Review

Detection of T cell cytokine production as a tool for monitoring immunotherapy

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1. Introduction

There are many complex relationships between tumour cells and effector cells in the immune system. These interactions are controlled predominantly by cytokines, either within the tumour environment, or systemically where the effector cells may be stimulated as a response to the presence of the tumour. Favourable clinical responses in cancer patients have been shown to be associated with enhanced cell-mediated immunity as well as T cell infiltration in tumours. This status is controlled in part by a predominantly Th1 cytokine profile e.g. IFNγ, TNFα and IL-12. Conversely, patients with advancing cancer may have impaired cell-mediated immunity as a result of an imbalance of Th1 and Th2 cytokines e.g. IL-4 and IL-10 [6,9,15]. Whilst cytokines have long been known to orchestrate the immune system by allowing communication between regulatory and effector cells, the pleiotropic nature of these molecules results in a very complex environment in which to study any single molecule's properties.

Several *in vitro* protocols have been developed, which aim to closely reflect cytokine production and T cell function *in vivo*. However, these assays have been developed in artificial settings and as such only allow conclusions to be drawn within a defined context [11].

The aim of this report is to outline the basic protocols and applications for the detection of intracellular cytokines by flow cytometry, in the context of disease monitoring.

2. Methodology

Junge and colleagues [7] detailed a method to study the intracellular cytokine production of single cells by flow cytometry, which has become the basis of many studies. This assay has become an indispensable means of evaluating immunotherapy in the patient, where cytokines responses are postulated to influence clinical outcome. The basic protocol for the detection of mitogen-induced cytokine production is outlined in Protocol 1. A variation on this method, which allows the detection of cytokines from antigen-specific cells, has been optimised and is outlined in Protocol 2.

2.1. Protocol 1

Mitogen-induced cytokine production can be detected by flow cytometry as described by Junge et al. [7] with slight modification [17]. Sodium heparinized venous blood was diluted with an equal volume of stimulation medium (RPMI 1640 containing 2 mM L-glutamine; 10% FCS; 100 μg/ml penicillin; 100 μg/ml streptomycin; 1 μg/ml Iomomycin; 2.5 ng/ml phorbol 12-myristate 13-acetate (PMA); 10 μg/ml Brefeldin A (BFA)). 1 ml of diluted blood was aliquoted into 15 ml conical polypropylene tubes and incubated in a humidified incubator at 37°C 5% CO$_2$ for 4.5 hr. 1 ml of unstimulated blood incubated with BFA alone was also set up as a control. 100 μl aliquots of stimulated blood were then surfaced stained with CD3-PerCP and CD8-FITC antibodies at room temperature for 15 min. Erythrocytes were lysed and leukocytes fixed by the addition of 2 ml of 1xFACS Lysing Solution (Becton Dickinson, Oxford, UK) and incubated at room temperature for 10 min. Remaining cells were washed in 2 ml wash buffer (PBS/0.5% BSA/0.1% NaN$_3$), before permeabilising with 500 μl of 1xFACS permeabilisation solution (Becton Dickinson, Oxford, UK) and incubating at room temperature for 10 min. The cells were then washed in 2 ml wash buffer before stain-

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ing cells with the appropriate anti-cytokine antibodies for 30 min at room temperature. Cells were washed with 2 ml wash buffer and fixed with 200 μl of CellFix (Becton Dickinson, Oxford, UK) before analysis on a FACScan (Becton Dickinson, Oxford, UK). Typically 10,000 events were collected.

2.2. Protocol 2

Antigen-specific cytokine production can also be detected by flow cytometry as described by Waldrop et al. [16] for PBMC and more recently by Dr. V.C. Maino (Personal communication) for whole blood. Sodium heparinized venous blood was aliquoted into 15 ml conical polypropylene tubes at 1 ml per tube. The appropriate antigen was added at the optimal concentration along with anti-CD28 (Becton Dickinson, Oxford, UK) and anti-CD49d (Becton Dickinson, Oxford, UK) antibodies both at 1 μg/ml final concentration. The blood was mixed gently before being incubated in 5%CO_2 for 2 hr. BFA (10 μg/ml final) was added and the diluted blood incubated at 37°C for a further 4 hours. EDTA (20 mM final) was added and vortexed vigorously to remove adherent cells and incubated at room temperature for 15 min. 100 μl aliquots of stimulated blood were taken, erythrocytes lysed and leukocytes fixed by the addition of 2 ml of 1×FACS Lysing Solution (Becton Dickinson. Oxford, UK) and incubated at room temperature for 10 min. Remaining cells were washed in 2 ml wash buffer (PBS/0.5% BSA/0.1% NaN_3), before permeabilising with 500 μl of 1xFACS permeabilisation solution (Becton Dickinson. Oxford, UK) and incubated at room temperature for 10 min. The cells were washed in 2 ml of wash buffer before being stained for surface CD69-PE, CD4-PerCP and intracellular cytokines simultaneously at room temperature for 30 min in the dark. The cells were then washed and fixed for analysis as outlined in the mitogen-stimulated protocol above. Typically, 50,000 events were collected.

3. Results

The following examples comprise of cytokine data obtained from 2 patients with malignant melanoma who received two different immune treatments over varying time schedules. Patient 1 was injected twice with BCG (Bacille-Calmette-Guérin), which is a non-specific adjuvant commonly used in the treatment of bladder cancer. Whilst its application in immunotherapy has been shown to correlate with cell mediated re-

sponses, there have been marked differences in clinical outcome of individual patients [15]. Figure 1 represents the mitogen-induced cytokine (protocol 1) profiles of the Th1-associated cytokine interferon-γ (IFNγ) and the Th2-associated cytokine interleukin-4 (IL-4) before and after the administration of BCG. It is apparent that there is a significant alteration in the general cytokine profiles with an increase in the number of IFN(producing cells and a decrease in IL-4 producing cells, this is typical of a Th2 to Th1 switch.

Patient 2 was treated with a HLA-A2* binding peptide, specific for a melanoma-associated antigen, over a period of 6 weeks. Blood samples were tested before and after treatment using the same peptide in an antigen-specific cytokine assay (protocol 2). The presence of peptide, as well as anti-CD28 and anti-CD49d antibodies, has induced activation of some CD4+ T cells and the production of IFNγ from a low frequency of responding cells (Fig. 2).

4. Discussion

To date, the majority of published studies evaluating cytokine production after immunotherapy have coupled mitogenic stimulation of lymphocytes to responses at either the mRNA level using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, or at the protein level by ELISA. Significant disadvantages are associated with both of these techniques. Firstly, RT-PCR requires isolation of peripheral blood mononuclear cells (PBMC), rendering the analysis of small blood volumes impossible. Secondly, translocational control methods involved in the regulation of cytokine gene expression mean that the levels of cytokine RNA may not accurately reflect the amount of protein synthesised [17]. Thirdly, RT-PCR gives rise to many false positive results within the context of detecting cytokine production.

The detection of cytokines at the protein level requires their specific isolation and concentration from serum or supernatants of experimental cell cultures. This can also be achieved by the use of specific monoclonal anti-cytokine antibodies employed in an ELISA. However, many molecules, such as β-2-microglobulin, produced by cells are capable of interfering with the detection of cytokines by binding to cytokines themselves, thus inhibiting their detection by ELISA [3]. Whilst ELISA studies are informative, they can only give an impression of the bulk cytokine production from a heterogeneous population of cells. They assume that
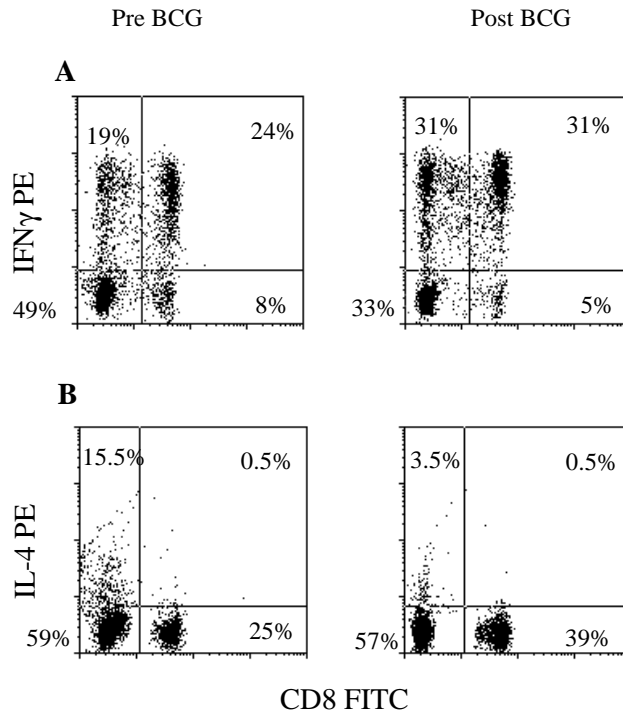
Fig. 1. Intracellular flow cytometry detection of cytokine synthesis from peripheral blood T lymphocytes stimulated following protocol 1 (see Methods). Cells were selected from a 'tight' lymphocyte gate set on the cells forward and side scatter properties, and T cells selected by staining positive for CD3. The percentages in each quadrant represent percentage of CD8+ (LR, UR) or CD4+ (i.e. CD3+/CD8- (LL, UL) T cells producing IFN((A) or IL-4 (B).

all cells of a given phenotype respond in a similar way under experimental conditions, which is not an accurate reflection of cytokine expression by individual effector cells [2,8].

Assays have therefore been developed to study cytokine production by single effector T cells – these include immunofluorescence microscopy [14]; ELISpot [1] and limiting dilution analysis [13]. However, even though all three assays offer additional benefits they involve labour intensive techniques requiring considerable technical expertise, the result of which is that the routine examination of single cells is still greatly restricted. The levels of cytokine production by individual T cells stimulated following these protocols is relatively low. This makes the subsequent discrimination between cytokine positive and negative cells prone to subjective discrepencies. The advent of flow cytometry has allowed the automated detection of many thousands of cytokine-secreting cells simultaneously. The employment of golgi transport inhibitors such as monensin and brefeldin A allow the concentration of cytokines within the cell amplifying the detection of cytokine producing cells. Furthermore, individual cells

may be assessed for secretion of more than 1 cytokine at once.

In patients receiving inert adjuvants such as BCG, there may be little point in trying to examine the response of lymphocytes to specific antigens. The very low frequencies of antigen-specific T cells compared to the vast heterogeneous T cell repertoire mobilised in response to adjuvants means that there is little chance of being able to detect subtle changes in their cytokine profile. A more appropriate assessment of the immune response to immunotherapy may be to examine alterations in the balance between Th1 and Th2 cytokine production as determined by mitogen-induced protocol. Likewise, where specific effector cells have been targeted by antigen-specific immunotherapy, simply screening for gross changes in the balance between Th1 and Th2 cytokine production gives us very little information, as the responding T cells occur at such low frequencies. The frequency and cytokine profiles of responding T cells can only be detected and compared when examined by antigen-specific intracellular flow cytometry, as the assay is designed to look for changes at the minute level of individual cells.

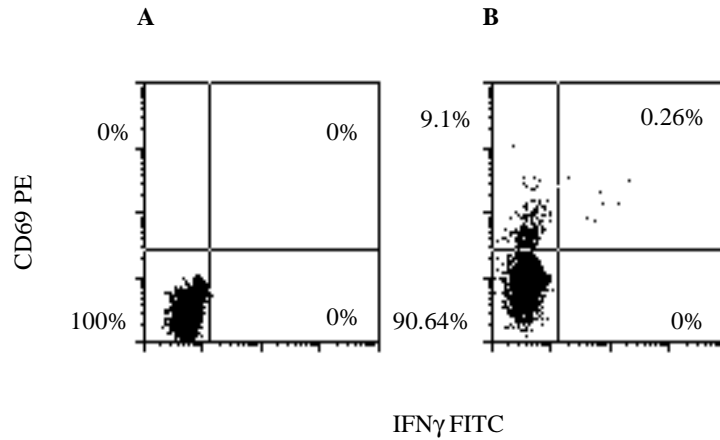**A**   **B**

Fig. 2. Intracellular flow cytometry detection of cytokine synthesis from peripheral blood T lymphocytes following protocol 2 (see Methods). Cells were selected from a 'tight' lymphocyte gate set on the cells forward and side scatter properties and T helper cells selected by staining positive for CD4. The percentages in each quadrant represent percentage of CD69+ (activated) T cells producing IFNγ of unstimulated (A) and peptide/CD28/CD49d stimulated cells.

When selecting which protocol for detecting cytokine production to use consideration must be made with respect to the heterogeneity of responding cells. Many researchers have concluded that, in response to selected antigens, cytokine synthesis varies between stimulated cells. They postulate that even these subtle differences may hold the key to detecting positive immunomodulation [12].

However, there are disadvantages associated with the detection of intracellular cytokines common to both protocols. Firstly, the necessity of blocking golgi transport prevents the ability to quantitate cytokine production at the single cell level. Secondly, the process of fixing and permeabilising cells prevents subsequent functional investigation.

Peptide-MHC tetramers have been developed which allow the detection and isolation of viable antigen-specific T cells by flow cytometry. This method can also be used in conjunction with cytokine detection [4] however, this approach is limited by the need for HLA and specific immunogenic peptide determination. Another recently developed technology is that of antibody-antibody conjugates as a cell surface matrix technology. The conjugates are specific for cell surface CD45 and cytokine and as such allow the detection of secreted cytokine from specific viable cells which can be isolated and characterised further [10].

In conclusion, there are now several established methods for the detection of cytokine production at the single cell level. Each assay has its own advantages and disadvantages making the selection of the most appropriate application dependant on the resources and expertise available. However, the major consideration in the selection should be the specificity of the assay in relation to that of the stimulation regime if cytokine production is to reflect positive clinical outcome, e.g. vaccination.

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