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Measuring human T-lymphocyte function

Julian K. Hickling

T lymphocytes (T cells) play critical roles in the regulation of immune responses, and are responsible for mediating many of the effector mechanisms of the immune system. For this reason, there has always been a need for assays to measure accurately the activity of populations of T cells, both in model (animal) systems and in humans. The expansion of the biotechnology industry has led to a dramatic increase in the number of novel immunotherapeutics that are being developed for the treatment of cancer, autoimmune disorders and infectious diseases. This increase in activity in the field of immunotherapy, coupled with the expense of clinical trials, has led to renewed interest in methods that accurately assess T-cell function, as researchers seek to maximise the amount of information that can be obtained from each clinical study. Assessing the quantitative and qualitative nature of a T-cell response, for example following vaccination or immunosuppressive therapy, can provide valuable information about the efficacy of a treatment, in place of a clinical endpoint. This article reviews some of the established methods that are used to monitor human T-cell activity, and describes some new approaches that are in development to increase the speed, sensitivity and relevance of such methods.

Because of the wide-ranging effects of T lymphocytes (T cells), which can be either beneficial or harmful, and their involvement in the vast majority of immune responses, immunologists are continually devising methods for the qualitative and quantitative analysis of T cells to aid their understanding of the immune processes that are involved.

T cells

T-cell subsets

T cells can be subdivided into several populations using various operational and phenotypic parameters. In particular, helper T lymphocytes (THLs) can be assigned to one of several subsets including Th1, Th2 and Th0 (Ref. 1; see Table 1). The classification can be made largely on the basis of the types of cytokines (soluble molecules, produced and secreted by lymphocytes, which then mediate or regulate immune responses) that the cells produce. In very broad terms, Th1 T cells are considered to be responsible for cell-mediated effector mechanisms, whereas Th2 T cells play a greater role in the regulation of antibody production. However, the divisions are not absolute and there is considerable overlap or

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Table 1. Human T-cell subsets (tab001jhc)

T-cell subset	Phenotype	Operational definition
Th1	$CD4^+$	Production of IL-2, IFN-γ and TNF-α/β
Th2	$CD4^+$	Production of IL-4, IL-5, IL-6, IL-10 and IL-13
Th0	$CD4^+$	Production of both Th1 and Th2 cytokines
CTL	$CD4^+$ or $CD8^+$	Ability to lyse target cells; production of IFN-γ and TNF-α/β

Abbreviations used: CD4 = a cell-surface marker that is found on a subset of T cells, and usually used as a marker of T-helper lymphocytes (THLs); CD8 = a cell-surface marker that is found on a subset of T cells, and usually used as a marker of cytotoxic T lymphocytes (CTLs); IFN-γ = interferon gamma; IL-2 – IL-13 = interleukin 2 – interleukin 13; Th1 = Th1 subset of T-helper lymphocytes (THLs); Th2 = Th2 subset of T-helper lymphocytes; Th0 = Th0 subset of T-helper lymphocytes; TNF-α/β = tumour necrosis factor α or β.

redundancy in function between the T cells that are assigned to the different subsets. Indeed, some researchers feel that the division of helper T cells into Th1 and Th2 subsets has been overplayed, and that in reality the situation is not so polarised, with many cells falling into the Th0 (intermediate) category (Ref. 2).

Antigen recognition by T cells

T cells recognise protein antigens in the form of peptide fragments that are presented at the cell surface by major histocompatibility complex (MHC) class I or MHC class II molecules (Fig. 1 and Fig. 2). When the antigen-specific T-cell receptor (TCR) on the T-cell surface (specifically the α/β chains of the CD3 complex) interacts with the appropriate peptide–MHC complex, it triggers phosphorylation of the intracellular domains of the CD3 ζ (zeta) chains. Subsequently, the zeta-associated protein 70 (ZAP-70) binds to the phosphorylated zeta chains, and is activated. Simultaneous co-ligation of the cell marker CD4 (or CD8) with the MHC class II (or class I) molecule results in the phosphorylation of the lck kinases. These events stimulate the activation of at least three intracellular signalling cascades. T-cell activation also requires a second co-stimulatory signal (such as the interaction between the cell markers CD28 on the T cell, and CD80 on the antigen-presenting cell; Fig. 3). This interaction also triggers several intracellular signalling pathways. Activation of T cells can lead to cell division, lymphokine secretion by the T cell and expression by the T cell of antigens associated with the activated state. Alternatively, in the case of cytotoxic T lymphocytes (CTLs), interaction with antigen via the specific TCR leads to destruction of target cells.

Antigen processing and antigen presentation to T cells

There are two major pathways of antigen processing within the antigen-presenting cell (APC) and target cell (Ref. 3). The endogenous pathway processes proteins that have been synthesised within the APC (Fig. 1). In this pathway, proteins in the cytoplasm are cleaved by proteasomes (a proteolytic organelle) into peptide fragments of ~ 20 amino acids in length. These fragments are then transported into the lumen of the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) complex, where they encounter newly formed heavy-chain molecules of MHC class I and their associated β_2 microglobulin (β_2m) light chains. The heavy chain, light chain and peptide form a trimeric complex, which is then transported to and expressed on the cell surface. T cells that express the CD8 cell-surface marker recognise antigens that are presented by MHC class I molecules. CD8 functions as a co-receptor in this process, binding to an invariant region of the MHC class I molecule (Ref. 4).

In the exogenous pathway, soluble proteins are taken up from the extracellular environment, generally by specialised or 'professional' APCs. The antigens are then processed in a series of intracellular acidic vesicles, called endosomes (Fig. 2). During this process, the endosomes intersect with vesicles that are transporting MHC class II molecules to the cell surface. $CD4^+$ T cells recognise antigens that are presented by MHC class II molecules. As with CD8, the CD4 molecule functions as a co-receptor, increasing the strength of the interaction between the T cell and the APC (Refs 5, 6).

For both systems of antigen presentation, the

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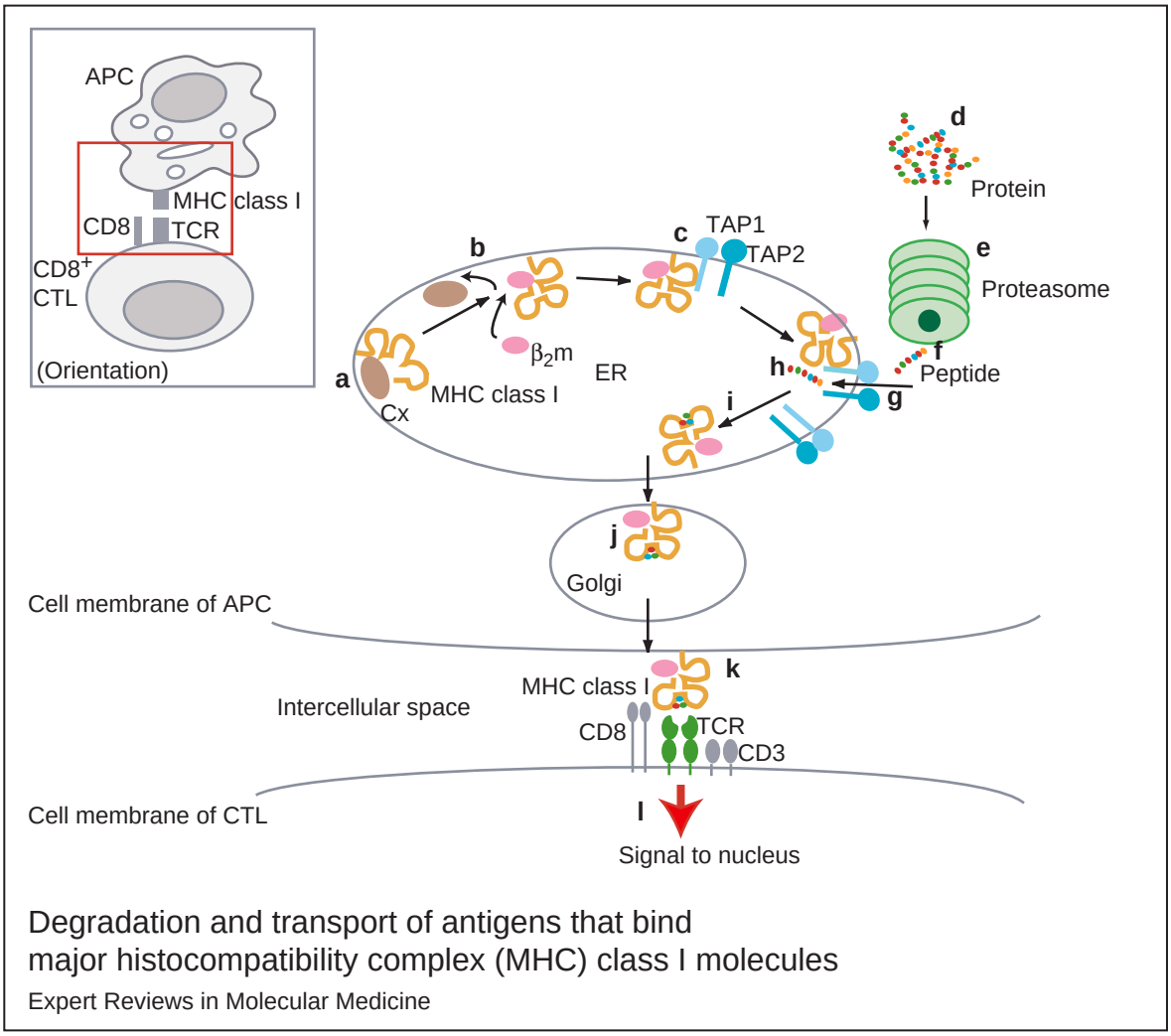


Figure 1. Degradation and transport of antigens that bind major histocompatibility complex (MHC) class I molecules. (a) In an antigen-presenting cell (APC), newly synthesised MHC class I molecules bind to calnexin (Cx), which retains them in a partially folded state in the endoplasmic reticulum (ER). (b) Binding of MHC class I molecules to β_2 microglobulin (β_2m) displaces Cx and allows binding of chaperonin proteins (calreticulin and tapasin; not shown). (c) The MHC class I–β_2m complex binds to the TAP complex (TAP1–TAP2), which awaits the delivery of peptides. (d) Peptides (e.g. from antigens) are formed from the degradation of cytosolic proteins ('self'-, pathogen- and tumour-derived proteins in the cytoplasm). (e) These are degraded by proteasomes into (f) short peptides. (g) Peptides are transported into the ER by the TAPs, where they meet the MHC class I–β_2m complex (h). This peptide binding in the antigenic groove of the MHC stablises the structure of the MHC class I molecule and (i) releases the TAP complex. (j) The fully folded MHC class I molecule with its peptide is transported to the cell surface via the Golgi apparatus. (k) Recognition of the MHC class I–peptide complex by the T-cell receptor (TCR) of an antigen-specific ($CD8^+$, $CD3^+$) cytotoxic T lymphocyte (CTL) takes place and (l) a signal transduction event activates effector functions in the MHC-class-I-restricted T cell; this requires co-stimulation to occur (not shown) **(fig003smc)**. First published in: Man, S. (1998) Human cellular immune responses against human papillomaviruses in cervical neoplasia. Exp. Rev. Mol. Med. txt001smc, 3 July 1998.

recognition of the antigen by the T cells is described as being MHC restricted; that is, the T cells recognise only antigen presented by self-MHC molecules. The nature of the process of antigen recognition by T cells has profound implications on the design of assays to measure T-cell function (see below).

The role of T cells in immune responses

Any immune response involves the interaction of

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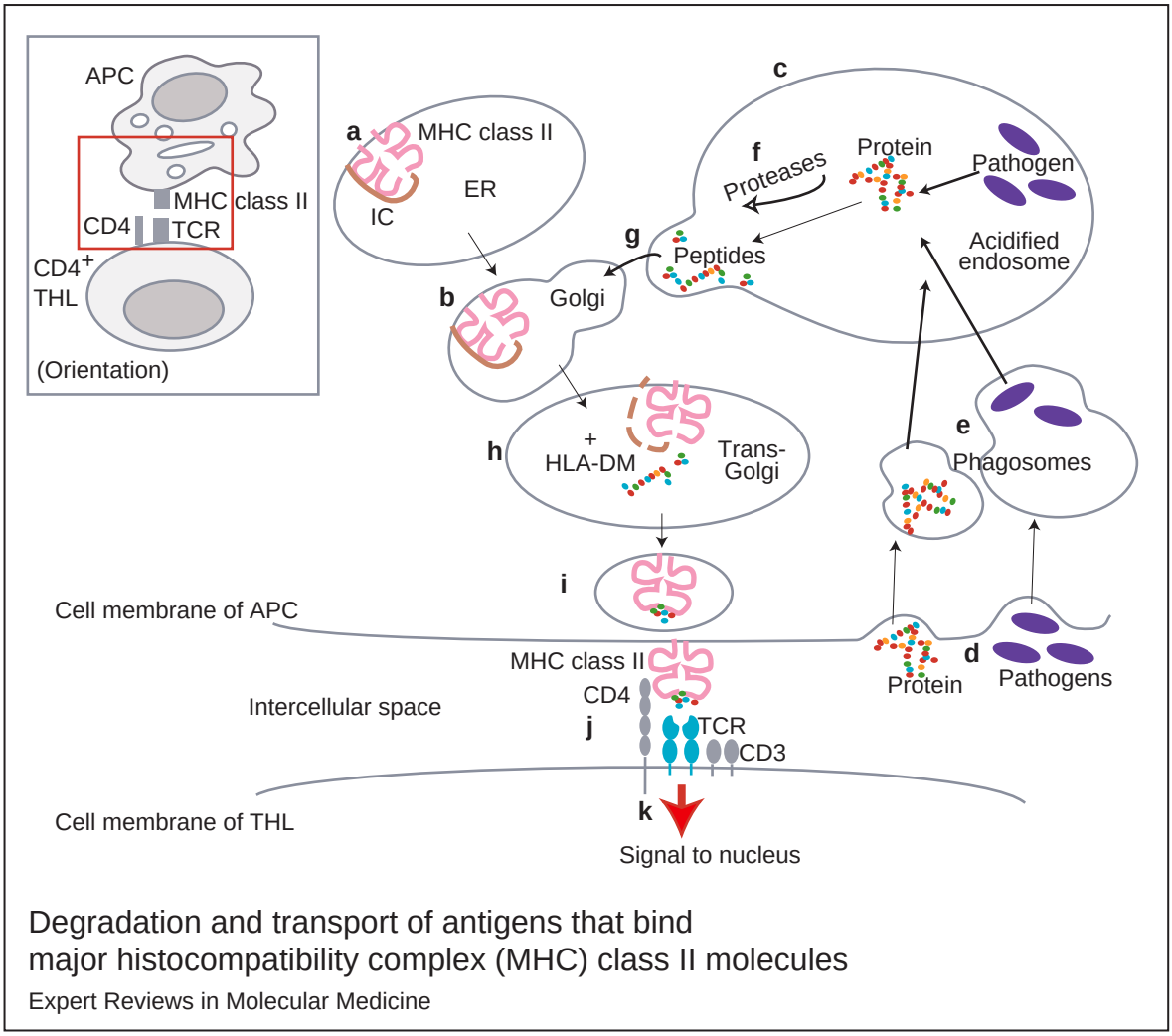


Figure 2. Degradation and transport of antigens that bind major histocompatibility complex (MHC) class II molecules. (a) In an antigen-presenting cell (APC), newly synthesised MHC class II molecules bind the invariant chain (IC), which prevents binding of peptides that are present in the endoplasmic reticulum (ER). (b) The IC allows transport of MHC class II molecules from the ER into the Golgi apparatus to acidified endosomes. (c) Endosomes contain peptides that are derived from either resident pathogens (e.g. bacteria) or (d) engulfed extracellular proteins (or pathogens) (e) in the phagosomes. (f) Proteases within the endosome degrade proteins into peptides. (g) The endosome fuses with the Golgi to form the trans-Golgi. (h) Here, the IC is cleaved and released from the MHC class II molecule. This allows the binding of peptides within the endosome to the peptide-binding cleft of the MHC molecules. An MHC-class-II-like molecule (HLA-DM) binds to MHC class II molecules to facilitate the release of the IC (not shown). (i) The MHC class II–peptide complex is then transported to the cell surface of the APC for (j) recognition by the T-cell receptor (TCR) of ($CD4^+$, $CD3^+$) T-helper lymphocytes (THLs) and (k) intracellular signalling for activation. Recycling of MHC molecules and co-stimulation are not shown **(fig004smc)**. First published in: Man, S. (1998) Human cellular immune responses against human papillomaviruses in cervical neoplasia. Exp. Rev. Mol. Med. txt001smc, 3 July 1998.

many different cell types, and it is not possible to consider cell-mediated responses and antibody-mediated responses entirely separately. However, T cells play an important role in the regulation of virtually all immune responses, providing 'help' for antibody production by B lymphocytes (B cells), and by providing growth factors for B cells, T cells, and several other cell types. Furthermore, CTLs carry out important effector functions, being one of the cell types that are responsible for destroying virally infected cells, tumour cells, and allogeneic transplant cells [ones

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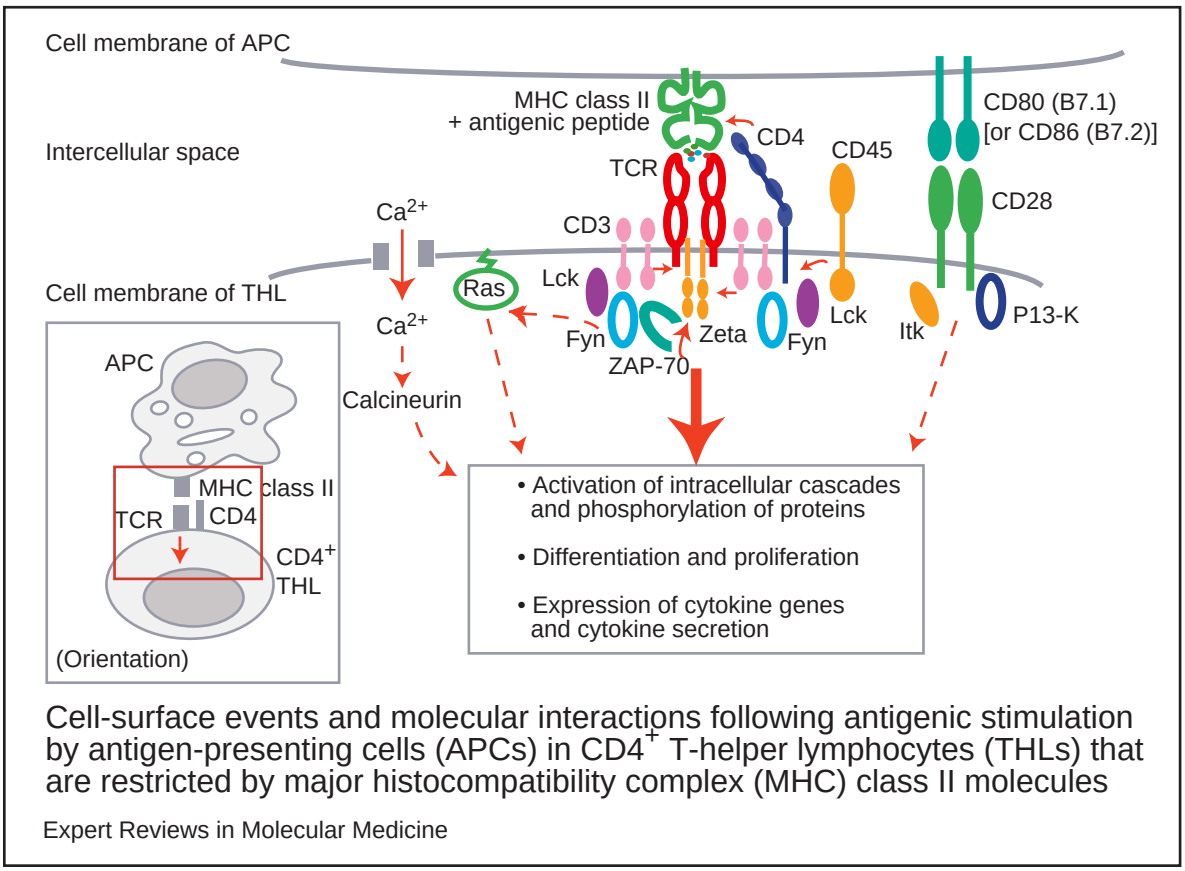


Figure 3. Cell-surface events and molecular interactions following antigenic stimulation by antigen-presenting cells (APCs) in CD4⁺ T-helper lymphocytes (THLs) that are restricted by major histocompatibility complex (MHC) class II molecules. Interaction of the antigen-specific T-cell receptor (TCR) on the T-cell surface (specifically the α/β chains of the CD3 complex) with the appropriate peptide–MHC complex triggers phosphorylation of the intracellular domains of the CD3 ζ (zeta) chains. Subsequently, the zeta-associated protein 70 (ZAP-70) binds to the phosphorylated zeta chains, and is activated. Simultaneous co-ligation of CD4 (or CD8) with the MHC class II (or class I) molecule results in the phosphorylation of the lck kinases. These events stimulate the activation of at least three intracellular signalling pathways. A second co-stimulatory signal (e.g. the interaction between CD28 on the T cell and CD80 on the APC) triggers additional intracellular signalling pathways **(fig001jhc)**.

that are not matched with the recipient for human leucocyte antigens (HLAs)].

Measurement of helper T-cell function

Isolation of T cells for in vitro assays

Most of the assays of T-cell function described below are carried out using peripheral blood mononuclear cells (PBMCs), which are obtained from venous blood by differential density centrifugation. In many situations, this level of cell separation is sufficient; however, if pure populations of T cells or a particular T-cell subset are required, then additional methods can be used. These include sorting individual cells (or populations of cells) into microtitre wells using flow cytometry (cell sorting), or the use of magnetic beads coupled to monoclonal antibodies to enrich or deplete subsets of cells in a population of cells (based on cell-surface markers).

Lymphoproliferation assays using ^{3}H-Thy

The measurement of the proliferation of lymphocytes that occurs following various stimuli (such as exposure to mitogenic agents, polyclonal stimuli or specific antigens) is a fundamental technique for assaying T-cell responses. However, simple enumeration of T cells before and after such stimulation is laborious, and in most cases is not possible because the cells that are responding represent only a small percentage of

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the total cell population at the start of the assay. This is certainly the case when assaying antigen-specific responses. Therefore, the incorporation of radiolabelled tritiated thymidine (^{3}H-Thy) into the DNA of dividing cells is commonly measured. This assay requires cells (usually PBMCs) to be incubated in the presence of the antigenic or mitogenic stimulus for 3–7 days, before the addition of ^{3}H-Thy for 6–18 hours. The total amount of the radiolabel that was incorporated into the cells in that time period is then measured, which provides a measure of the rate of synthesis of DNA by the entire population of cells. In a 'standard' assay, the cells are co-cultured with a range of concentrations of stimulating antigen, and at least one other antigen to act as a control to determine the specificity of the response. Results are usually expressed as a stimulation index (SI), which is the ratio of the scintillation counts (as a result of the incorporated ^{3}H-Thy) obtained in the presence of the test antigen, divided by the counts obtained in the presence of the control antigen (or culture medium alone).

Incorporation of ^{3}H-Thy provides a good correlate of T-cell division, although it does not necessarily reflect the overall size of the final cell population because some stimuli can induce rapid division and yet be toxic in the longer term to many of the cells exposed to them. Furthermore, no information about the synthesis of DNA by individual cells is obtained (Ref. 7).

Analysis of the expression of activation markers on the surface of T cells by flow cytometry has also been investigated as a method for evaluating T-cell proliferation. Cell-surface markers (molecules) that have been used include: CD25, CD69, CD71 and HLA-DR (a subtype of human MHC class II molecule), all of which are upregulated following T-cell activation. This approach has the advantage that the expression of other cell-surface molecules can be analysed simultaneously, allowing further characterisation of the responding-cell population. However, the correlation between the percentage of T cells that stain positively for the markers listed above and the incorporation of ^{3}H-Thy is not always good, suggesting that the two techniques might be used to complement one another, rather than be considered as alternatives (Ref. 8).

Although lymphoproliferation assays require several days of cell culture, they are reasonably straightforward and can be used to analyse relatively large numbers of samples. The degree of proliferation observed is proportional to the number of antigen-specific cells that are present in the original population. However, in its simplest form described above, the assay might not always be sufficiently sensitive or quantitative to compare the relative numbers of antigen-specific lymphocytes in a series of PBMC samples, such as might be drawn over a period of time to follow the effect of vaccination, or the response to an infection.

Limiting-dilution analysis

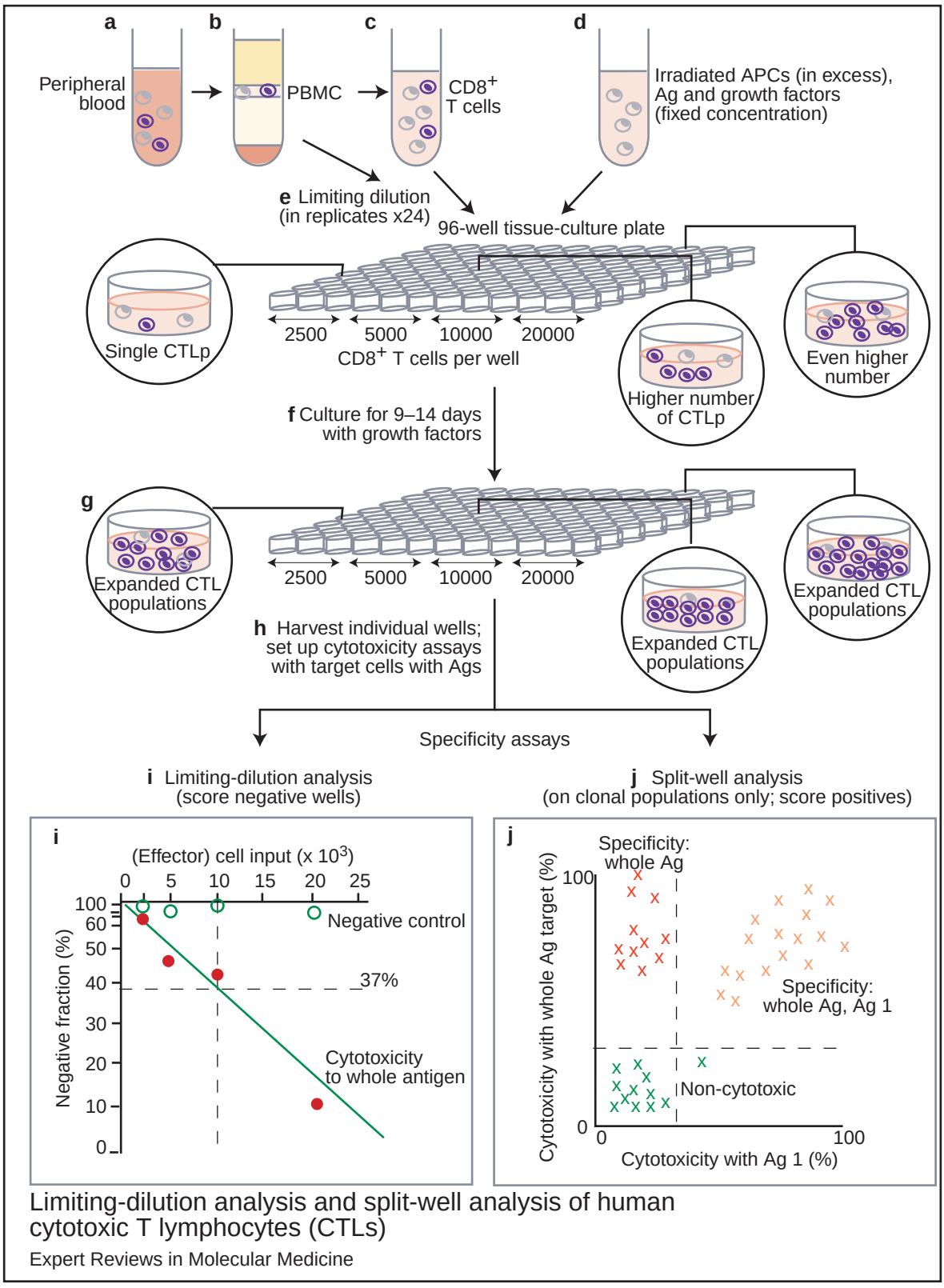
To provide quantitative estimates of the number or frequency of T cells present in a given PBMC population that are specific for a particular antigen, limiting-dilution assays (LDAs) are required (Refs 9, 10). These assays provide an estimate of the 'precursor frequency' of a given cell type. Positive results in this assay (proliferation or cytotoxicity, see below), indicate the presence of antigen-specific precursor cells in the PBMC population at the start, which have become activated and have subsequently divided during the period of cell culture. The function of these cells is then measured in the assay by either proliferation, cytokine production or cytotoxicity (Fig. 4).

In LDAs, many micro-lymphoproliferation assays are prepared in vitro, using a range of dilutions of the cell population under investigation, with at least 24 replicate cultures at each dilution. Other factors (such as growth factors, antigen and APCs) need to be added to the microtitre wells in excess, so that the only parameter that is limiting is the number of responding antigen-specific cells that are present in the cell population at the start. Under these conditions, and assuming that 'single-hit' kinetics apply, the number of non-responding cultures follows the Poisson distribution (Fig. 4). A semi-log plot of the percentage of non-responding cultures plotted against the number of input cells per culture should produce a straight line, and the input number of cells at the start that contained on average one specific precursor cell can be calculated from the zero term of the Poisson distribution. Several different statistical approaches for calculating the precursor-cell frequency have been described, including minimum χ^2 (Ref. 11) and maximum likelihood analysis (Ref. 12). Detailed comparisons of these and other methods have already been published (Refs 13, 14).

A variation on the lymphoproliferation

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Limiting-dilution analysis and split-well analysis of human cytotoxic T lymphocytes (CTLs)

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Figure 4. Limiting-dilution analysis and split-well analysis of human cytotoxic T lymphocytes (CTLs)
See next page for figure legend **(fig002jhc)**.

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Figure 4. Limiting-dilution analysis and split-well analysis of human cytotoxic T lymphocytes (CTLs). (a) Peripheral blood mononuclear cells (PBMCs) are obtained from the original peripheral (venous) blood sample by differential centrifugation over (b) a sucrose-based cushion. Total PBMC populations can be used as the responding-cell population in the assay, or (c) an additional purification step [such as selection of $CD8^+$ T lymphocytes (T cells) using magnetic beads] can be performed [the specific precursor cells that are to be detected are indicated in (darker) purple]. (d) A fixed concentration of antigen (Ag), extraneous growth factors and an excess of irradiated antigen-presenting cells (APCs) are added to each of the 96 wells of a microtitre cell culture plate. (e) Dilutions of the population of responder cells are made, and added to the microtitre wells. At least 24 replicates for each cell concentration are set up. As the input number of 'responder' cells increases, then, on average, the number of specific cytotoxic T-lymphocyte precursor cells (CTLp) in each well also increases. (f) The cells are cultured for 9–14 days to allow differentiation and expansion, which results in sufficient numbers for detection in the cytotoxicity assay (g). (h) To assay the samples for cytotoxicity, the content of each well is equally divided (split) for testing against a panel of target cells that are labelled with ^{51}chromium (typically, 2–5 different types of target cells). The number of microtitre-well cultures that fail to lyse the specific target cells is then recorded for each set of replicates with the same cell input number. (i) A plot of the log (base 10) of negative cultures against the number of cells that were started with allows determination of the concentration of cells in a well that would, on average, have contained a single, specific precursor cell. (j) Split-well analysis; cells in wells that were seeded at a cell concentration lower than that of the precursor frequency, can be assumed to be clonal in origin. Cells in these wells can be tested for cytotoxicity to a range of antigens **(fig002jhc)**.

LDA, which can be used to assay helper T-cell frequencies, is one that measures cytokine production from each micro-culture. Supernatant is harvested from the stimulated cultures, and assayed for the presence of cytokine, either by enzyme-linked immunosorbent assay (ELISA) or by bioassay. This assay has the advantage that it can be faster than assessing cell division by the incorporation of ^{3}H-Thy. This approach has been used to enumerate the frequency of T cells that are specific for the antigen tetanus toxoid, and the infectious agents human cytomegalovirus (HCMV) and herpes simplex virus (HSV; Refs 15, 16)

LDAs provide more information than the standard lymphoproliferation assay, but also require significantly larger quantities of PBMCs (at least 50 ml of blood is often required for each assay). Furthermore, the assay is critically dependent on a clear distinction being made between 'positive' and 'negative' cultures. This usually involves defining a threshold, typically two or three standard deviations above the mean value of the background proliferative response. However, in practice, the background counts are not always evenly distributed about the mean, with the right-hand 'tail' being considerably longer than the left-hand 'tail' (Ref. 17). In such situations, reliance on a threshold can be problematic, with minor differences in the definition of the threshold leading to very different estimates of the frequency of precursor cells.

Broman and co-workers (Ref. 17) have developed an alternative assay to address these drawbacks. Their method is more sensitive than the standard lymphoproliferation assay, and it might provide a more efficient alternative to the standard LDA. In this assay, the population of cells at the start are diluted into microtitre wells at a single (carefully chosen) cell density, rather than being used at a series of dilutions; at the end of the incubation period, the amount of thymidine incorporated in each well is used to estimate the absolute number of responding cells per well, rather than using an arbitrarily defined threshold to estimate which wells have cells that responded. This assay has been used successfully to monitor frequencies of antigen-specific T cells in individuals who had been immunised with a sub-unit vaccine consisting of glycoproteins B and D of HSV-2 (Ref. 18).

In summary, lymphoproliferation assays can be a very useful tool for monitoring immune responses; they are relatively easy to perform, and can be used to process relatively large numbers of samples. The further development of (often cumbersome) LDAs means that quantitative information on cell frequencies can now be obtained on a larger scale than before; however, one drawback of lymphoproliferation assays is that they do not provide a measure of the effector function of the responding cells. Indeed, it is not entirely clear which T-cell function or subset of T cells is being measured in lymphoproliferative assays, and so these assays are used solely to

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provide a general indicator of T-cell reactivity.

Measurement of cytokine production by T cells

Assessing cytokine production using immunoassay or bioassay

As described above, helper T cells can be subdivided into Th1 and Th2 populations, based on the types of cytokines that are produced following antigen-induced activation. It can be invaluable to know the relative proportions of these two populations of cells that are circulating in vivo following immunisation, or during an attempt to use an immunotherapeutic agent to modify a disease process. In some cases, a Th1-dominated response can be beneficial, whereas a Th2-dominated response might be ineffective, or even detrimental. Cytokine levels in body fluids can be measured directly; alternatively (as discussed here), populations of T cells can be stimulated and cultured in vitro, and the quantities of cytokines that are produced can be determined. In either situation, both bioassays and immunoassays can be used. Immunoassays are often favoured because they can be faster and, unlike bioassays that involve tissue culture, do not require the use of as many items of specialised equipment (Ref. 19). These bioassays have the advantage that they measure the biological effects of cytokines, and so only functional cytokines are measured. However, cytokines that are bound to soluble cytokine receptors (which can be present in plasma or serum) will not be detected. Another disadvantage of bioassays is that of lack of specificity; all of the reporter cell lines that are used in these assays will respond (and can give a similar response) to at least one cytokine, and several cytokines can be present in the culture supernatant during the assay. This problem can largely be overcome by the incorporation in the assay of monoclonal antibodies to bind to (and inactivate) the 'cross-reactive' cytokines. A comprehensive review of cell lines and bioassays available has been published by Mire-Sluis et al. (Ref. 20).

The simplest means to estimate Th1 and Th2 T-cell responses at the level of the total cell population is to culture PBMCs or T cells in vitro in the presence of the appropriate antigen or stimulus. After a specified period of time, the supernatant from these cultures is removed for testing and assayed for the presence of cytokines that are indicative of the Th1 and Th2 subsets [e.g. interleukin 2 (IL-2) and interleukin 4 (IL-4), respectively] using an ELISA or bioassay.

Assaying cytokine production at the level of the total cell population has the advantage that the cytokines are produced in measurable amounts. However, the amount of cytokine measured might not accurately reflect the total quantity of cytokine produced, because some of the cytokine might have bound to, and been 'used up' by, cells that are present in the culture. One solution to this problem is to measure cytokine messenger RNA (mRNA) levels, which can be done quantitatively using competitive polymerase chain reaction (PCR) assays. However, this then relies on the (not always valid) assumption of a direct relationship between the amount of mRNA that encodes cytokines and the amount of cytokine protein secreted.

More information can be gained if cytokine production is assayed at the level of the individual cell (Ref. 21). As with lymphoproliferation assays, LDAs allow the frequency of cytokine-producing precursor cells to be determined, but again the assays are unwieldy and time-consuming.

Cytokine measurement by ELISPOT assays

The enzyme-linked immunospot (ELISPOT) assay is an adaptation of the ELISA, which measures the local concentration of cytokines that are released from an activated T cell. In the ELISPOT method, cells that have been stimulated with antigen in vitro were incubated in nitrocellulose-lined microtitre wells, which have been pre-coated with anti-cytokine antibody. After incubation (for several hours or days), the local production of cytokines around 'producing cells' can be visualised by adding a second antibody that is labelled with the enzyme alkaline phosphatase or horseradish peroxidase, and then adding a substrate that is enzymatically converted into an insoluble coloured product (Fig. 5). Cytokine-producing cells can then be visualised as 'spots'. This assay is up to 200 times more sensitive than conventional ELISAs, is non-radioactive, and provides quantitative data as accurately as can conventional LDAs (Ref. 22).

The major disadvantage of the ELISPOT method is that scoring the wells for positive reactions involves the manual enumeration of large numbers of coloured spots, which can vary greatly in size and shape; thus, this method is subject to operator bias. Despite this drawback,

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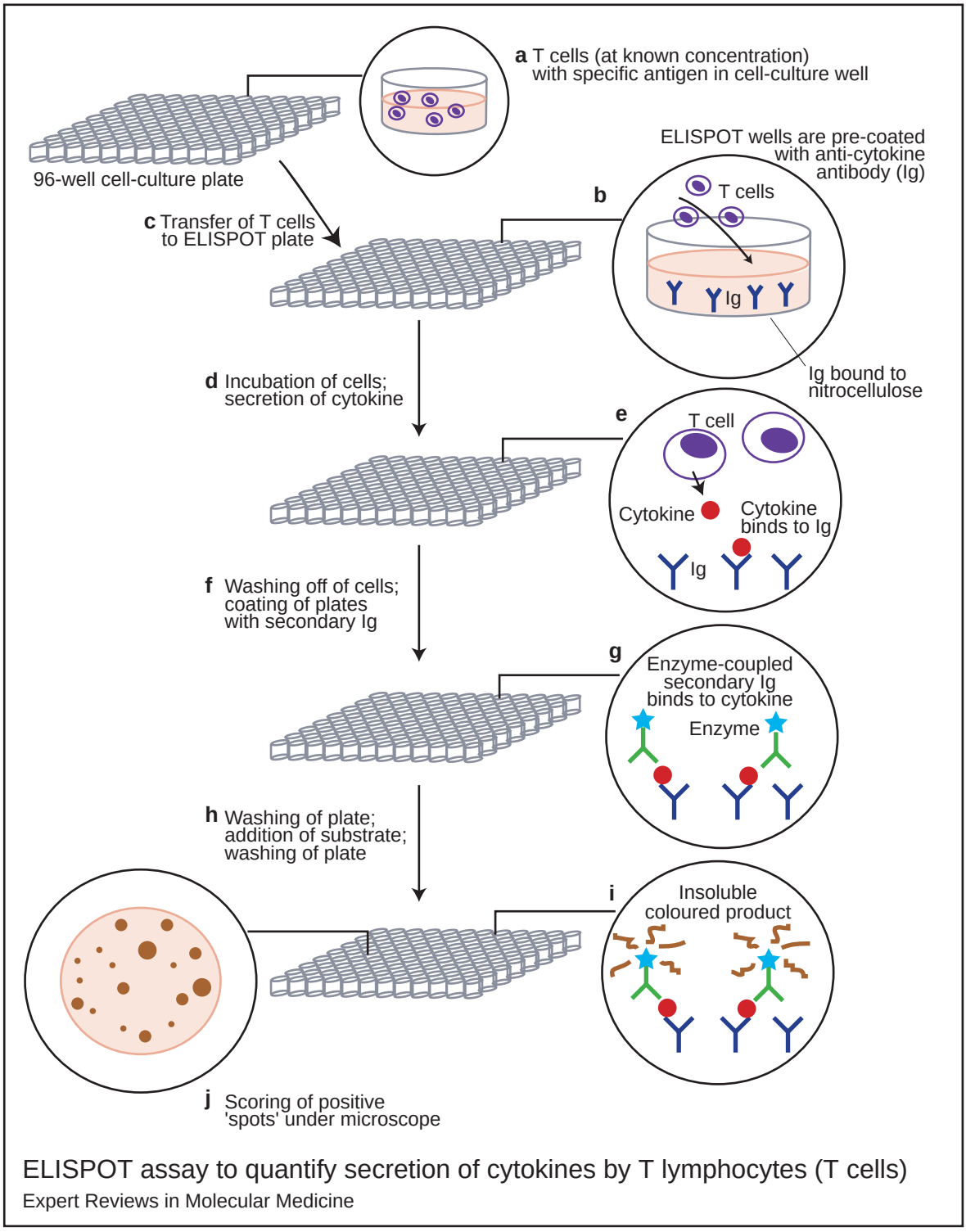


Figure 5. ELISPOT assay to quantify the secretion of cytokines by T lymphocytes. See next page for figure legend **(fig003jhc)**.

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Figure 5. ELISPOT assay to quantify the secretion of cytokines by T lymphocytes. (a) The T lymphocytes (T cells) are activated in vitro by being co-cultured with antigen. (b) The wells of the ELISPOT plate are coated with antibody (immunoglobulin; Ig) that is specific for the cytokine that is being assayed for. The Ig binds to the nitrocellulose base of the ELISPOT plate. (c) The activated T cells are transferred to the ELISPOT plate, and (d) cytokines are released during the incubation period. (e) Those cytokines that are released locally around each T cell bind to, and are therefore 'captured' by, the specific antibody. (f) The cells and any excess cytokines are washed off. (g) A second antibody that is also specific for the cytokine of interest is added; this antibody is coupled to an enzyme that is capable of converting a substrate into an insoluble coloured product. (h) The plates are washed once more, and the enzyme substrate is added. (i) The substrate is converted into the insoluble product, forming spots of colour that represent the areas of captured cytokines that were secreted by adjacent T cells. (j) The coloured spots are counted using a microscope or digital-imaging system **(fig003jhc)**.

ELISPOT assays have been used extensively and successfully to characterise cytokine profiles and define responding subsets of THLs in several human diseases (Refs 23, 24, 25). Computer-based image-analysis systems are now being developed to increase the accuracy and speed of scoring ELISPOT assays (Refs 26, 27).

Intracellular-cytokine staining

Cytokine production at the level of the single T cell can be analysed using flow cytometry to detect pools of intracellular cytokines. T cells are stimulated in vitro [typically with phorbolmyristate acetate (PMA) and ionomycin], and for at least some of the stimulation period monensin or brefeldin A are present to block the transport of cytokines through the Golgi apparatus, and therefore prevent the secretion of cytokines. The T cells are then fixed, permeabilised (to allow cytokine-specific antibodies to enter the cell), and stained for the presence of intracellular cytokines using directly conjugated anti-cytokine antibodies (Ref. 28).

Intracellular-cytokine staining offers several advantages to the researcher: large numbers of T cells can be analysed in a short period of time; also, the use of multiple antibodies that are coupled to different fluorochromes allows the co-production of more than one cytokine to be analysed, and/or the determination of the phenotype of the cells that are producing the cytokine. This method has been used successfully to characterise cytokine production by circulating $CD4^+$ T cells in the peripheral blood of individuals who are infected with human immunodeficiency virus (HIV; Refs 29, 30).

Measurement of T-cell cytotoxicity

CTLs are believed to play critical roles in the control of many viral infections, and also in the destruction of tumour cells. For this reason, there has for many years been much interest in developing assays to detect the presence of CTLs. Classically, $CD8^+$ T cells have been regarded as the T-cell population that mediates cytotoxic activity. This view is consistent with the ability of $CD8^+$ T cells to recognise antigens that are processed via the endogenous pathway, which would be the pathway for the presentation of peptides that are derived from viral proteins synthesised within a virally infected cell. However, cytotoxic activity is not the exclusive preserve of $CD8^+$ T cells. For example, $CD4^+$ T cells have been reported to be responsible for cytotoxic activity in several human diseases and animal models of disease; moreover, $CD4^+$ CTLs are believed to be a significant, if not the dominant, cytotoxic population of cells in some virus infections such as HSV-2 (Ref. 31).

Chromium-release assay

For many years, the standard assay for cytotoxic function has been the chromium-release assay. First described by Brunner and co-workers (Ref. 32), the assay involves the radiolabelling of 'target cells' with sodium chromate ($Na_2{}^{51}CrO_4$). The radiolabelled target cells are then incubated with the test effector-cell population for a short period (4–6 hours). The amount of ^{51}Cr released into the supernatant is then quantified, to provide a measure of target-cell lysis. ^{51}Cr in the form of $Na_2{}^{51}CrO_4$ offers the advantage of being taken up by live cells in its hexavalent form, but is released from lysed cells in its trivalent form, which is not re-utilised (Ref. 33).

It has been estimated that each CTL effector cell is capable of lysing up to five target cells in the same microtitre well, during the course of a 4-hour assay. To achieve lysis of 50% of the target cells (of which there are usually 2000–5000 in each microtitre well), therefore, requires 200–500 CTLs. The effector-cell population is tested at a range of

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Table 2. Comparison of the sensitivity of detection of $CD8^+$ T cells in currently available assays (tab002jhc)

Assay[a]	Detection limit (CTL frequency)[b]
^{51}Cr release (direct assay)	1 in 1000
^{51}Cr release (after in vitro re-stimulation)	1 in 50 000
ELISPOT	1 in 100 000
Tetramer analysis[c]	1 in 50 000

[a] The assay used to measure human T-cell responses in vitro; this might follow an extended period of re-stimulation and expansion in vitro. [b] The lowest frequency of specific T cells in the cell population at the start of the assay that can be detected using the assay (if pre-culture and re-stimulation have been used, the cells might have been expanded in relation to their frequency in the peripheral blood). [c] In tetramer analysis, a complex of four recombinant major histocompatibility complex (MHC) class I molecules with β_2 microglobulin and a peptide is labelled first with biotin, and then with streptavidin to allow the complex to be further labelled with a fluorochrome. Binding of this complex can be detected using a flow cytometer and is a measure of the number of specific T cells in a cell population (such as peripheral blood) that can bind to the MHC–antigen-peptide complex.

Abbreviations used: ^{51}Cr = radiolabelled chromium, which is used to label target cells; it is released as a result of cytotoxicity and can be measured in the cell-free supernatant; CTL = cytotoxic T lymphocyte (T cell); ELISPOT = enzyme-linked immunospot assay, a colorimetric assay based on the enzyme-linked immunosorbent assay (ELISA), which measures 'spots' on a filter that represent the cytokines that are produced by individual cells during cytotoxicity.

concentrations, up to 100 times the number of target cells (e.g. 5000 x 100 effector cells per well). Therefore, the CTLs must be present at a frequency greater than, or equal to, approximately one per thousand in the effector-cell population at the start of the assay in order to be detected, because of limitations in the total number of effector cells that can be placed in the microtitre well (Ref. 34).

In the vast majority of cases, the activated CTLs that are under investigation are present in the peripheral blood at too low a frequency to be detected simply by using freshly isolated PBMCs as effector cells in the ^{51}Cr-release assay (see Table 2). Exceptions to this rule include the measurement of specific CTLs in measles (Ref. 35), cytomegalovirus (CMV; Ref. 36), mumps (Ref. 37) and HIV^+ subjects before the onset of acquired immunodeficiency syndrome (AIDS); in some such individuals, the CTLs can be detected directly (Ref. 38). Most other situations require the CTLs to be re-stimulated and expanded in number in vitro, typically for 10–14 days before carrying out the assay. Technically, this can be problematic because in many cases it requires both a source of antigen and a source of autologous APCs to present the antigen. Furthermore, the manner in which the antigen is provided can profoundly affect the nature of the responding T cells that will be detected in the assay. For example, the provision of soluble or particulate antigen in the culture will lead to the preferential expansion of $CD4^+$ CTLs. To generate $CD8^+$ CTLs, antigens need to be processed via the endogenous pathway. This requires the antigen to be provided in the form of infected autologous cells (for example those that are infected by either the pathogen under study or a recombinant virus that expresses the target protein). Alternatively, synthetic peptides that represent a previously identified and characterised CTL epitope can be used; this is because such peptides can bind directly to MHC class I molecules at the cell surface of the APCs.

The influence of the nature of the antigen that is presented during the in vitro re-stimulation process has been demonstrated in several cases. For example, LDAs of HSV-specific CTLs showed that either $CD4^+$ T cells or $CD8^+$ T cells predominated, depending on whether inactivated virus or virus-infected stimulator cells, respectively, were used (Ref. 39). Similarly, re-stimulation with inactivated whole influenza virus lead to the generation of $CD4^+$ CTL lines, whereas cells infected with (live) influenza virus stimulated predominantly $CD8^+$ CTL lines (Ref. 40).

Finally, after the re-stimulation phase, an additional source of autologous (or HLA-matched) APC and antigen are often required for use as target cells in ^{51}Cr-release assays. Thus, while such assays are proving to be a useful research tool, they are not suitable for screening large numbers of samples, because they can

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require up to three weeks of in vitro culture, up to two different autologous cell lines, and at least one source of antigen. Despite these shortcomings, CTL assays of this type have been used successfully to monitor phase I clinical trials of several vaccines (Refs 41, 42, 43).

The data obtained from ^{51}Cr-release assays that use cells from such short-term 'bulk' T-cell cultures as effector cells are only semi-quantitative. The specific lysis of the target cells can reflect the relative proportions of specific precursors in the starting population, but the assay does not allow estimates of frequency of CTLs to be made. ^{51}Cr-release assays have been successfully adapted to the LDA format (Fig. 4; Refs 44, 45, 38), although these assays are large and somewhat unwieldy, and require at least 50–100 ml of peripheral blood per assay. Some of these disadvantages can be overcome by automating some of the operations such as setting up the original cultures or the ^{51}Cr-release assay (Ref. 46). Once established though, LDAs of CTLs can be very powerful; furthermore, if the appropriate target cells are available, they also enable the relative frequencies of CTLs with different antigen specificities to be analysed (Fig. 4). This has been achieved with several virus infection systems, including HCMV (Ref. 45). However, although the data are quantitative, it must be remembered that what is measured is the number of cytotoxic T-lymphocyte precursor cells (CTLp) that are able to divide a sufficient number of times during the period of culture, and then carry out specific lysis of the target cells. CTLs that have the appropriate antigen specificity but are at a different stage of differentiation might not be detected using this method.

Alternative cytotoxicity assays that avoid the use of ^{51}Cr

Methods that are able to measure cell lysis but do not require the use of (radioactive) ^{51}Cr have been of interest for some time, and include colorimetric assays, which are available as kits from some manufacturers. More recently, assays that use the technology of time-resolved fluorometry have been developed (Ref. 47). In this case, target cells are labelled by forming a complex with europium diethylenetriaminopenta acetate (Eu^{3+} DTPA). After the release of the Eu^{3+}-complex from lysed cells, a highly fluorescent complex is formed in an enhancer solution. After laser excitation, the fluorescence decay of the complex is relatively long (100–1000 ns), and can be distinguished from background fluorescence by time-resolved fluorometry. This assay has been used successfully in place of the standard ^{51}Cr-release assays by some groups (Ref. 48). However, there appears to be more variability in the ability of different target-cell types to be labelled and to retain the label with $^{3+}$Eu, than with ^{51}Cr, and for some users who have a wide range of target-cell types, this might limit its utility.

Matzinger and co-workers have developed the JAM assay, which is based on events that occur during cell death by apoptosis (Ref. 49). In this assay, the target cells should, ideally, be a rapidly dividing cell type. The target cells are labelled for up to 18 hours with ^{3}H-Thy, which is incorporated into the DNA, before the target cells are co-cultured with the effector T cells. Apoptosis, which is induced by CTL-mediated killing during the assay, induces laddering of DNA in the target cells. The entire contents of the microtitre well (T cells and target cells) are then harvested onto glass-fibre filters, as in lymphoproliferation assays; intact DNA (with its associated radiolabel) is trapped on the filter, whereas fragmented (laddered) DNA passes through into the waste. Thus, an 'absence of (scintillation) counts' indicates cytotoxic activity in the effector T-cell sample.

ELISPOT assays for CD8$^+$ T-cell activity

Assays that measure cytokine production by activated CD8$^+$ T cells can be used as an alternative means of investigating CD8$^+$ T-cell activity. In particular, ELISPOT assays to detect interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) have been developed as alternatives to the ^{51}Cr-release assays as a measure of CD8$^+$ T-cell activity (Refs 22, 50, 51). The method for detecting CD8$^+$ T cells using the ELISPOT assay is essentially the same as that described above, but in this case, the antigen that is used is typically a short synthetic peptide, representing an epitope that is known to be recognised by CD8$^+$ CTLs. As before, the ELISPOT method can be used to provide a quantitative measure of CD8$^+$ T cells that produce IFN-γ and/or TNF-α. Studies of human CTLs that are specific for influenza virus, using ELISPOT and conventional ^{51}Cr-release assays, suggest a good correlation between the two methods (Ref. 52). However, the ELISPOT assay might be more sensitive than ^{51}Cr-release assays for studying populations of T cells that are present at low frequencies (Ref. 53).

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Tetramer analysis for direct 'visualisation' of CD8⁺ T cells

Recent technological advances have now made possible one of the long-term goals of T-cell assays, namely to identify individual T cells on the basis of the specificity of binding to the MHC–peptide complex. This had not been possible previously, owing to the complex nature of recognition of antigens by T cells, which requires the interaction of the TCR, the antigenic peptide, and both the heavy and the light chains of MHC class I molecules. Furthermore, the interaction of this complex is low affinity, with a fast 'off-rate' (the TCR dissociates rapidly from the MHC–peptide complex; Ref. 54). Several groups have now shown that it is possible to generate tetrameric forms of MHC–peptide complexes, which are able to interact specifically with T cells that bear TCRs with a corresponding specificity (Ref. 55).

In the tetramer-analysis method, the restricting MHC molecule is synthesised in a soluble form by *Escherichia coli*. At the carboxyl (COOH) terminus of the molecule, a sequence that can be recognised by the enzyme BirA is incorporated. At this stage, the MHC molecule is not correctly folded for peptide binding but the correct conformation can be induced by the addition of light chain (β_2m) and the peptide that represents the appropriate peptide epitope. The enzyme BirA is then used to attach the polypeptide biotin to the biotin-recognition sequence. The complex (via biotin) can then be attached to another polypeptide streptavidin, which has previously been tagged with a fluorochrome. Because each streptavidin molecule has four biotin-binding sites, a tetrameric complex is produced, which contains four MHC class I molecules; this complex has a greater affinity for T cells than monomeric MHC class I molecules. The presence of the fluorochrome allows T cells that have bound the tetramer to be detected by flow cytometry (Fig. 6).

The tetramer-analysis method has already been used to study CD8⁺ T-cell responses in mouse models of acute virus infection, such as lymphocytic choriomeningitis virus (LCMV; Refs 56, 57), as well as in virus infections of humans, such as Epstein–Barr virus (EBV; Ref. 58) and HIV (Ref. 59). Interestingly in these cases, tetramer analysis has revealed that the expansion of antigen-specific CD8⁺ T cells during the acute phase of the response is far greater than previously thought. The large numbers of CD8⁺ T cells do not appear to be due to a 'bystander effect' (proliferation of T cells that are not antigen-specific but are driven by local cytokine production; reviewed by McMichael and O'Callaghan in Ref. 60).

One of the main limitations of the tetramer-analysis method is that it is applicable only in situations where the CD8⁺ T cells that are being quantified recognise a well-defined peptide epitope in conjunction with a known MHC class I molecule. Therefore, at present, it can be applied only to the analysis of a relatively small number of T-cell responses. However, tetramer analysis offers many potential advantages over some of the more 'traditional' CTL assays. This method is quantitative, it does not require the use of radioisotopes, and it is fast, so that fresh blood (or tissue-derived) samples can be analysed, and large numbers of samples can be processed. Because the analysis is performed using a flow cytometer, cells can be stained with the tetramer and at the same time with fluorescently labelled monoclonal antibodies that are specific for other cell-surface molecules; this allows additional characterisation of the responding cells. However, unlike the intracellular-cytokine staining method (described above), tetramer staining does not kill the labelled cells; therefore, the cells can be cell sorted into homogenous cell populations by flow cytometry, and placed into additional assays (such as the ELISPOT assay) to confirm their functional ability. Finally, unlike LDAs, specific T cells can be analysed from blood samples without the prerequisite of in vitro culture; some populations of CTLs that have been expanded in vivo might have limited growth potential in vitro, and therefore might not be detected by LDAs. Instead, the LDAs might detect only a sub-population of CTLp that are able to grow preferentially in vitro.

Clinical implications and practical applications

Problems associated with measuring T-cell function in humans

The methods that are available to measure T-cell activity in humans are clearly limited by several factors. The most obvious limitation is the inability (for ethical and practical reasons) to conduct experiments in vivo. Thus, all assays of human T-cell function rely on in vitro systems that have been developed to mimic in vivo situations, or at least to detect cells that have been activated in vivo.

The in vitro manipulation of human T cells can

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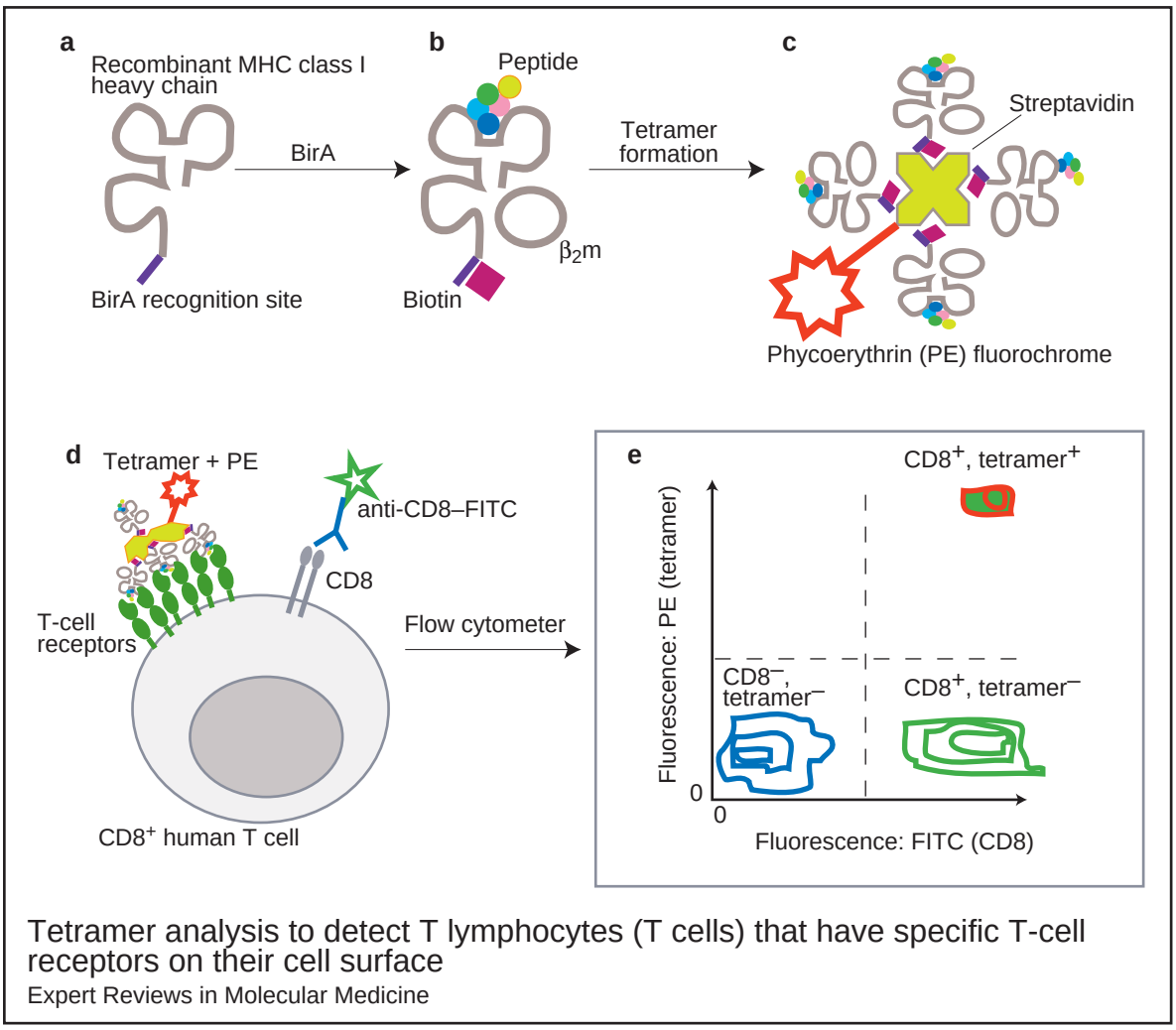


Figure 6. Tetramer analysis to detect T lymphocytes (T cells) that have specific T-cell receptors on their cell surface. (a) Soluble versions of the heavy chain of major histocompatibility complex (MHC) class I molecules are synthesised in *Escherichia coli* bacteria. (b) The molecules adopt an appropriate conformation following the addition of β_2 microglobulin (β_2m) and a synthetic peptide that represents the epitope that is recognised by the T-cell receptor (TCR) of interest. This peptide is able to bind to the MHC molecule. In addition, the enzyme BirA is used to attach a biotin molecule to the specific BirA-recognition sequence, which has been incorporated into the carboxyl terminus of the MHC molecule. (c) Four MHC–biotin complexes are linked to a single streptavidin molecule, using the specific biotin–avidin interaction, to form a tetramer. The streptavidin molecule is 'tagged' with a fluorochrome (e.g. phycoerythrin; PE). (d) Tetramers are mixed with the cell population that is to be analysed [e.g. total peripheral blood mononuclear cell (PBMC) populations or $CD8^+$ T lymphocytes (T cells)]. Only T cells that have TCRs that are capable of binding to the particular MHC–peptide combination that is present in the tetramer are able to bind the tetramer; thus, such cells will become labelled with the PE fluorochrome [shown in red on the graph in (e)]. A monoclonal antibody that is specific for a T-cell marker and is tagged with a different fluorochrome [e.g. fluorescein isothiocyanate (FITC), shown in green] can also be used. (e) The cells are then analysed using flow cytometry; the proportion of the $CD8^+$ T-cell population that stains positively with the tetramer can be determined (top, right-hand quadrant) **(fig004jhc)**.

lead to many misleading artefacts; thus assay methods that minimise the total culture time and number of in vitro manipulations are preferred. For example, LDAs that require the cells to be cultured in vitro for 2–3 weeks before they are assayed essentially measure only those cells that were able to grow under these experimental conditions. Furthermore, the phenotype and

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function of the cells can be influenced by factors such as antigen concentration, the source of serum used for cell culture, and (in particular) the concentration of the cytokines that are present in the culture. In addition, for practical reasons, it is often necessary to cryopreserve (freeze) PBMCs before assaying them for function. Procedural variation and cell losses associated with the freezing and subsequent thawing of PBMCs add further variability to the assay methods.

The usefulness of the assays is also constrained further by the fact that in the vast majority of cases the only 'body compartment' that can be sampled and used as a source of T cells (and APCs) for assay is peripheral blood (which contains circulating T cells). However, in some cases, limited numbers of cells can be obtained from some mucosal surfaces; for example, cytobrush sampling has been used to detect HIV-specific CTLs from the cervix (Ref. 61). In the case of some tumours or infectious diseases in humans, biopsy material has been used to study tumour- or lesion-infiltrating lymphocytes (Refs 31, 62). This approach might be important, because CTLs that are mediating an in vivo effect in an ongoing disease process might be expected to be concentrated at the site of the lesion, tumour or draining lymph node, rather than circulating in the blood.

T-cell responses as surrogate markers in clinical trials

For several reasons, the development of novel immunotherapeutic agents for the treatment of infectious diseases, cancer, autoimmunity and allergy has highlighted the need for new approaches to the design of clinical trials, and the incorporation of assays that determine immune function into the protocols.

Many of the new immunotherapeutic approaches that are currently being developed are highly disease specific. Often, there is no suitable animal model that can be used for pre-clinical studies to predict the efficacy of an agent in humans. In some cases, such as sub-unit vaccines containing one (or a very few) T-cell epitopes, there is no guarantee that a mouse will be able to mount an immune response to the same peptide or protein that will be used in humans. Thus, questions that might previously have been addressed in pre-clinical studies might now be answered only by studying the immune responses of subjects during phase I clinical trials.

In some serological responses, a threshold titre of antibody that is reactive against a specific virus protein can be said to 'correlate with protection' (i.e. it is a surrogate marker); however, in infectious disease immunology, tumour immunology or autoimmunity, there is usually no one assay of T-cell function that can be used as a validated surrogate marker. In many cases, such as recovery from a virus infection, clinical observations indicate that cell-mediated immune responses are important. In some situations, this is backed up with evidence from the laboratory; for example, the presence of detectable CTL responses can correlate with a good recovery from infection, or survival from virus reactivation after an organ transplant. Thus, at present, all that assays of T-cell function can do in the clinical setting is provide data to support other observations made. The time and expense involved in designing and running clinical trials provide an incentive to maximise the amount of information that can be obtained from each study. In phase I clinical trials (which are not designed to measure efficacy) of vaccines or therapies to modulate immune responses, monitoring of the immune responses of trial subjects can provide information at an earlier stage about which dose level or dosing regimen might be optimal in subsequent trials of efficacy. The ability to assay easily and rapidly following treatment an immune response that is defined as a valid surrogate marker (because it correlates with a favourable outcome) is a valuable goal.

Research in progress and outstanding research questions

In the near future, we are likely to see the continued development of robust and reliable quantitative assays of T-cell function that can be used to analyse relatively large numbers of samples.

In association with such assay development work, there is a need for researchers studying a particular disease process to identify precisely which T-cell function(s) correlate with protection or survival; such information could be of value for future clinical studies and used to provide validated surrogate markers for determining the effectiveness of a therapy.

Analysis of relevance of T-cell subsets in human disease

In the same way that a threshold titre for protective antibody responses can be defined in

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some diseases, specific information will be required for each pathology under investigation, to demonstrate which T-cell subsets and/or cytokines are capable of mediating protection or recovery. Ideally, the numbers of T cells that are required for protection or recovery also need to be determined.

Studies of surrogate markers

As indicated above, there are several situations when investigators would like to be able to use the presence of a T-cell response of a certain type and magnitude as a surrogate marker for clinical benefit. For example, several novel tumour therapies are based on the concept of inducing T-cell responses that are specific for tumour antigens. Trials of anti-tumour therapies can be very lengthy, if the only endpoint is a clinical effect, such as delayed time to tumour recurrence. Before the measurement of T-cell responses can be accepted as true surrogate markers, two crucial issues need to be addressed: first, a clear understanding of the T-cell subsets that are involved in various disease processes needs to be developed, and second, truly quantitative assays that can be performed reproducibly in different laboratories (i.e. equivalent to ELISAs for the measurement of antibody responses) need to be developed. The recent advent of tetramer analysis makes the establishment of reproducible quantitative assays a realistic goal in T-cell analysis for the first time.

Sampling technology for T cells

Although peripheral blood will remain the most accessible source for sampling T cells, investigators are becoming more concerned with understanding the responses of T cells at the site of the lesion or disease process. Novel assays that require fewer T cells for analysis need to be developed, along with improved culture methods to allow the expansion in number and subsequent assay of small numbers of precursor cells.

Assays for use in clinical trials or for clinical monitoring

Further research will define the differences in the $CD8^+$ T-cell populations that are detected by LDAs, ELISPOT assays and tetramer analysis. In particular, the ELISPOT assays and tetramer analysis do not measure cell killing by $CD8^+$ T cells; should $CD8^+$ T cells, therefore, continue to be described in functional terms (as CTLs)? Also, it is clear that the different assay methods are detecting cell populations with very different potentials for in vitro growth. It will be interesting to see whether the large, expanded $CD8^+$ T-cell populations that have been detected by tetramer analysis in some human infections (e.g. with HIV) are also expanded in other acute and chronic infections. In addition, work is under way to adapt the method for the detection of MHC class-II-restricted $CD4^+$ T cells. Tetramer analysis has many attributes that should make it the 'assay of choice' for monitoring responses to vaccines or other immunotherapies undergoing testing in clinical trials, providing the necessary reagents can be made.

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Features associated with this article

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Table 2. Comparison of the sensitivity of detection of $CD8^+$ T cells in currently available assays (tab002jhc).

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Figure 1. Degradation and transport of antigens that bind major histocompatibility complex (MHC) class I molecules (fig003smc). First published in: Man, S. (1998) Human cellular immune responses against human papillomaviruses in cervical neoplasia. Exp. Rev. Mol. Med. txt001smc, 3 July 1998.
Figure 2. Degradation and transport of antigens that bind major histocompatibility complex (MHC) class II molecules (fig004smc). First published in: Man, S. (1998) Human cellular immune responses against human papillomaviruses in cervical neoplasia. Exp. Rev. Mol. Med. txt001smc, 3 July 1998.
Figure 3. Cell-surface events and molecular interactions following antigenic stimulation by antigen-presenting cells (APCs) in $CD4^+$ T-helper lymphocytes (THLs) that are restricted by major histocompatibility complex (MHC) class II molecules (fig001jhc).
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Animations

Degradation and transport of antigens that bind major histocompatibility complex (MHC) class I molecules (swf001smc).
Degradation and transport of antigens that bind major histocompatibility complex (MHC) class II molecules (swf002smc).

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