# Preface

The immune system is a complex network in which different cell types and soluble factors interact to efficiently eliminate various kinds of microorganisms as well as aberrant cell clones. The roots of immunologic investigations reach far into the past. In 430 BC, Thucydides reported that survivors of the plague did not present a second time with similar symptoms. The first report of a successful immunotherapy was made by Edward Jenner in 1798 who found a protective effect of cowpox vaccination against human pox. Since then, much knowledge has been accumulated; today, investigations of the molecular mechanisms of immune regulation are of central research interest. The novel insights into gene polymorphisms and gene regulation gathered from this work has improved our knowledge of individual immune reactions and risk factors in overcoming infections. Strategies to use the immune system for cancer treatment have been propelled by the discovery of divergent immunoregulatory cytokines and the introduction of new gene therapy strategies to modify immune responses. Recently, the discovery of various dendritic cells has focused attention on these cell types as central elements of the immune response and to the possibility of dendritic cell expansion, maturation, and consecutive stimulation with immunoreactive tumor-specific peptides. Similarly, methods for ex vivo expansion of various stem cell-derived cell types have led to an improved therapeutic management of various benign and malignant diseases. However, hope for greater therapeutic success in the clinical setting, and therefore patient benefits, has been quite limited with these methods. In the field of immunoregulation, enormous effort is still required to increase fundamental understanding and the therapeutic benefit. Only basic science and further research will provide for means to achieve this goal.

*Cytokines and Colony Stimulating Factors: Methods and Protocols* is intended to promote all the present knowledge and understanding generated by the many novel technologies described here in the hope of spreading awareness of its useful applications. Our book aims to address the needs of novice investigators hoping to work in this field who need profound and practical information to get started, as well as seasoned investigators seeking to extend their technical range. The book not only provides detailed descriptions of methods, but also includes a section on troubleshooting in each chapter indicated in the Notes section. With the spreading use of these methods and the growing activity in the field, many qualified scientists can work on the therapeutic benefit of these studies for future patients suffering from these diseases.

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# 2

# Intracellular Detection of T-Cell Cytokines

## *Differentiation of TH1 and TH2 Cells*

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## 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 suspective protein. In addition,

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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, Nurenberg, Germany).
2. Phosphate-buffered saline (PBS): 8 g NaCl, 1.42 g $Na_2HPO_4 \cdot H_2O$, 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. Bicoll (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 µg/mL streptomycin
   2 m*M* 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<br>IgG2a surface |
| 2 | IgG1 cytoplasmic | IgG1 surface | IgG1 surface | IgG1 surface<br>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; isoytype: mouse IgG1)
   Anti-CD4-PE (clone: 13B8.2; isoytype: mouse IgG1)
   Anti-CD8 FITC (clone: B9.11; isoytype: mouse IgG1)
   Anti-CD8 PE (clone: B9.11; isoytype: mouse IgG1)
   Anti-CD14 PC5 (clone: RMO52; isoytype: mouse IgG2a)
   Anti-CD56 PC5 (clone: N901-NKH1; isoytype: mouse IgG1)
   Anti-IFN-γ FITC (clone: B27; isoytype: mouse IgG1)
   Anti-IL-4 PE (clone: 8D4-8; isoytype: 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 800*g* without break.
4. Transfer mononuclear cell fraction to a new tube and wash with PBS to remove residual Ficoll solution (600*g*, 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/streptomycine at a final density of $10^6$/mL (*see* **Note 2**).

### 3.2. Stimulation

1. Transfer 200 µL 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 µg/mL) for 4 h.
3. Add GolgiStop (0.6 µL 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% $CO_2$ (*see* **Note 3**).

### 3.3. Staining of Cells

1. After stimulation, transfer cells from two wells (approx $4 \times 10^5$ 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 600*g* for 5 min.
4. Discard the supernatant and resupend the cells in 100 µL PBS.
5. For cell surface analysis, stain the cells with 10 µL 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 µL 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 µL PBS.
11. After the addition of 10 µL 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 µL 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!

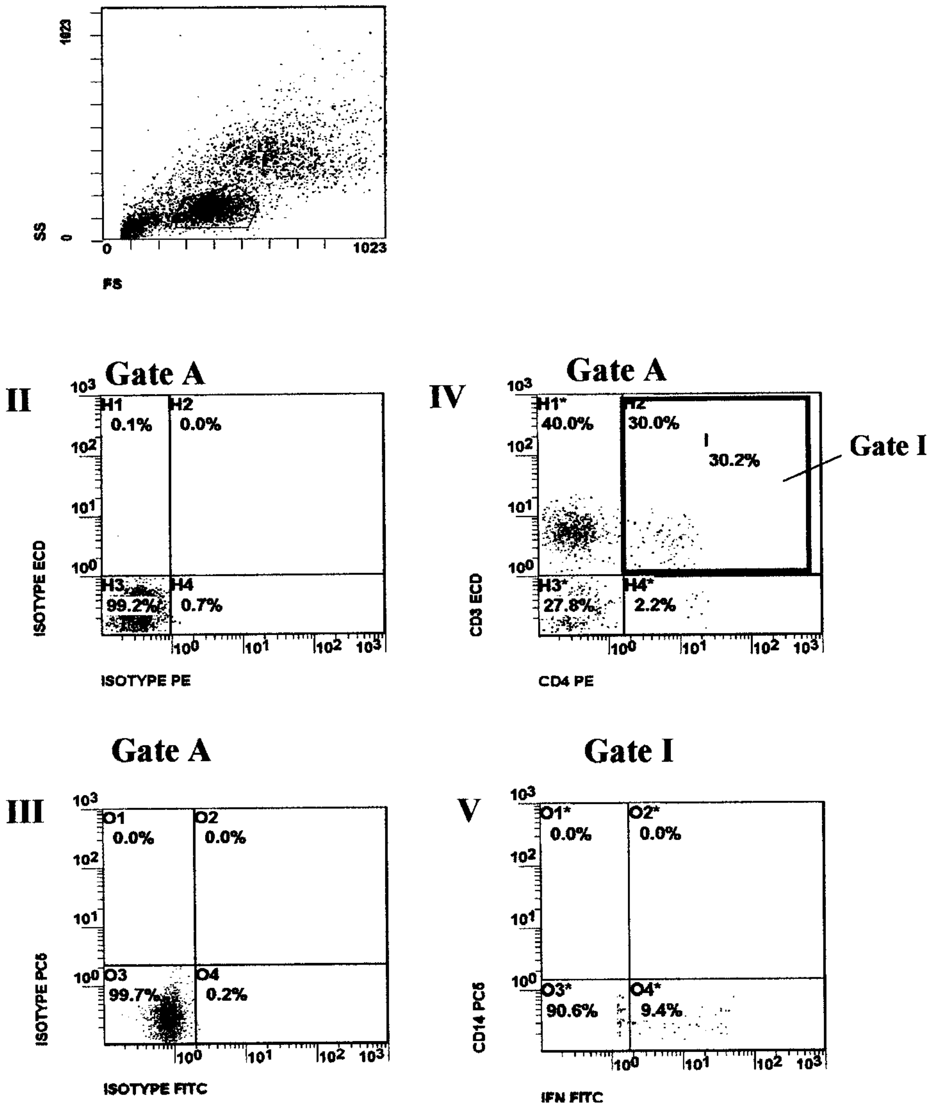


Fig. 1. IFN-γ content of CD3-positive lymphocytes. Shown is a cytoplasmic staining of cells from a patient after allogeneic transplantation. Lymphocytes are gated in gate A after FSC/SSC dot plot. **(I)**. Left panel shows isotype control **(II–III)**; right panel shows specific staining: **(IV)** CD4 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.

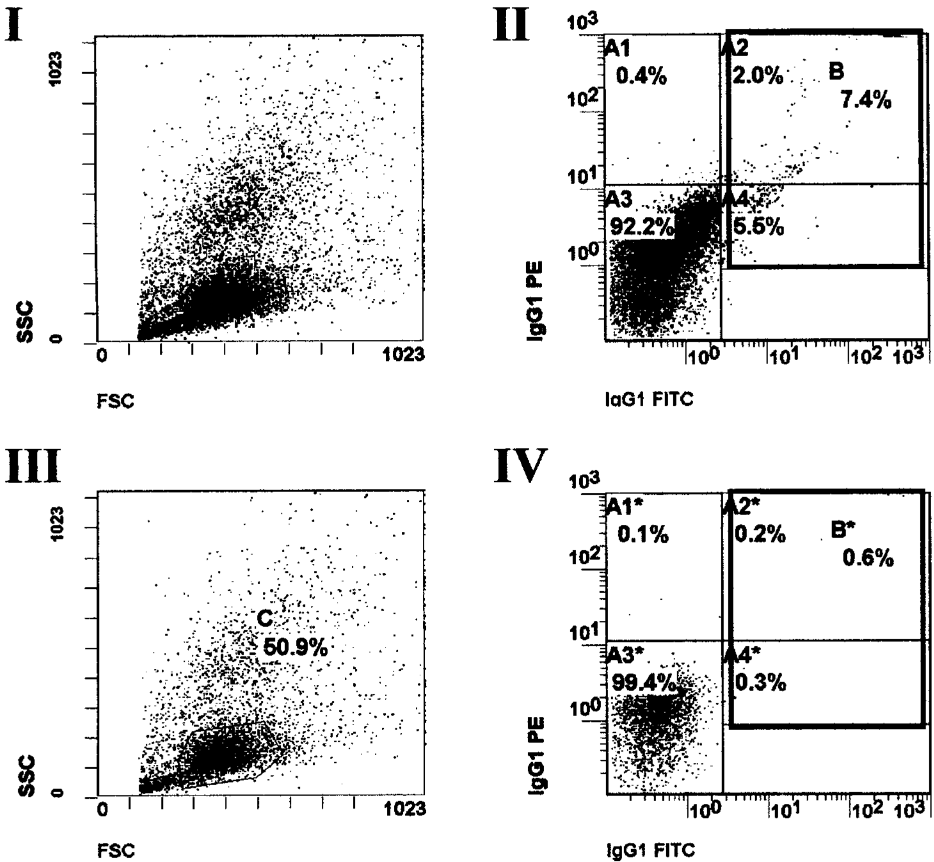


Fig. 2. Reduction of nonspecific staining by gating. Shown is a cytoplasmic isotype control staining of cells from a patient after allogeneic transplantation. Left panel shows FSC/SSC dot plot (**I** + **III**), right panel shows fluorescence signals in FL1 and FL2 (**II** + **IV**). A great portion 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 interupt 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**).

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