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Analysis of functional polarization of T lymphocytes by flow cytometry

Cytometryczna ocena funkcjonalnej polaryzacji subpopulacji limfocytów T

INTRODUCTION

Effective reactions of organism against different types of pathogens require various mechanisms of immunological responses. T-cell functions are one of the most important among effector mechanisms and their regulations are essential for host survival. Shortly after their development in the thymus, T cells enter peripheral immune system where are stimulated by antigens together with major histocompatibility complex (MHC) molecules. At this stage, T cells produce interleukin 2 and clonally expand and differentiate into mature effector cells. T helper lymphocytes express the CD4 antigen and can differentiate into at least two subpopulations of cells — Th1 and Th2, distinguished by pattern of cytokines that they produce [5, 9]. The identification of functionally distinct CD4 cells was presented in 1986 by Mosmann *et al.* [8]. Cytokines produced by Th1 cells (INF-γ, IL-2) are involved in cell-mediated reactions, they activate cytotoxic and phagocytic function of macrophages, NK cells, cytotoxic T lymphocytes. Th1 cells participate in inactivation of intracellular pathogens (e.g. viruses, yeast) and, if uncontrolled are able to mediate autoimmune diseases such as type-1 diabetes or multiple sclerosis. Th2 cytokines (IL4, IL-5, IL-13) encourage IgE antibody production, proliferation and function of eosinophils and mast cells and elimination of extracellular parasites. Dysregulations of Th2 cytokines can lead to strong antibody and allergic responses. Unlike mouse T cells, productions of IL-6 and IL-10 are not restricted to a single subpopulation of Th cells in human lymphocytes.

The mechanisms underlying polarization of naive Th cells are complex and remain partly unclear. The principal factors include: nature and dose of antigen, affinity of ligation between TCR receptor and antigen-MHC complexes, presence of signals provided by the interaction of CD80-CD28, ICAM-1-LFA-1, CD40-CD40L. Many studies suggest that IL-4 and IL-12 provide dominant signals inducing the differenti-

ation of Th1 and Th2 cells. IL-12 derived from antigen presenting cells (APC) plays a major role in differentiation and clonal expansion of Th1 cells due to activation of the transcriptional factor STAT-4. Interleukin-4 produced by Th2 lymphocytes, mast cells, basophiles, CD4+NK1.1 cells is a potent factor inducing Th2 response.

Recent data indicate that dendritic cells (DC) that originate from the place of pathogen activity provide naive Th cells with proper signals to differentiate into Th1 or Th2 cells.

Despite the fact that presence of effector Th1 and Th2 subpopulations is well documented, many CD4 cells do not simply match a Th1/Th2 dichotomy. T cells producing cytokines of both patterns have been called Th0 cells [1, 9]. Heterogeneity in expressions of cytokines among T cells may represent distinct phenotypes, differences at development stages or variability in responses to activation stimuli.

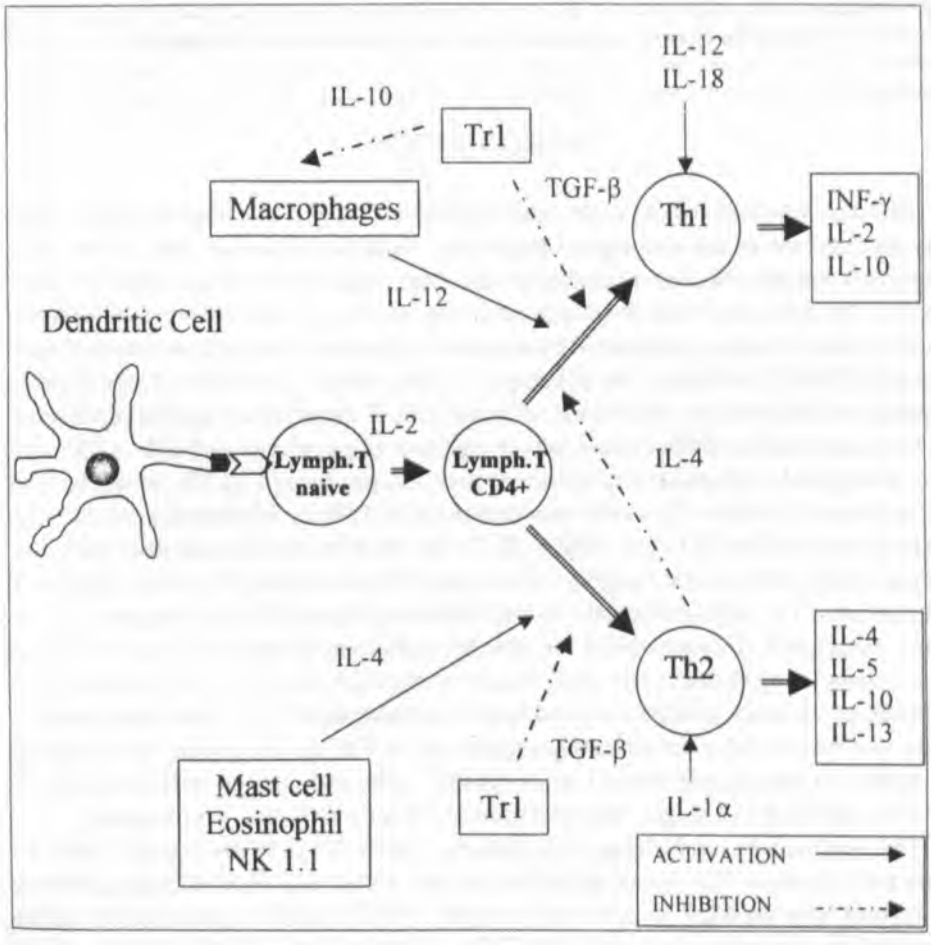

Fig 1. Differentiation of Th subpopulations

In addition to well-known Th1 and Th2 subsets, recent progress has been made in recognition of regulatory T cells (Th3/Tr1). These cells constitute 10% of mature T lymphocytes and play critical role in generation and maintenance of tolerance to self and foreign antigens [13]. Tr1 cells suppress immune response via mechanism dependent on the production of IL-10 and TGF-β. These cytokines are able to render the APC tolerogenic and at the same time directly induce anergy of T cells.

Analogous to CD4 subpopulations, cytotoxic T lymphocytes are characterized by similar functional polarization [14]. In addition to their role in eliminating of infected cells, CD8 cells have regulatory function in proliferation and differentiation of Th cells. They act on CD4 cells directly or by production and secretion of cytokines. Similar to Th cells, two distinct CD8 subsets, Tc1 and Tc2 are identified by their cytokines, INF-γ and IL-4, respectively. IL-12 strongly promotes growth of Tc1 clones, whereas IL-4 suppresses proliferation of this clone.

ANALYSIS OF INTRACELLULAR CYTOKINE PRODUCTION

T lymphocytes and the other cells of immune system play they regulatory and effector function mainly by means of production and secretion of cytokines. Many cytokines are pleiotropic and acting in paracrine or autocrine way they regulate activation, proliferation and differentiation of hematopoietic and non-hematopoietic cells. The cytokine production can by analyzed at level of protein or mRNA, intracellularly or after secretion into extracellular fluid.

In variety of different experimental techniques, including enzyme-linked immunoabsorbent assay (ELISA) or polymerase chain reaction (PCR), cytokine production is measured in bulk stimulated cultures and non-specifically for individual cell. The ELISPOT method and *in situ* hybridization method allow to evaluate cytokines at a single cell level but they are laborious and time consuming [2].

Flow cytometry is a rapid technique of multiparameter analysis of heterogeneous populations without prior cell sorting. After sample preparation, cells are labeled directly or indirectly with combination of different fluorochromes conjugated to antibodies. Blood, bone marrow or lymph node suspensions are focused in fluid stream and reach the place of laser activity. Emitted fluorescence signals are collected by light scatter and fluorescence detectors, amplified, digitized and stored in computer. Data presentation is possible in form of single parameter histogram or 2–, 3–D contour, dot, and density plots.

Pioneering method of detection of intracellular cytokines productions in flow cytometry was presented by Jung *et al.* [5] but has been significantly modified in order to improve its sensitivity and simplicity [10, 11, 12]. Assessments of cytokine expression capabilities combined with analysis of other superficial or intracellular antigens provide excellent tool to qualitative and quantitative measurement. Cytokine expression patterns can be used to characterized normal and pathological cell function of different immune situations or type of responsiveness to many stimuli (e.g. bacteria, drugs).

Briefly, this technique is based on the stimulation of cells *in vitro* in the presence of a pharmacological inhibitor of secretion — monensin or brefeldin A (BFA). Mo-

nensin disrupts ion gradients in cell membranes, causing transport arrest at the Golgi complex, whereas brefeldin stops transport in a pre-Golgi compartment. The addition of these substances for last 3–5 h of stimulation increase the number of cytokine positive cells and signal/noise ratio. In longer cell cultures this chemicals are toxic for cells. Activated cultures are subsequently harvested for staining. After cell surface staining leukocytes are subsequently fixed and permeabilized in order to enable penetration of anti-cytokine antibodies inside cells [6].

Detection of intracellular cytokines by flow cytometry could be used to evaluate immune regulation of human individuals and animal models. Simultaneous detection of cell surface and intracellular antigens provides opportunity for cytokine measurement in cells collected from peripheral blood (whole blood and separated mononuclear leukocytes), bone marrow aspirates, lymph nodes and other biological fluids. This method offers a wide range of scientific possibilities, but also creates many obstacles and problems. There are various parameters that have to be optimized in each studied model, as well as issues of specificity and appropriate controls [7, 10].

Stimulation

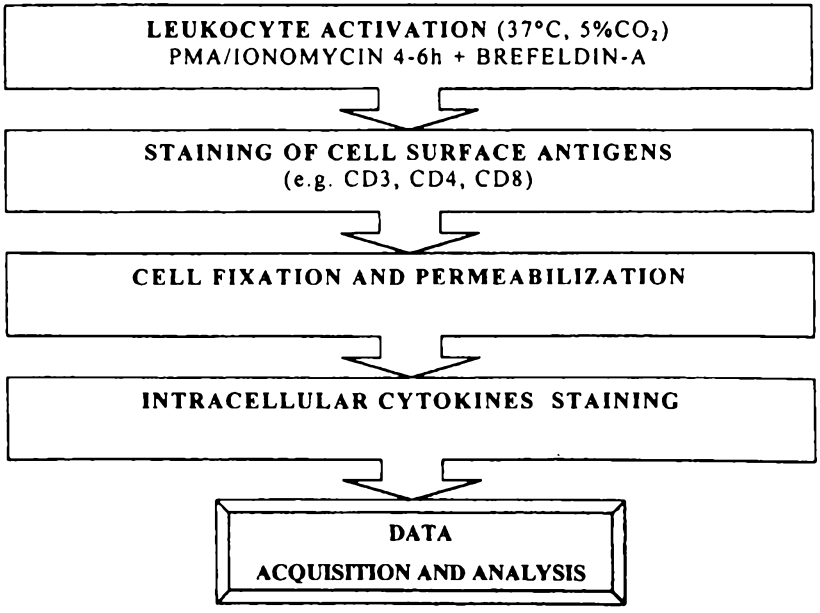

Fig 2. Schematic diagram of intracellular cytokine detection by flow cytometry.

In vivo cytokine production is transient and depends on contact of antigen presenting cell with T lymphocytes. Non-stimulated lymphocytes spontaneously express only a small amount of cytokines and without proper stimulation it is very difficult to detect cytokines intracellularly. There are two groups of *in vitro* activators [3]. The first one consist of widely applied polyclonal activators — phorbol myristate acetate

(PMA) and ionomycin, anti-CD3 and anti-CD28 antibodies, lectins (e.g. phytohaemagglutinin). Antigens (viruses, bacterial antigens) compose the second group of more "physiological" stimulators [10], mostly useful for monitoring of infectious immune challenges. It is necessary to determine the kinetic profiles of cytokine production in order to establish "window of time" of cell harvesting. Optimal period of activation for IL-2, IL-4, INFγ, TNFα is 4–6 hours at 37°C, the time of the highest frequencies of cytokine producing cells. Monensin or brefeldin have to be added for last 3–4 hours of activation. Successful activation is critical to achieve good results and CD69 antigen, marker of early lymphocyte activation, is control tool of this step of protocol. Unfortunately, PMA/ionomycin lymphocyte activation is associated with down regulation of some surface antigens. In case of PMA/ionomycin stimulation, expression of CD69 above 90% is proof of proper activation and analysis of intracellular cytokines should be carried out in CD69 positive leukocytes. Selection of activation model should be in any case individually considered according to experimental model.

Fixation and permeabilization

Permeabilization of cytoplasmatic membrane of lymphocytes is necessary step for detection of intracellular antigens. Among chemicals increasing cell membrane permeability is saponin, widely used nonionic detergent. Saponin activity is reversible and does not result in autofluorescence and cells aggregation. Many commercial kits containing saponin solutions are available. In addition, they contain fixatives (paraformaldehyde), preserving cell morphology. These solutions have to be added before intracellular staining and together with anti-cytokine antibodies in order to maintain cells in permeabilized state.

Monoclonal antibodies

Expressions of INFγ and IL-4 are routinely measured in order to assess polarization of T cells participating in Th1 (CD4+/INFγ+) or Th2 (CD4+/IL-4+) cytokine responses. In addition, analysis of cytokine production should be carried out only in CD69 positive cell (if 4-colour detection is available). A lot of murine anti-cytokine antibodies directly conjugated with fluorochromes are commercially available. According to the need of investigator, fluorochromes are selected from among: fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC). Intracellular cytokines staining is less sensitive than cell surface staining due to poorer discrimination between antigen specific and nonspecific binding. When performing multicolor analysis, the high intensity of fluorescence emission makes phycoerythrin (PE) an ideal dye of anti-cytokine antibodies. Cell surface staining usually precedes permeabilization and subsequent intracellular labeling but it is also possible to make all stainings at the same time during the intracellular staining step (if surface epitopes of interest antigen resist fixation and permeabilization).

Controls

Nonspecific binding of antibodies to intracellular proteins is a significant issue related to permeabilization and fixation of cells, autofluorescence or presence of cy-

tokines in surface-bound forms. Therefore, it is of great importance to use appropriate controls of experiments [4]. Several types of controls are performed to confirm the specificity of cytokine staining:

- Isotype control seems to be less useful as opposed to surface antigen staining. The percentage of cytokine positive leukocytes is counted after subtracting fluorescence of Ig isotype control.
- Preblocking of fluorochrome conjugated antibody with purified cytokine proteins or unconjugated monoclonal anti-cytokine antibody, enables blocking of specific staining. This control is very useful and appropriate but rather expensive.

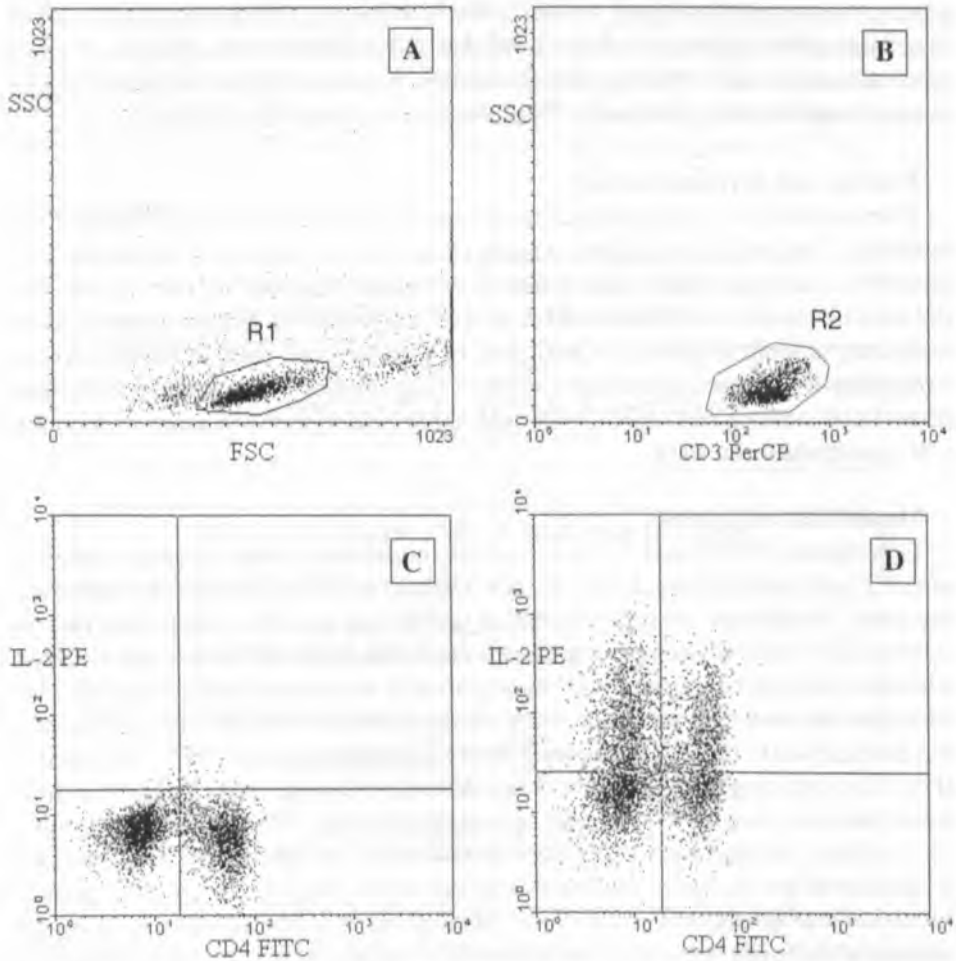

Fig 3. Flow cytometric evaluation of IL-2 expression in Th lymphocytes (CD4+).
A — cells were gated on region (R1) on the basis of cell size (FSC — *forward light scatter*) and cellular granularity (SSC — *side scatter*).
B — within R1 additional region (R2) was created of CD3 positive population.
C/D — analysis of IL-2 expression in unstimulated (C) and stimulated (D) lymphocytes within R2 region (CD4+ and CD4– cells).

- o Activation control is formed by stimulated cells with PMA/ionomycin but without brefeldin. Proper activation is distinguished by surface expression of CD69 greater than 90%.
- o Permeabilization control is estimated by intracellular staining of CD69 in cells activated with PMA/ionomycin in the presence of brefeldin-A.
- o Positive control is very useful to verify the staining procedure or adequacy of culture condition for cytokine assay. The control consist of cell population with known frequency of cytokine producing cells.

CONCLUSION

The simplicity of method and relatively short time of intracellular cytokine detection by flow cytometry, as well as availability of many anti-cytokine monoclonal antibodies makes this technique applicable to clinical assessment of Th1/Th2 balance. Flow cytometry gives capabilities of simultaneous multi-parameter analysis of surface and intracellular antigens and offers unique tool for evaluation of immune processes. It is possible to measure thousands of molecules within seconds combined with subsequent quantitative and qualitative analysis. Intracellular cytokine determination can be connected with analysis of cell cycle, processes of proliferation and apoptosis. These technique is applicable in many studies related to disturbance in the Th1/Th2 polarization, e.g. allergy, autoimmune diseases or infections.

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STRESZCZENIE

Limfocyty T są jednymi z najważniejszych komórek odpowiedzi immunologicznej. Limfocyty pomocnicze Th charakteryzują się ekspresją antygenu CD4 i można je podzielić, na co najmniej dwie subpopulacje Th1 i Th2. Cytokiny produkowane przez komórki Th1 (np. INF-γ) są istotne w odpowiedzi komórkowej, podczas gdy cytokiny charakterystyczne dla komórek Th2 (np. IL-4) mają decydujące znaczenie w odpowiedzi humoralnej. Dzięki użyciu cytometrii przepływowej możliwa jest ocena wewnątrzkomórkowej produkcji cytokin przez pojedynczą komórkę. Metoda ta udostępnia wieloparametrowy pomiar niejednorodnej populacji komórek, dając w ten sposób narzędzie obserwacji złożonych fizjologicznych i patologicznych procesów immunologicznych. Ocena polaryzacji produkcji cytokin przez limfocyty T dostarcza danych o stanie układu odpornościowego w przebiegu m.in. nowotworów, zakażeń, chorób alergicznych i autoimmunologicznych.