Methods to measure T-cell responses

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A successful vaccine for immunotherapy, particularly for solid tumors or viral infections, requires a suitable target antigen and the production of a cytotoxic T-cell response. In addition, CD4 T cells play an important role in cellular immunity. Here, we briefly discuss methods by which T cells are measured *in vitro* after vaccination.

KEYWORDS: antigen delivery • cellular responses • cytotoxic T lymphocyte • cytotoxic T lymphocyte precursor • immunotherapy • OTI • OTII • proliferation • T cell • vaccine

There is an active interest in tumor immunotherapy. Studies over 30 years ago involving tumor cell lysates with or without adjuvant have now re-emerged, and patients have been shown to generate immune responses to tumor antigens. Currently, great emphasis for tumor immunotherapy is based predominantly on genetic engineering techniques, which have made difficult/impossible techniques a reality and successful tumor immunotherapy a realistic goal. Many tumor-specific or -associated antigens have been identified (e.g., melanoma antigens, p53, MUC1, Her2/neu and carcinoembryonic antigen), which can be produced in large amounts by recombinant techniques either as fusion proteins in bacterial or other systems, or as soluble molecules in eukaryotic systems. Furthermore, synthetic peptides can be made to parts of the tumor antigen that are presented by either class I or class II MHC molecules. Cytokines have been described and, nowadays, there is much more knowledge of how the immune system functions, in particular how cellular immune responses are generated and how peptides can be presented by class I or class II MHC molecules. This knowledge has led to the 'peptide approach' to tumor immunotherapy. Dendritic cells (DCs) have also emerged as playing a central role in generating immune responses in patients with cancer and other diseases. Various other methods have also been applied for the generation of optimal immune responses, such as DNA vaccines, combination gene therapy, hybrid-cell vaccination, tumor-cell vaccination and cell-free vaccines. In addition, there are now multiple methods for measuring cellular immunity: cytotoxic T lymphocyte (CTL) activity assays, CTL precursor

(CTLp) frequency assessment, T-cell proliferation, delayed-type hypersensitivity, intracellular and extracellular cytokine production by cells in culture assessed by flow cytometry or in ELISpot assays, cytokine bioplex assays and the use of class I/II peptides bound into a tetrameric MHC complex that bind to T cells bearing the appropriate T-cell receptors. Thus, there is now an enormous volume of information and reagents available for successful vaccination procedures. As the major focus in immunotherapy is on the induction of CTL, here we will briefly assess methods, for measuring *in vitro* and *in vivo* CTL and CTLp. We also note that often immune assays of T-cell function do not correlate with each other.

Measurement of CTL function

Measurement of target cell lysis is the fundamental measure of CTL function. CTLs are usually CD8⁺ T cells. The gold standard for CTL lysis has been the ⁵¹Cr-release assay in which ⁵¹Cr is added to target cells and the amount of ⁵¹Cr released by lysed cells is measured. Detection of mouse or human CTL activity usually relies on cytotoxicity assays where the peripheral blood mononuclear cells (PBMCs) or spleen cells are stimulated with their cognate ligand (usually an MHC class I-restricted peptide) and expanded by addition of IL-2 over 1 week, and then tested for their ability to lyse ⁵¹Cr-loaded cells. Correlative studies between such bulk PBMC cultures tested in standard CTL assays and assays that measure the CTLp frequency, have shown that the latter have a sensitivity to detect greater than one in 80,000 initial CTLs able to be reactivated from peripheral blood, whereas

the former only detect CTLs reliably if they are in frequencies greater than one in 20,000 [1]. More recent studies have attempted to use a modification of the ELISpot method to detect low frequencies of CTLs as they secrete cell-lysing molecules such as granzyme B and perforin [2]. It is, however, still unclear how strongly these *ex vivo* assays would correlate across vaccine and disease models with CTL and CTLp detection, given that the latter represent assays that require cells to be reactivated in the presence of peptide and IL-2.

A CTL method has been developed that requires only 150 μ l of blood from mice without sacrificing the animal. PBMCs are stimulated *in vitro* with a combination of recall peptide or protein, cytokines, costimulatory molecules and irradiated feeder cells for 7 days plated under limiting dilution conditions [3]. This assay is very sensitive and antigen specific, and is more efficient than conventional CTL assays. The advantage of this assay is that blood samples can be isolated from individual mice at various time points throughout the course of an *in vivo* study and CTL responses can be monitored. Another approach for CTL measurement is the use of flow cytometric analysis, or the fluorescent-antigen-transfected target cell-CTL assay. These approaches do not require radioactive isotopes to label CTL target cells as do conventional CTL assays. Instead, plasmid vectors encoding antigen–green fluorescent protein fusion proteins are used to nucleofect target cells. Elimination of antigen–green fluorescent protein-expressing cells by CTL, *in vitro* sensitized PBMCs or *ex vivo* PBMCs is quantified following a 4–18 h coculture period by flow cytometry [4].

During CTL activation, CTLs release cytoplasmic granules that contain serine esterases. The amounts of enzyme released during CTL activation could be quantitated by spectrophotometric analysis of the colored product of the enzymatic degradation of a synthetic substrate [5–8]. Measurement of granzyme B and perforin has also been used as an alternative to ^{51}Cr -release assays [9,10]. Furthermore, a flow cytometry-based assay for CTL lysis without the use of ^{51}Cr , which is based on specific binding of antibody to activated caspase-3 in target cells, is commonly used [11]. This assay is convenient and has enhanced sensitivity compared with conventional ^{51}Cr -release assays. Furthermore, lactate dehydrogenase (LDH) release is being used as an end point for cytotoxicity assays as an alternative to using ^{51}Cr . LDH is used as a marker for necrotic cell death [12]. Most cells contain LDH and when cells are lysed, LDH could be measured in the culture media [13]. More recently, administration of 5-bromo-2-deoxyuridine (BrdU), a thymidine analog, to activated T cells has been used to measure CTL activity. BrdU is taken up by cells during the S-phase of the cell cycle, which is then detected by an anti-BrdU-fluorochrome antibody by flow cytometry [14,15].

Measurement of CTL precursor

Cytotoxic T lymphocyte precursor assays determine the frequency of effector CD8⁺ T cells in a population. An inverse correlation has been shown between CTLp and tumor protection in mice [16]. CTLp assays are dose–response assays that allow for detection of all positive or negative CD8⁺ T-cell responses in each individual culture within replicates that vary in the number of responder cells

tested. The frequency of positive cultures cannot be demonstrated as it is not clear if one or more precursors in the culture well are generating the positive response (i.e., lysis of the target cells). However, the negative response indicates that there are no precursors of a given specificity. Thus, the measurement of the CTLp in the original population is possible by determining the number of wells that are negative in the experiment. Multiple cultures are set up at different cell concentrations, usually replicates of 32 of at least six effector cell doses (ranging from 1×10^3 to 5×10^5 cells per well) are used. If the percentage of negative cultures is converted to its negative logarithm, the results can be plotted graphically. A linear relationship should exist between the dose of effector cells, represented on a linear scale, and the frequency of negative wells on a logarithmic scale. CTLp frequencies are determined as the inverse of effector cell dose required to generate 37% negative wells (FIGURE 1A). In general, a CTLp of one in 10,000 is very high, one in 20,000 is high, one in 50,000 is moderate, one in 100,000 is weak and less than one in 200,000 is negative.

OT T-cell method for assessing CD4 or CD8 responses

The OT T-cell system is widely used to evaluate ovalbumin (OVA)-specific T-cell responses. It encompasses OVA-epitope-specific CD8⁺ and CD4⁺ T cells derived from OTI and OTII transgenic mice of the C57BL/6 background, respectively. The OT T-cell proliferation assay represents a powerful tool to evaluate the efficacy of vaccines in which OVA is used as the model antigen [17]. The method for *in vitro* OTI and OTII T-cell proliferation uses OTI and OTII T cells that are seeded with varying concentrations of purified DCs, which have been preloaded with peptide (CD4 and CD8), protein OVA control or the titrated OVA-containing vaccine. Proliferation of T cells is monitored by addition of [^3H]-thymidine. The efficacy of the vaccine is, therefore, represented by labeled T-cell proliferation. An example of an expected result is shown by our previous studies, in which a novel OVA vaccine is evaluated (FIGURE 1B & 1C) [18].

The incorporation of 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) into cells has recently become a popular method to monitor cell division and differentiation, and cell proliferation versus apoptosis both *in vivo* and *in vitro*. The incorporation of CFSE into cells has also been used to analyze responses of lymphocytes (T cells) *in vivo* [19]. Purified OTI and OTII T cells are labeled with CFSE and become responsive 'reporter cells' to achieve evaluation of vaccine immunogenicity elicited after immunization [20]. Specifically, CFSE-labeled OT cells are introduced into C57BL/6 mice preimmunized with the OVA vaccine. The extent of splenic OT T-cell proliferation should correlate with the efficacy of OVA presentation by DCs. The level of T-cell proliferation is visualized by the descending CFSE fluorescence. An example from our previous study of OTI T-cell proliferation by OVA plus CpG immunization is shown (FIGURE 1D).

Parameters for effective CD4 & CD8 T-cell induction

The route of immunization is an important factor for efficient CTL generation. Intraperitoneal, intradermal, intravenous and intramuscular routes are commonly used in mice. Subcutaneous

injections of antigen usually give low CTLp frequencies in mice [16,21,22]. The dose of antigen used to inject is also important; lower doses usually stimulate cellular (CD8) immunity whereas higher antigen doses stimulate humoral (antibody) immunity [16,23]. Increases in immunogenicity can be achieved by formulating antigen with adjuvants (e.g., glutamyl muramyl dipeptide, muramyl dipeptide, incomplete Freund's adjuvant and AdjuPrime™ [Pierce, IL, USA]) [16], making it into a particle [24–26] or by the addition of cytokines (e.g., IL-4, IFN- γ , IL-12 and GM-CSF) [27–33].

IL-2 is strictly required in the process of cytotoxic CD8⁺ T-memory induction and survival. Less is known regarding its role in memory CD4⁺ T-cell generation. This is important not just in the context of the CD4⁺ T cells themselves, but also because their activity is, in turn, necessary to support effective recall cytotoxic CD8⁺ T-cell and antibody responses. Adoptive transfer studies of *in vitro*-primed T cells into IL-2-knockout mice suggest that IL-2 is necessary for CD4⁺ T-cell priming to enable long-term survival of CD4⁺ memory T cells *in vivo* [34]. In addition to promoting both CD8⁺ and CD4⁺ T-cell division, IL-2 keeps T cells alive through induction of the anti-apoptotic molecule Bcl-2 [34]. It has also been proposed that CD4⁺ T cells that secrete IL-2 during a primary response subsequently preferentially differentiate into effector and memory cells [35]. Indeed, intermittent administration of IL-2 in patients with HIV helps sustain CD4⁺ T-cell baseline levels [36]. Importantly, although effector memory cells are expanded, central memory cells exhibit prolonged survival. CD4⁺ memory T cells further usually express high levels of IL-7 receptor (IL-7R), and IL-7 can promote resting CD4⁺ memory T-cell survival *in vitro* and *in vivo* [37]. IL-2 can also regulate IL-7R expression on CD4⁺ T cells, perhaps programming memory cells to enhance their survival *in vivo*. Another proproliferative cytokine, IL-15, can induce proliferation of effector memory CD4⁺ T cells and promote their migration to extralymphoid effector sites [38]. IL-21, produced by Th2 CD4⁺ T cells, also has the ability to costimulate CD4⁺ T-cell

Figure 1. Example of CTLp, OTI, OTII and CFSE experiments. (A) Typical example of cytotoxic T lymphocyte precursor (CTLp), illustrating a linear relationship between the dose of effector cells, represented on a linear scale, and the frequency of negative wells on a logarithmic scale. CTLp frequencies are determined as the inverse of effector cell dose required to generate 37% negative wells. Representative CTLp frequency is 1/10,000. (B) Vaccine OVA-pulsed DCs induce high levels of MHC class I and II presentation, leading to CD4⁺ and CD8⁺ T-cell proliferation. Titrated DCs preloaded with control peptides, vaccine OVA and OVA were coinoculated with OTI or OTII T cells. (C) Coimmunization of CpG with OVA induces the CD8⁺ T-cell response. CFSE-labeled OTI T cells were transferred into a mouse preimmunized with OVA or OVA/CpG. At day 3, splenocytes are harvested and analyzed for division of CFSE-labeled T cells. CFSE: Carboxyfluorescein diacetate succinimidyl ester; DC: Dendritic cell; OTI: Ovalbumin-specific CD8⁺ T cells from OTI T-cell receptor transgenic mice; OTII: Ovalbumin-specific CD8⁺ T cells from OTII T-cell receptor transgenic mice; OVA: Ovalbumin.

proliferation and to enhance CD4⁺ memory responses. On the basis of the aforementioned evidence and other similar studies, common γ -chain cytokine-expressing plasmids are being incorporating in vaccines to promote expansion of memory T cells [39]. Based on these cytokines, one would predict that strong protective CD4⁺ T-cell and cytotoxic CD8⁺ T-cell immunity could be elicited. Results from malaria vaccine studies in humans support this hypothesis. However, the nature of the protective immunity to malaria vaccines tested in human trials has yet to be confirmed. The *ex vivo* ELISpot assay (IFN- γ) fails to correlate with protection in a range of malaria vaccine studies in humans. However, malaria antigen-specific IFN- γ responses elicited following 2 weeks coculture with IL-2 (the cultured ELISpot method) correlates with protection in humans, such as in the case of the leading malaria vaccine RTS,S/AS02 [40] as well as heterologous prime–boost regimens involving DNA, modified vaccinia virus Ankara or fowlpox virus 9 [41], highlighting the importance of measuring the induction of central memory T cells.

Expert commentary & five-year view

A central question in vaccine development is whether the immune responses being measured after immunization (usually in the short term) will be able to predict the induction of long-term memory that can be elicited by the challenge pathogen and protect against disease. Many studies have now shown that

diverse assays such as the widely used *ex vivo* ELISpot often do not correlate in mice or humans with proliferative assays [42] or assays where central memory CD4⁺ or CD8⁺ T cells are expanded first with antigen in the presence of growth cytokines such as IL-2 followed by assessment of their capacity to kill (CTL assay) or secrete cytokines [42–44]. Indeed, the capacity to secrete a specific cytokine often does not correlate with other cytokines, regardless of the assay used [45,46]. Finding immune correlates of protection, particularly in diseases where T cells play a key role in mediating it, such as cancer, HIV and malaria, is a difficult task. However, although each disease is different and, thus, the nature of protective immunity may also differ, we suggest that assessment of common factors and cytokines that promote the survival and expansion of both CD8⁺ and CD4⁺ memory T cells and therefore long-term immunity may offer a way forward for predicting long-term vaccine efficacy.

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Key issues

- Conventional ⁵¹Cr-release assays to measure cytotoxic T lymphocyte (CTL) activity is an effective measure of CD8⁺ effector-cell development. Other current assays detect CD8 T cells that produce cytokines (ELISpot, intracellular cytokine staining and cytokine bioplex assay) and are useful, but often do not correlate with CTL activity.
- However, even in diseases where CTLs are associated with protection, the measurement of cytokines that promote long-term CD8 and CD4 T-cell survival is likely to be critical in predicting long-term protection.
- CTL precursor is the measure of the frequency of CD8⁺ T-cell generation to a specific antigen, and is more sensitive to conventional CTL assays.
- New methods for CTL measurement have been devised, some of which include lactate dehydrogenase, caspase-3, granzyme B, serine esterases, bromodeoxyuridine uptake and fluorescence detection, which are more sensitive to conventional ⁵¹Cr-release assays and more user-friendly than the highly sensitive CTL precursor method. In addition, carboxyfluorescein diacetate succinimidyl ester dilution has been used to measure T-cell division using flow cytometry after antigen immunization both *in vitro* and *in vivo*.
- *In vivo* and *in vitro* OTI and OTII cells have been used to determine the induction of effective CD4 and CD8 T cells.
- Route, dose and cytokines play important roles in the generation of effector T cells, and the release of such cytokines is important to determine long-lasting immunity.

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