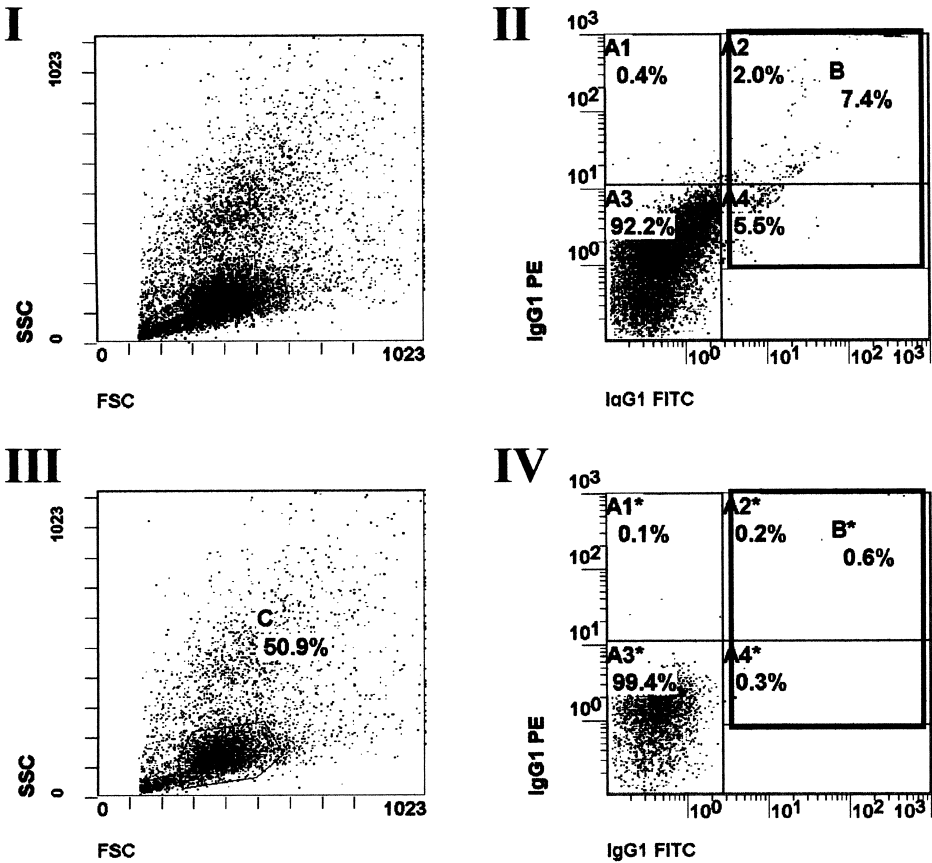


Fig. 2. Reduction of nonspecific staining by gating. Shown is a cytoplasmic isotype control staining of cells from a patient after allogeneic transplantation. Left panel shows FSC/SSC dot plot (I + III), right panel shows fluorescence signals in FL1 and FL2 (II + IV). A great portion of unspecific staining occurred (II, gate B). Nonspecific stained cells in gate B can be reduced by gating lymphocytes, as shown in (III), and analysis of gate C, shown in IV.

2. It is possible to keep the isolated mononuclear cells at 4°C overnight in medium and proceed on the next day. It is also possible to interrupt the staining procedure after fixation of the cells. However, best results are obtained without interruptions.
3. The stimulation time depends on the observed cells and the cytokines of interest. Therefore, kinetic experiments might be necessary to determine the optimal time for stimulation.
4. It is important to control the cells that are in the antibody containing solution after vortexing (sometimes cell pellets move while vortexing)!

5. Unfortunately, nonspecific staining by isotype control antibodies may occur. This might be reduced by blocking of the cells with Fc block (e.g., from BD Pharmingen) prior to staining. However, background staining can also be reduced by gating strategies. Most of the background results from cell debris or cell aggregates. Gating the population of interest by FSC/SSC characteristics can reduce the nonspecific staining dramatically (e.g., see **Fig. 2**).

References

1. Griffioen, A. W., Rijkers, G. T., and Cambier, J. C. (1991) Flow cytometric analysis of intracellular calcium: the polyclonal and antigen-specific response in human B lymphocytes. *Methods* **2**, 219.
2. Reddy, S., Rayburn, H., and Vonnemelcher, H. (1992) Fluorescence-activated sorting of totipotent embryonic stem-cells expressing developmentally regulated lac z fusion genes. *PNAS* **89**, 6721.
3. Leary, J. F. (1994) Strategies in rare cell detection and isolation. *Methods Cell Biol.* **42**, 331.
4. Young, J. S., Varma, A., and Diguisto, D. (1996) Retention of quiescent hematopoietic cells with high proliferative potential. *Blood* **87**, 545.
5. Wieder, E. D., Hang, H., and Fox, M. H. (1993) Measurement of intracellular pH using flow cytometry with carboxy-SNARF-1. *Cytometry* **14**, 916.
6. Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein-labelled Annexin V. *J. Immunol. Methods* **184**, 29.
7. Vermes, I., Haanen, C., and Reutelingsperger, C. (2000) Flow cytometry of apoptotic cell death. *J. Immunol. Methods* **243**, 167.
8. Lecoecur, H., Fevrier, M., Garcia, S., Riviere, Y., and Gougeon, M. (2001) A novel flow cytometric assay for quantitation and multiparametric characterization of cell-mediated cytotoxicity. *J. Immunol. Methods* **253**, 177.
9. Heine, J., Jaeger, K., Osthaus, A., Weingaertner, N., Munte, S., Piepenbrock, S., et al. (2000) Anaesthesia with propofol decreases FMLP-induced neutrophil respiratory burst but not phagocytosis compared with isoflurane. *Br. J. Anaesth.* **85**, 424.
10. Thomson, A. W. (1998) *The Cytokine Handbook*, 3rd ed. Academic, New York.
11. Schroff, R. W., Bucana, C. D., Klein, R. A., Farrell, M. M., and Morgan, A. C., Jr. (1984) Detection of intracytoplasmic antigens by flow cytometry. *J. Immunol. Methods* **70**, 167.
12. Jacob, M. C., Favre, M., and Bensa, J. C. (1991) Membrane cell permeabilization with saponin and multiparametric analysis by flow cytometry. *Cytometry* **12**, 550.
13. Sander, B., Andersson, J., and Andersson, U. (1991) Assessment of cytokines by immunofluorescence and paraformaldehyde-saponin procedure. *Immunol. Rev.* **119**, 65.
14. Verheyen, J., Bönig, H., Kim, Y. M., Banning, U., Mauz-Körholz, C., Kramm, C., et al. (2000) Regulation of interleukin-2 induced interleukin-5 and interleukin-13

- production in human peripheral blood mononuclear cells. *Scand. J. Immunol.* **51**, 45.
15. Chalmers, I. M. H., Janossy, G., Contreras, M., and Navarrete, C. (1998) Intracellular cytokine profile of cord and adult blood lymphocytes. *Blood* **92**, 11.
 16. Mosmann, T. R. and Sad, S. (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today* **17**, 138–145.
 17. Jung, T., Lack, G., Schauer, U., Uberruck, W., Renz, H., and Gelfand, E. W. (1995) Allergens, IgE, mediators, inflammatory mechanisms. Decreased frequency of interferon γ and interleukin-2-producing cells in patients with atopic diseases measured at the single cell level. *J. Allergy Clin. Immunol.* **96**, 515.
 18. Meyaard, L., Hovenkamp, E., Keet, I. P. M., Hooibrink, B., Jong, I. C. H., Otto, S. A., et al. (1996) Single-cell analysis of IL-4 and IFN- γ production by T cells from HIV-infected individuals. *J. Immunol.* **157**, 2712.
 19. Carayol, G., Bourhis, J. H., Guillard, M., Bosq, J., Pailier, C., Castagna, L., et al. (1997) Quantitative analysis of T Helper 1, T Helper 2, and inflammatory cytokine expression in patients after allogeneic bone marrow transplantation. *Transplantation* **63**, 1307.
 20. Murphy, W. J. and Blazar, B. R. (1999) New strategies for preventing graft-versus-host disease. *Curr. Opin. Immunol.* **11**, 509.
 21. Pan, L., Delmonte, J., Jalonen, C. K., and Ferrara, J. L. (1995) Pretreatment of donor mice with granulocyte colony stimulatory factor polarizes donor T-lymphocytes towards type 2 cytokine production and reduces severity of experimental graft versus host disease. *Blood* **86**, 4422.

Cytokines and Colony Stimulating Factors

Methods and Protocols

Körholz, D.; Kiess, W. (Eds.)

2003, XV, 478 p., Hardcover

ISBN: 978-1-58829-035-9

A product of Humana Press