

Review

Antigen-specific cytometry

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Introduction

From its origins in the 16th century, microscopy has allowed the cell, as the basic unit of eukaryotic life and disease, to be identified and analyzed. Today, quantitative cytometric technologies, either microscope based or flow cytometric, are the most powerful tools to analyze the proliferation, physiology and differentiation of cells generally, and are particularly useful in immunopathology. In combination with monoclonal antibodies (which recognize specific gene products) conjugated to sensitive fluorescent dyes, cell types can be identified according to the genes they express. They can also be isolated using either fluorescence-activated cell sorting (FACS) or magnetic cell sorting (MACS). In the past 20 years, immunofluorescence-based cytometry and cell sorting have become 'state of the art' technologies, mostly serving to identify subsets of lymphocytes and systemic changes in the immune system. Although it is certainly of value for diagnosis and analysis of immunopathology, cytometry did have one major limitation; except in a few experimental situations, it was not possible to focus analysis on those lymphocytes that specifically recognize the relevant antigens in a normal or pathological immune reaction. This drawback has recently been overcome both for B and T lymphocytes, using antigen to identify the cells. Today, a number of exciting new technologies make it possible to analyze and isolate specifically those lymphocytes that are directly involved in the immune reaction to given antigens. These advances will spur research in arthritis considerably.

Why did this take so long? The problem is twofold. First, the diversity of the immune system means that lymphocytes recognizing a particular antigen are rare. Estimations of the frequencies of cells specific for one antigen used to

range from 10^{-5} to 10^{-6} , based for example on limiting dilution analyses. For a number of biological and physical reasons immunofluorescence, either with antigens or antibodies, shows considerable variation in intensity. This makes it technically difficult to identify accurately rare cells of interest at frequencies below 10^{-3} to 10^{-4} . Apart from that basic limitation, it is extremely time consuming to analyze a sufficient number of rare cells to obtain a reliable result.

Nevertheless, experimental work [1–4] has shown that it is possible cytometrically to identify and analyze B memory lymphocytes and plasma cells that occur at very low frequencies and that recognize a particular antigen with high affinity, using native antigens conjugated to haptens or fluorochromes. The decisive technological advance in those experiments was the use of a 'parallel' cell-sorting technology (MACS), providing a nonoptical (in this case magnetic) label to enrich antigen-binding cells to make them detectable by flow cytometry and to isolate them for proof of specificity. Today, the cytometry of B lymphocytes according to antigen specificity is not so much a problem of technology as of biology, because B cells that bind to one particular antigen often occur at frequencies of 1–10/ml blood, thus making the availability of sufficient blood for analysis a limiting factor.

A second challenge for antigen-specific cytometry has been the fact that the antigen receptors of T lymphocytes recognize fragments of antigen only in the context of either major histocompatibility complex (MHC) class I or class II molecules. Initial attempts to use recombinant and labelled MHC molecules, and to load them with peptides of interest to stain peptide-specific T cells failed in the late 1980s. Recently, not only has the direct labelling of

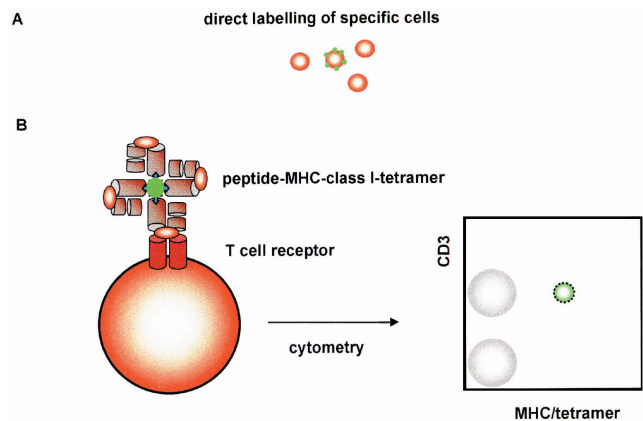
T cells with MHC–peptide complexes finally been achieved, but also alternative technologies have been developed that identify T cells that react to particular antigens by DNA synthesis, proliferation or cytokine expression. In combination or alone, those technologies now offer unique options to analyze antigen-specific T lymphocytes directly *ex vivo*, and to isolate them for molecular and functional studies. Innovative diagnostic and therapeutic strategies based on the identification and isolation of antigen-reactive lymphocytes can now be developed, targeted at the smallest functional unit of immunological disease: the cell. These technologies will have a profound impact not only in arthritis research, but also on research of numerous other diseases.

Peptide–major histocompatibility complex multimers

In 1996, Altman *et al* [5] were the first to show that tetramers of peptide–MHC class I (HLA-A2) complexes can label cytotoxic T cells specifically, providing sufficient avidity to obtain a clearcut cytometric signal (Fig. 1). The advance over previous attempts was the introduction of biotin groups at the C-terminal of the recombinant MHC molecules, allowing four-peptide–MHC monomers to be complexed with avidin, which happens to have four binding sites for biotin. As the authors state in their abstract, ‘This approach should be useful in the analysis of T cells specific for ... autoantigens’. This seems very true, although no studies have been published to date that use tetramer technology for the analysis of autoimmune diseases. One reason might be that tetramer technology requires recombinant MHC molecules and defined peptides. Although some MHC class I molecules are already available (most notably HLA-B27 [6]), the selection is still sparse, and is even more so for MHC class II tetramers, which would allow identification of T helper lymphocytes. Also, a relevant peptide has to be known beforehand (eg from analysis of specificities of T cells expanded *in vitro*), information that is not available for many autoantigens. It is clear, however, that MHC multimers for most MHC haplotypes will be developed soon, which will make this technology the method of choice for the direct identification of T cells according to their peptide specificity and MHC restriction. Relevant peptides can be easily defined by functional assays directly *ex vivo*, as described below.

Already, the available tetramers have provided extensive data on the frequencies and phenotypes of specific T cells for peptides from human immunodeficiency virus, influenza virus, Epstein–Barr virus (EBV) and hepatitis C virus, and for peptides from melanoma antigens [5,7–10]. This has shown that previous estimates based on *ex-vivo* expansion of virus-reactive T cells by limiting dilution had underestimated the frequencies of specific T cells by at least 10-fold. For example, Callan *et al* [7] showed that, during acute EBV infection, CD8⁺ T cells specific for a

Figure 1

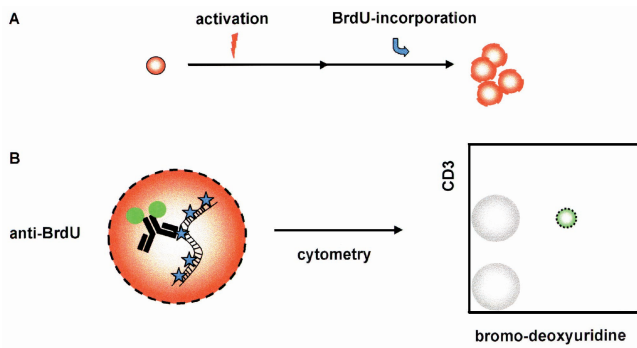


T-cell staining with peptide–major histocompatibility complex (MHC) multimers. (a) Specific cells can be labeled directly *ex vivo*. (b) The peptide–MHC multimer consists of a complex of four peptide–MHC–biotin monomers complexed with avidin and directly conjugated to fluorescein. Counterstaining for CD3 helps to ensure specificity of the staining, but can be replaced for any other parameter (eg secreted cytokines; see Fig. 5).

single EBV epitope are actually not rare at all, but can comprise up to 44% of the total CD8⁺ cells in peripheral blood. The vigorous T cell activation in antiviral immune responses thus seems to be antigen-specific, and not driven by bystander activations, as had been speculated earlier.

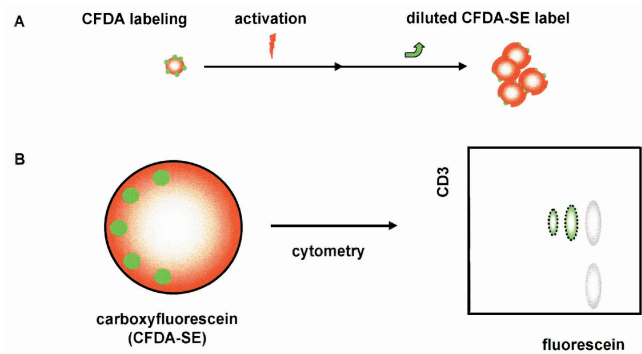
For the analysis of autoimmunity, the application of the tetramer technology provides a few additional challenges. One is epitope spreading (ie the sequential involvement of additional peptide epitopes during the course of disease). The simultaneous assessment of MHC multimers with different peptides, which would be required to analyse an array of peptides, may be limited by competition for the same T-cell receptors and the fact that current flow cytometers can analyze four fluorescent colours per cell maximally only. It can only be hoped that future development of instruments will take up the challenge and make more parameters available. That this is possible has been demonstrated by Bigos *et al* [11], who provided up to 11 parameters in their experimental setup. Future instruments may also provide better resolution for analysis of the cells identified by peptide–MHC multimer staining for phenotype and function. This is the important information. In view of the present limitations of the tetramer technology, a number of alternative strategies have been developed, all of which convert the reaction of T cells to antigen into a cytometric readout. Although information is lost about the cells that bind to the antigen but do not react to it, these technologies offer the decisive advantage that physiological or pathological reactions can be studied on the level of individual cells, directly *ex vivo*, for complex antigens and for any MHC haplotype restriction.

Figure 2



Cytometric analysis of DNA synthesis. (a) During antigenic challenge *ex vivo* (2–5 days) bromodeoxyuridine is incorporated into the DNA of cells performing DNA synthesis. (b) Fixed cells are stained intracellularly with fluorescent antibodies that recognizing bromodeoxyuridine-containing DNA.

Figure 3



Cytometric analysis of cell proliferation. (a) Cells are labelled with carboxyfluorescein (carboxyfluorescein-diacetate-succinimidylester) before antigenic challenge *ex vivo* (2–5 days). Cells reacting to the antigen by proliferation will divide their fluorescent label among their daughter cells on each cell division. (b) Carboxyfluorescein is directly analyzed on viable or fixed cells, and cells can be counterstained for any other parameter.

T-cell reactions I

DNA synthesis (bromodeoxyuridine incorporation)

Classically, and at the level of bulk cell populations, the reaction of a few T cells within populations has been (and in many laboratories still is) measured by their incorporation of radioactively labelled [³H]-thymidine into DNA during DNA synthesis, which is triggered by the antigen from antigen-presenting cells. [³H]-thymidine incorporation is often mistaken as a measure for proliferation, although it actually measures DNA synthesis. [³H]-thymidine incorporation has a number of severe limitations, such as lack of sensitivity caused by the low frequency of responsive cells, and background DNA synthesis in other cells. Also, it does not provide information on the frequencies of responding cells, or on the intensity of DNA synthesis per cell or its phenotype, nor does it allow isolation of such cells for further analysis.

Flow cytometric analysis of DNA synthesis provides a number of advantages. For instance, cells that react to antigenic challenge by DNA synthesis can be identified according to incorporation of bromodeoxyuridine into the DNA [12,13]. Cells with bromodeoxyuridine-containing DNA can then be detected with bromodeoxyuridine-specific fluorescent antibodies (Fig. 2). This technology requires fixation of the cells, however, and thus does not allow further functional studies. The cells can be isolated by FACS for molecular analysis though, and their cytometric analysis directly provides information on frequency and phenotype, and allows analysis of functional molecules such as intracellular cytokines.

Proliferation (carboxyfluorescein)

Cytometry offers a unique option to directly analyze proliferation of cells in response to an antigenic challenge.

Cells are labelled with carboxyfluorescein-diacetate–succinimidylester (CFDA-SE), which binds at random to secondary amino groups of cellular proteins. This provides a uniform fluorescent label to all cells before the antigenic challenge. Cells reacting to the antigen by proliferation will lose 50% of their fluorescent label upon each cell division (Fig. 3) [14]. This allows not only identification and enumeration of antigen-reactive cells, but also supplies information on the proliferative history of each of the cells. Moreover, the cells can be isolated intact for functional studies and molecular analysis. Needless to say, CFDA-SE staining can be combined with stainings for phenotype and effector molecules, such as cytokines. In immune responses to *Candida* antigens, the CFDA-SE technology compared favourably with the classical [³H]-thymidine uptake assay [15]. An intrinsic limitation of the CFDA-SE technology is that cells are not identified that react with effector function rather than with proliferation, or that die by apoptosis during the time of analysis, which is a legitimate reaction of T cells to antigenic challenge.

Intracellular cytokine expression

Apart from DNA synthesis and proliferation, the reaction of T cells to antigen is reflected by calcium influx, a change in membrane potential and by the expression of genes that respond to activation or that are required for effector functions. Although the biochemical changes occur fastest, their analysis in cytometry has been hampered by the fact that current technologies provide too little resolution to identify responding cells accurately if they are rare. The situation is different for activation-induced gene expression, in which cytokines have drawn most attention. In T cells, their expression is transient and depends on antigenic challenge. Although induction of

their expression is dependent on specific costimulatory signals and DNA synthesis [16], the expression of cytokines upon restimulation of the T cell is dependent on antigenic challenge, and is independent of the inducing costimuli and occurs with fast, uniform kinetics within 5–10 h. Cytokines provide a direct measure of the effector function of T lymphocytes. The only disadvantage for cytometric analysis has been that they are secreted, but are not cell surface bound, at least not abundantly, and thus are not readily available for antibody staining of viable cells [17]. Only recently have technologies been developed for the cytometry of secreted proteins of viable cells (Secretion of cytokines, below). Currently, the prevalent approach is the immunofluorescent staining of cytokines within fixed cells before secretion.

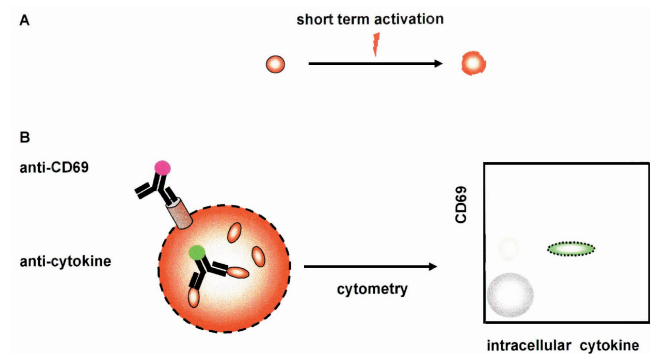
For the detection of antigen-reactive T cells, intracellular cytokine staining has been pioneered by Suni *et al* [18]. They have developed the technology into a whole-blood assay, adding antigen and CD28 costimulation for a few hours, then lysing red blood cells and staining the lymphocytes for intracellular cytokines and the fast activation marker CD69 (Fig. 4). The short activation period and the use of CD69 efficiently reduce background (staining) and exclude bystander cells from the analysis. The efficient use of autologous antigen-presenting cells, supported by CD28, allows analysis either of antigens that have to be processed or of peptides of antigens that may replace endogenous peptides on MHC molecules of any cell. In fact, the technology has been used to identify the dominant T-cell epitopes of cytomegalovirus from a peptide library, as recognized by T cells that express interferon- γ upon challenge, directly *ex vivo* [19], and in a similar way to identify the major autoepitopes recognized by nucleosome-specific T cells in human systemic lupus erythematosus [20]. Currently, candidate autoantigens are evaluated by this direct cytometric analysis of antigen-specific T cells in autoimmune diseases such as systemic lupus erythematosus, reactive arthritis and rheumatoid arthritis.

Secretion of cytokines

The immunofluorescent detection of cytokines within T cells before secretion requires fixation of the cells and permeabilization of their membranes, usually by saponin, in order to get the staining antibodies inside the cell. This has the obvious disadvantage that the cell is killed and the less obvious disadvantage that the cell can no longer be sorted by MACS technology. The additional magnetic parameter provides essential discriminatory resolution for the identification of cells at frequencies below 10^{-5} to 10^{-4} , and T cells reactive to many antigens are less frequent than this. It is also required for their isolation by combined MACS and FACS.

For the cytometry of viable cells according to proteins secreted by them, and their availability for MACS, our

Figure 4



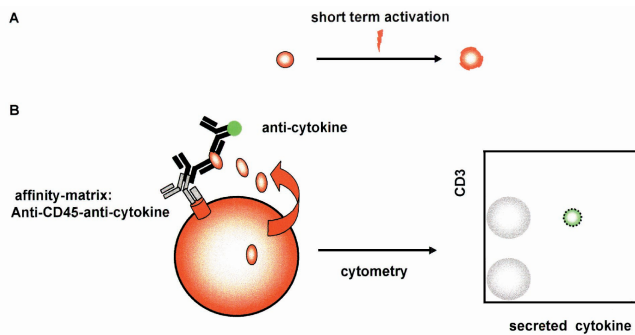
Cytometric analysis of intracellular cytokine expression. (a) Cells can be analyzed after short-term activation *ex vivo* (5–10 h). (b) Specifically activated T cells are able to react with upregulation of the early activation marker CD69 and effector functions (eg cytokine expression). The cytometric analysis has to be performed on fixed cells with permeabilized cell membranes to allow the staining antibodies to enter the cell.

group developed cell-surface affinity matrix technology [21]. This technology has been employed successfully in cytometric identification, analysis and isolation of plasma cells secreting antibodies specific for a particular antigen [3]. For the detection of secreted cytokines and in its most advanced version, the technology uses conjugates of antibodies specific for a cell-surface protein (CD45) to antibodies specific for a given cytokine (eg interferon- γ). Cells are labelled with the bispecific conjugate, allowed to secrete cytokines and are then stained for cytokines bound to the bispecific conjugates (Fig. 5). Initially used in experimental set-ups for murine T cells, the cellular affinity matrix technology has recently, and with success, been applied to the direct *ex vivo* analysis of human cytotoxic and helper T lymphocytes that react to tetanus, influenza and cytomegalovirus antigens by secretion of interferon- γ (Brosterhus H *et al*, personal communication, 1999). In many cases, magnetic cell sorting proved to be essential for the clearcut identification of reactive cells, because they occurred at frequencies of below 10^{-5} among peripheral blood cells. Moreover, isolation of interferon- γ -secreting CD8⁺ T cells helped to demonstrate that those cells specifically killed target cells that displayed the antigenic peptide.

Conclusion

In terms of arthritis research, the emerging technologies for the cytometric identification, analysis and direct isolation of B and T cells binding or reacting to defined antigens have provided exciting and valuable tools in our quest to identify the role of pathogens and autoantigens in the pathogenesis of rheumatic diseases. Most promising appears to be a combination of the tetramer- and cytokine-secretion technologies, allowing simultaneous identifica-

Figure 5



Cytometric analysis of secreted cytokines. (a) Cells can be analyzed after short-term activation *ex vivo* (5–10 h). (b) A bispecific cytokine affinity matrix, specific for a cytokine of choice and a cell-surface molecule, is attached to the viable cells. During a short culture period (15–30 min), secreted cytokines are bound and then stained with a second cytokine-specific antibody.

tion of antigen-specific and antigen-reactive cells. To that end, considerable advances will be required in both technologies, but even now they can help answer questions that long remained unanswered.

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