Published in final edited form as:

Expert Rev Vaccines. 2012 December ; 11(12): 1459–1470. doi:10.1586/erv.12.125.

Pushing the frontiers of T-cell vaccines: accurate measurement of human T-cell responses

Fadi Saade^{1,‡}, Stacey Ann Gorski^{1,2,‡}, and Nikolai Petrovsky^{1,3,*}

¹Vaxine Pty Ltd, Bedford Park, Adelaide 5042, Australia

²Department of Microbiology, University of Virginia, Charlottesville, VA 22908, USA

³Department of Diabetes & Endocrinology, Flinders Medical Centre/Flinders University, Adelaide 5042, Australia

Abstract

There is a need for novel approaches to tackle major vaccine challenges such as malaria, tuberculosis and HIV, among others. Success will require vaccines able to induce a cytotoxic T-cell response – a deficiency of most current vaccine approaches. The successful development of T-cell vaccines faces many hurdles, not least being the lack of consensus on a standardized T-cell assay format able to be used as a correlate of vaccine efficacy. Hence, there remains a need for reproducible measures of T-cell immunity proven in human clinical trials to correlate with vaccine protection. The T-cell equivalent of a neutralizing antibody assay would greatly accelerate the development and commercialization of T-cell vaccines. Recent advances have seen a plethora of new T-cell assays become available, including some like cytometry by time-of-flight with extreme multiparameter T-cell phenotyping capability. However, whether it is historic thymidine-based proliferation assays or sophisticated new cytometry assays, each assay has its relative advantages and disadvantages, and relatively few of these assays have yet to be validated in large-scale human vaccine trials. This review examines the current range of T-cell assays and assesses their suitability for use in human vaccine trials. Should one or more of these assays be accepted as an agreed surrogate of T-cell protection by a regulatory agency, this would significantly accelerate the development of T-cell vaccines.

Keywords

clinical trials; CTL; CYTOF; ELISPOT; flow cytometry; T-cell assay; vaccine

© 2012 Expert Reviews Ltd

*Author for correspondence: Tel.: +61 882 044 572, Fax: +61 882 045 987, nikolai.petrovsky@flinders.edu.au.

‡Authors contributed equally to this manuscript

For reprint orders, please contact reprints@expert-reviews.com

Disclaimer

This paper's contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH or the National Institute of Allergy and Infectious Diseases.

Financial & competing interests disclosure

This work was supported in part by contracts U01 AI061142 and HHSN272200800039C from the National Institute of Allergy and Infectious Diseases, NIH, Department of Health and Human Services. N Petrovsky and F Saade are affiliated with Vaxine Pty Ltd, an Australian company developing vaccine adjuvants. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Immunization has been the single most successful medical intervention for the global prevention of infectious disease. However, there remains a need for vaccines with novel modes of action, to tackle important pathogens such as malaria, tuberculosis and HIV [1]. An additional challenge is to develop vaccines against noninfectious diseases such as cancer or autoimmunity. These will require the creation of vaccines able to induce strong T-cell responses, a weakness for existing vaccine approaches. T-cell vaccine development has been hindered by the difficulty of accurately and reproducibly measuring human T-cell responses. This contrasts with the relative ease of measuring neutralizing antibody titers in human peripheral blood. Vaccine trials that rely upon assessment of clinical outcomes generally require very large sample sizes, take many years to complete and are extraordinarily expensive. Hence, easily measurable surrogates of T-cell immune protection would be of major benefit. Thus, there is a need for well-validated T-cell assays suitable for use as proxies of vaccine efficacy, in the same way that neutralizing antibody titers are used as surrogates of humoral protection. Unfortunately, T-cell assays that closely correlate with host protection have proved difficult to develop. As detailed in Table 1, despite the many T-cell assays now available, there is a paucity of data on the utility of such assays in and across large-scale human clinical trials.

In animal models, T-cell function is conveniently measured using cells obtained from lymphoid tissues such as spleen, bone marrow and lymph nodes. However, in humans, measurement of T-cell function is hindered by restricted access to lymphoid tissues, with peripheral blood mononuclear cells (PBMC) the main source of cells for use in assays. Unfortunately, the function of T cells in peripheral blood may not mirror the major lymphoid compartments or disease-affected tissues. Another disadvantage of PBMC for assessment of T-cell function is that PBMC isolation and processing, as well as cryopreservation, can have profound modifying effects on T-cell function [2,3]. These factors all need to be borne in mind when selecting assays for measurement of T-cell vaccine responses in human samples. This review summarizes recent assay advances that have made monitoring of T-cell function practicable in large-scale human vaccine trials (Figure 1). This capability will greatly assist the development of T-cell vaccines.

T-cell proliferation assays

PBMC proliferation in short-term culture after stimulation with antigen or peptide has traditionally been used as an integrated measure of cellular immunity. ^3H -thymidine incorporation has high sensitivity and was the gold standard for measuring proliferative responses [4]. However, ^3H -thymidine incorporation assays are only semiquantitative, require the use of radioisotopes, and do not provide information on the identity or function of the proliferating cells. New dye dilution-based cytometry assays eliminate the need for radioactivity and have greater flexibility, allowing staining with additional antibody markers to identify the proliferating cell types and the cytokines they produce. Among these assays, the carboxyfluorescein succinimidyl ester (CFSE) assay has been widely used to evaluate antigen-specific T-cell proliferation (Figure 2A) [5–7]. PBMC can be easily stained with CFSE and T-cell proliferation, following antigen stimulation, is measured through the halving of fluorescence in daughter cells. The sequential loss of CFSE intensity can identify divided cells up to approximately eight divisions. However, CFSE assays require careful optimization with potential cell toxicity and culture biases resulting in modulation of activation markers [8]. An alternative method for measuring T-cell proliferation is 5-bromo-2-deoxyuridine incorporation into nascent DNA, as detected with a 5-bromo-2'-deoxyuridine-specific antibody [9]. Yet another method for measuring T-cell proliferation utilizes the Ki67 nuclear antigen that is expressed in the nucleus throughout the proliferative phase of the cell cycle and can therefore be used as a marker of recently or actively dividing

cells [10]. Ki67 can be used to detect human PBMC proliferation directly *ex vivo* and has successfully been used to evaluate T-cell responses after human vaccination [11].

T-cell proliferation assays offer advantages including sensitivity and the ability to phenotype responding cells, but suffer from high intraindividual and interindividual variability even when performed by the same laboratory, on the same subject, but at different times [12]. The source of this variation likely includes differences in initial cell count, and media and culture conditions. Hence, T-cell proliferation assays remain at best semiquantitative.

Cytokine-based T-cell assays

Cytokine-based assays constitute the largest class of T-cell assays and take many different forms. At their simplest, these assays represent modifications of old-style proliferation assays where instead of measuring ^3H -thymidine incorporation they directly quantitate IL-2 produced in response to antigen stimulation [13,14]. Ready availability of ELISA allows a wealth of different cytokines to be measured. For example, IFN- γ , TNF- α and IL-2 are commonly used as markers of a Th1 response [15] and many other cytokine combinations can help define other T-cell subsets such as Th2, Treg and Th17 [16,17]. An alternative to PBMC cytokine assays is the whole-blood assay [18]. Whole blood most closely approximates the state of circulating immune cells *in vivo* and contains physiological concentrations of factors such as cortisol and melatonin that profoundly influence T-cell function and are responsible for the large diurnal variation seen in cytokine secretion [18]. In general, cytokine production per mononuclear cell in response to lipopolysaccharide or phytohaemagglutinin is higher in whole blood and within-assay variation is lower, making such assays more sensitive and reproducible than PBMC cultures [18]. The major practical limitation of the whole-blood cytokine assay is that as whole blood cannot be cryopreserved the assay must be performed on fresh blood.

Cytokines themselves can be measured using a variety of assay platforms. While conventional ELISA assays are the 'gold standard', they are only able to interrogate one cytokine per assay and require large sample volumes. Newer methods have been developed that can measure multiple cytokines in small sample sizes. Cytometric bead array (CBA) is a flow cytometry-based method for measuring multiple cytokines and chemokines in a small volume of serum or cell supernatant. CBA assays utilize antibody-coated beads, in which each bead has a fluorescent signature to indicate the cytokine to which the bead is specific [19]. Multiple beads can then be multiplexed to detect different cytokines in as little as a 25 μl sample. Fluorescently-labeled detection antibodies are then added and the sample is analyzed on a flow cytometer. Prepackaged and custom bead arrays are available. A commercial form of the CBA, the Luminex xMAP platform, has become popular both in the research and clinical settings owing to its ease of use. Although Luminex requires purchase of expensive specialized instrumentation that is dedicated to multiplexing analysis, it is compatible with many bead manufacturers. The Luminex technology can also be used to measure cytokine and other mRNAs from a single sample, including small volumes of whole blood [20,21]. The various CBA assays show a good correlation with ELISA, although sensitivity is slightly higher with the CBA format [22–24]. Although absolute values in cytokine concentration can differ between bead manufacturers, comparisons between laboratories can be made if the same bead vendor is used [25,26]. Once optimized, CBA assays can have coefficients of variation of less than 30% [27], and have been used in human vaccine studies to assess cytokine responses following whole blood or PBMC restimulation with antigen [27–29]. In addition to CBA, there are other multiplex cytokine measurement formats, such as chemiluminescence and electrochemiluminescence assays, the latter of which has the benefit of minimal signal decay as the readout is based on electrical stimulation of an excitable tag on the detection antibody and thus has higher

sensitivity than the CBA in human serum [30]. The convenience of these multiplex cytokine assays, plus the low sample volume requirement, have led to them being implemented in various clinical settings. However, these assays only allow a bulk measurement of cytokines produced by multiple cells, may be insufficiently sensitive for the detection of cytokine produced by low-frequency antigen-specific T cells, and they provide no information about the nature of the cytokine-producing cells.

Quantification of antigen-specific T cells

ELISA and CBA assays measure the total cytokine present in the culture supernatant and do not account for the level of cytokine production per cell. The cytokine ELISPOT and intracellular cytokine staining (ICS) are alternative methods that have the advantage of measuring cytokine production on a per-cell basis, and are therefore useful in identifying low-frequency T-cell responses [31]. During ICS, PBMC are stimulated with antigens in the presence of a Golgi inhibitor to prevent secretion of cytokines, which are thereby trapped intracellularly (Figure 2B). After the cells are fixed and permeabilized, they are stained with fluorescent-labeled anticytokine antibodies and analyzed by flow cytometry [32,33]. Multiple cytokines can be analyzed simultaneously alongside traditional cell surface markers, thereby allowing extensive phenotyping of the cytokine-producing cells [34]. This allows quantitation of specific functional populations of antigen-specific memory T cells. ICS can be performed both on whole blood and on cryopreserved PBMC but showed lower interlab coefficient of variation with the latter [35]. ICS has good sensitivity and precision when compared with ELISPOT, except in very low frequency responses where the ELISPOT is preferable for its higher sensitivity [36,37].

Yet another cytokine-based assay that can be used for the identification of individual antigen-specific T cells is the cytokine-secretion assay (CSA). In this assay, a cytokine-specific antibody matrix is bound to the T-cell surface so that when the cells secrete cytokine, it is captured on the surface, and can be identified with a cytokine-specific detection antibody by flow cytometry [38]. This method can be of benefit for enriching cytokine-producing cells (such as antigen-specific T cells) through the use of magnetic beads that bind the detection antibody [39] and has been successful in detecting transient production of IL-2 and IFN- γ from CD4⁺ T cells after *in vitro* stimulation [40]. CSA has the advantage that it does not require cell permeabilization and the detected cells can thereby be kept alive for further study.

The ELISPOT assay is a variant of the ELISA that, like ICS and CSA, allows detection and quantification of antigen-specific T cells producing a particular secreted product, for example, IFN- γ (Figure 2C). In brief, PBMC are cultured on an anticytokine capture antibody-coated membrane in the presence of an antigen. Following stimulation, each antigen-specific cell will make the relevant cytokine that will bind to the capture antibody on the membrane. The cells are then washed away and their secreted products can be detected as colored spots on the membrane by use of an enzymatically-labeled antibody and insoluble chromogenic substrate [41]. ELISPOT assays currently rank among the most validated assays for clinical trials, have good reproducibility and high sensitivity, particularly in samples with low-frequency responses [31,36,42]. ELISPOT assays have been used to measure infection or vaccine-induced T-cell responses, as well as to quantitate autoreactive T cells in patients with autoimmunity [43–45]. Recent studies have shown a good correlation between ELISPOT responses induced by cancer vaccines and disease outcome, suggesting this method as a valuable biomarker to predict clinical benefit following immunotherapy [46,47]. ELISPOTs allow the size and intensity of the spots to be calculated and this correlates with the amount of cytokine secreted by each cell. In addition, ELISPOT is well suited for clinical trial samples as it can be performed on both fresh and

frozen samples (Figure 2C) [48]. The major limitation of ELISPOT is that it provides no information on the phenotype of the responding T cell beyond the cytokine secreted. However, because the ELISPOT assay does not involve killing the stimulated cells, they can be recovered and restudied in subsequent assays. The IFN- γ ELISPOT after 10–14 days of PBMC culture (the ‘cultured ELISPOT’) has been shown to correlate with a memory T-cell response. This, in turn, has been shown to correlate with protection against malaria [49], suppression of viral rebound in hepatitis B virus carriers [50] or low viraemia in HIV [51]. A recent HIV prime-boost vaccine trial showed cultured ELISPOT to be the most sensitive assay for detecting HIV T-cell responses [52]. A limitation of the traditional ELISPOT is that it can only analyze one or two parameters per cell, as compared with ICS that can detect multiple parameters. An improvement of the ELISPOT is the FLUOROSPOT, which allows the simultaneous detection of multiple cytokines per cell [53], although current commercial kits have only been validated for two cytokines per cell. The FLUOROSPOT assay principle is similar to conventional ELISPOT, with the only difference being that cytokines are detected through the use of fluorescently labeled antibodies and enumerated with a fluorescent ELISPOT reader enabling the simultaneous use of multiple colors [54]. The ability of T cells to simultaneously secrete two or more Th1 cytokines like IFN- γ , IL-2 or TNF- α has been shown to be a useful predictor of HIV control [55], making FLUOROSPOT’s ability to detect multiple cytokines at a single-cell level a significant advance. Efforts have been made by several groups including the Cancer Vaccine Consortium group to establish ELISPOT harmonization guidelines for use within clinical trials [56,57].

A more recent method to quantitate antigen-specific T cells is tetramer staining. Tetramers are synthetic structures made from HLA molecules, four or more identical versions of which are linked together to form a multimeric complex that is then loaded with antigen-specific peptide [58]. Tetramers are designed to bind the T-cell receptors of antigen-specific cells with high avidity, allowing the detection of labeled cells through fluorescent molecules conjugated to the tetramer structure. Human tetramers have been successfully used to identify both CD8 and CD4 antigen-specific T cells in a variety of models. For example, influenza-specific CD8 T cells in HLA-A2⁺ PBMC were quantified before and after influenza vaccination of human subjects using an HLAA2 tetramer loaded with an influenza M1 peptide [59]. Similarly, a HLA-DRB1*1501 tetramer complex was used to define pneumococcal-specific T cells in human PBMC [60]. Tetramers are also useful in identifying and enriching rare cell populations, such as naive, antigen-specific T cells through the use of magnetic beads that are specific for the labeled tetramer [61]. Tetramer staining of antigen-specific CD8 T cells showed the highest precision, when compared with ICS and ELISPOT, with respect to intra-assay variation when using cryopreserved cells [37]. The downside to the use of tetramers is that they are HLA-specific and antigen epitope-specific as only T cells recognizing a specific HLA-peptide pair can be detected by a single tetramer. Hence, tetramer design requires considerable prior knowledge of the major pathogen epitopes recognized by human T cells and also the HLA type of each subject being studied. The use of pan-genome epitope mapping, the method of identifying those peptides that are most immunogenic and likely to be conserved within a pathogen (and hence less likely to undergo immune escape), has been greatly assisted by the development of epitope prediction algorithms and high-throughput HLA binding assays to screen for the best peptides for use in tetramer construction [62]. However, for many vaccine studies, specific information regarding HLA types and immunogenic epitopes is unlikely to be readily available, leaving tetramers as a research tool rather than routinely applied in human trials. Where tetramers have been used in human studies, for practicality they have generally been limited to a single antigenic peptide bound to a common human HLA allele such as HLA-A2, such that only HLA-A2-positive subjects are studied [63]. This means that the data

obtained cannot be generalized to predict the efficacy of the vaccine in a more general population.

In this context, the development of humanized mouse models transgenic for human HLA are a valuable tool to test the impact of HLA subtype on the outcome of vaccination or the impact of vaccination on a particular HLA type and to validate candidate tetramers [64,65]. These humanized models help to define the immunogenicity and protective efficacy of candidate vaccines in the context of human HLA polymorphism, thereby helping overcome the variation in the immune response in a genetically heterogeneous human outbred population.

CTL assays

While all the above assays provide useful information on T-cell proliferation or cytokine secretion, they do not directly measure T-cell cytotoxic ability. Cytotoxic T lymphocyte (CTL) responses are important, as the ability of CTL to destroy infected or malignant cells may best correlate with vaccine efficacy. For example, HIV-specific CTL activity, as measured by lytic granule loading and delivery of granzyme to target cells, was correlated with HIV long-term nonprogression [66]. CTL are generated from precursor T cells following stimulation with cognate peptide presented on target cells in the presence of adequate costimulatory signals and CD4 T-cell help. Lymphoblasts, tissue culture cells or tumor cells are among the most popular target cells used in CTL assays [67]. CTL kill target cells through either of two mechanisms: release of lytic granules containing perforin and granzymes, or triggering by CTL of target cell death-receptors such as Fas [68–70]. CTL activity was traditionally evaluated with the chromium (^{51}Cr) release assay (CRA). The CRA is based on the ability of CTL to lyse ^{51}Cr -labeled target cells loaded with an antigen of interest [71]. Briefly, following incubation of target cells with an MHC class I-restricted peptide, the target cells are labeled with ^{51}Cr . Labeled target cells are then incubated at different effector to target cell ratios with CTL effector cells that have been expanded in the presence of IL-2. Target cells that are recognized and lysed by the CTL subsequently release ^{51}Cr into the supernatant, which is then quantitated by scintillation γ counting. Although the CRA has been considered ‘the gold standard’ to measure CTL activity, it has many drawbacks including the use of radioisotopes, high interexperiment variability in labeling efficacy, and high background due to nonspecific release of ^{51}Cr from target cells; also, it is only semiquantitative unless a limiting dilution component is incorporated into the test [72].

Several nonradioactive tests have been developed to measure CTL activity as alternatives to the CRA. These assays are based on the detection of markers of degranulation of effector CTL or indicators of apoptosis or necrosis of target cells. Among the latter type of assays is the colorimetric measurement of lactate dehydrogenase (LDH) activity in the culture medium. LDH is present in the cytoplasm of all eukaryotic cells and is released into the culture media after cell damage [73]. LDH release assays correlate with the ^{51}Cr release assay but are less sensitive [74]. Alternatively, target cells can be labeled with compounds like europium, which, after lysis, can be quantified by fluorescence measurement [75]. The advantage of this assay as compared with CRA is its higher sensitivity, and cells can be labeled and frozen prior to performing the assay. Cytotoxicity can also be evaluated by colorimetric assays or luminescence following transfection of target cells with reporter genes [76,77]. For example, assessment of CTL function was reported using 3T3 cells transfected with a bicistronic vector expressing HBV core and EGFP/Fluc, which were incubated with CTL and killing quantified by measuring the luciferase reporter in the remaining viable target cells, which inversely correlated with target cell death [78]. In CTL assays, CTL release cytolytic granules directed against the target cell. These granules

contain a family of serine proteases including granzyme B and cytotoxic proteins such as perforin [68,70]. ELISPOTs can be used to detect either granzyme B or perforin release, and show a good correlation with CRA assays [79,80]. Granzyme B ELISPOT has been used in several clinical trials to monitor vaccine responses in cancer or AIDS patients [55,81].

The drawback of the above CTL assays is their inability to determine other phenotypic features of the responding cells. The use of flow cytometry for evaluation of CTL activity offers several advantages in terms of ease of use and ability to further characterize the CTL phenotype [72]. Flow cytometry can be used to detect CTL activity by using an antibody against cleaved caspase (active form) in target cells, detection of apoptotic target cells using labeled Annexin V, the uptake of fluorescent DNA probes (7-AAD or propidium iodide) or the measurement of effector cell degranulation using anti-CD107a antibody staining. Caspases are activated in target cells following CTL-mediated cytotoxicity [82,83]. A sensitive CTL assay uses an antibody to detect cleaved caspase-3 in target cells that have been labeled with a tracker dye, which distinguishes target from effector cells. This assay has been validated in a number of human systems, including mixed lymphocyte reaction and human peptide-specific T-cell responses *in vitro*, and has shown strong correlation with CRA [84]. The use of antiannexin V staining, together with a DNA probe, is one of the most popular cytometry assays used to evaluate CTL activity. Membrane phosphatidylserine (PS), which is normally on the inner leaflet of the membrane in healthy cells, translocates to the outer leaflet of apoptotic cells, thereby exposing PS to the external environment [85]. Annexin V binds PS with high affinity in the presence of calcium ions and can thereby be used to detect apoptotic cells [86,87]. Since annexin V staining precedes the loss of membrane integrity that accompanies the latest stages of cell death, a vital dye such as propidium iodide (PI) or 7-amino-actinomycin (7-AAD) can be used in conjunction with annexin V to allow investigators to distinguish early (PI –ve, annexin V+ve) from late (PI +ve, annexin V +ve), apoptotic cells. CD107a, the lysosomal associated membrane protein, has been used as a marker of both T-cell and NK-cell degranulation [88,89]. Following release of cytolytic granules by these cells, CD107a is transiently expressed on the cell surface as the endosomal membrane fuses with the plasma membrane. CD107a surface expression correlates inversely with intracellular perforin expression and can be detected by flow cytometry as a measure of CTL activity [90]. Immunostaining for both surface CD107a and subsequent ICS for perforin and/or granzyme B is a strong system for demonstrating CTL function and correlates with the granzyme B ELISPOT [91].

T-cell immuno phenotyping

The overall number of memory T cells following initial antigen exposure often correlates with a protective immune response [92,93]. Thus, the frequency of memory T-cell subsets can be a good indicator of vaccine efficacy. In conjunction with tetramer staining or IFN- γ ICS to identify antigen-specific T cells, it is useful to phenotype T-cell memory subtypes via flow cytometry. Although it is increasingly apparent that memory T cells exist as a continuum of different phenotypes [15,94], memory T cells are generally classified into subcategories, including central memory (Tcm) and effector memory (Tem [95]). Tcm resemble naive T cells in terms of surface marker expression except that Tcm express CD45RO, rather than the CD45RA isoform of the protein tyrosine phosphatase present on naive T cells [96]. Tcm are identified by CD62L and CCR7, whereas absent or dim expression of these markers identifies Tem [97,98]. Tcm are largely restricted to lymphoid tissue, whereas Tem are present in nonlymphoid tissues and are quicker to respond to a secondary challenge. Depending on the pathogen, higher numbers of Tcm or Tem are correlated with better protection. For example, higher numbers of Tem are associated with better protection in animal models of HIV and having Tem in the mucosa is essential for limiting early HIV replication [99]. Conversely, Tcm are associated with protection during

systemic infections [100]. Thus, the ability of a given vaccine to induce one or both of these T-cell subsets *in vivo* could be a good correlate of vaccine efficacy. However, as the STEP HIV trial demonstrated, overall numbers of antigen-specific T cells does not always predict vaccine mediated-protection [101,102]. Tcm and Tem phenotyping can also be combined with ICS to identify the functional capabilities of each memory T-cell subset. Higher mean fluorescence intensity of IFN- γ or IL-2 in Tem and Tcm, respectively, is usually indicative of a higher quality memory response. Cellular proliferation assays, such as CFSE or Ki67 staining, can also be combined with memory subset phenotyping to further assess the magnitude and quality of the T-cell response. CD154 and CD107a can also be used to identify antigen-specific CD4 and CD8 T cells, respectively, in the absence of available tetramers and these markers can also give researchers clues as to their functional potential [103,104].

CyTOF

The new technology of cytometry by time-of-flight (CyTOF) mass spectroscopy has recently generated much excitement in immunology for its ability to measure up to 60 parameters at a single-cell level, including surface markers, intracellular molecules and activation markers, such as phosphoproteins [105]. As opposed to traditional flow cytometry that uses fluorescently labeled antibodies to stain cells, CyTOF utilizes antibodies labeled with heavy metal isotopes that can be detected with a mass spectrometer. The use of metal isotopes eliminates the issue of spectral overlap that frequently occurs with the use of fluorescent antibodies. Initially used to describe lineages of hematopoietic stem cells, CyTOF can also be used to identify antigen-specific T cells and analyze combinatorial cytokine expression [94]. In this type of assay, a whole continuum of different T-cell phenotypes can be identified in one sample. Although CyTOF technology is only in its infancy, its ability to integrate many of the other assays described in this review could provide a very powerful means, by which to more comprehensively phenotype T-cell responses following vaccination.

Intracellular staining for phosphoproteins

Flow cytometry can also be used to assess phosphorylation events during T-cell signaling. Phosphorylation of ERK, ZAP70 and NF κ B can be assessed in human cells and has been used to assess T-cell responsiveness to cytokines such as IL-2 [106]. The strength of signal induced through the TCR has known outcomes on the differentiation potential of memory cells and thus analyzing levels of phosphorylation of T-cell signaling molecules could provide early clues as to the effectiveness of a given vaccine. Phosphorylation of molecules such as the STAT family can also be used to analyze responses to specific cytokines. For example, phosphorylated STAT5 has been used to assess the ability of CD4 T cells to respond to IL-7 [107] and thus infer the memory potential of such subsets. When combined with traditional flow cytometry or CyTOF, investigators can assess phosphorylation events in specific cell populations.

DTH assay

The delayed-type hypersensitivity (DTH) test is an *in vivo* assay that measures the activation of antigen-specific T cells in the skin. DTH tests require intradermal injection of antigen with the resulting T-cell response being quantified by the diameter of erythema 48–72 h later, this reflecting cytokine production by antigen-specific T cells at the site of injection. The central role of T cells in DTH is illustrated by the fact that patients with AIDS have an impaired DTH response against intracellular pathogens such as *Mycobacterium tuberculosis* associated with the loss of CD4⁺ cells [108]. Following intradermal testing with hepatitis B surface antigen (HBs), the DTH response was positive in subjects with positive anti-HBs

antibodies or with acute hepatitis B, but not in chronically infected patients [109], suggesting that DTH is a good predictor of the ability of T cells to clear virus and allow seroconversion. However, the DTH assay has relatively high variability and poor specificity. Considering the ease of this test and its historic application in clinical trials, DTH may still have utility to measure overall responsiveness to a vaccine antigen, although due to its inherent variability, it would be best used to support rather than substitute for more specific *in vitro* T-cell assays.

Expert commentary & five-year view

T-cell vaccines are required to protect against infections for which neutralizing antibodies are difficult to induce (e.g., HIV), are unable to cope with the multiplicity of serotypes (e.g., staphylococci or streptococci [110–112]), or where the pathogen is intracellular and thereby needs to be targeted by CTL, for example, HIV or TB. Two major barriers lie in the path to development of successful T-cell vaccines; the first being the difficulty in inducing robust and long-lived T-cell responses, and the second being lack of a clear regulatory pathway for approval of T-cell vaccines in the absence of a well-validated T-cell assay that predicts protection. The HIV STEP trial is an example of failed protection, despite induction of specific T-cell responses to vaccine antigen [101]. T-cell assays must be sufficiently robust and reproducible to be useful in a clinical trial setting. To this end, multiple groups including the FOCIS Human Immunophenotyping Consortium [113] and the Minimal information about T cell assays Project [114], have made significant strides in standardizing both cytometry-based and other T-cell assays. The challenges of designing and implementing T-cell assays for use in large-scale clinical trials are many and major, ranging from the inherent variability of cell-based assays to the logistics of collecting clinical samples in a consistent manner from multiple clinical trial sites, and then either shipping them under controlled conditions to a centralized laboratory or alternatively having them assayed at many different locations, with the inherent problem of lab and assay standardization. In particular, most older-style T-cell assays are labor intensive and difficult to standardize. Many of these problems have been resolved with modern T-cell assays relying upon an additional step of cryopreservation of PBMC at the clinical trial site, thereby allowing T-cell assays to be more conveniently scheduled or for clinical trial samples to be shipped to central reference labs for assay. However, cryopreservation may not always be a panacea, as some lymphoid cells, for example, dendritic cells, are less robust under cryopreservation [113]. Nevertheless, putting these difficulties aside, the evaluation of T-cell responses may provide valuable information on the beneficial effects of immunization and allow an early decision on whether a particular vaccine candidate is worthwhile to pursue further. To this end, it is important to have as many different readouts of the immune response as possible, although this may be limited by the amount of patient blood that can be obtained. Fortunately, this latter problem has largely been solved by the availability of multiparameter assays exemplified by CBA assays, flow cytometry assays, gene expression arrays and the latest CyTOF technology.

An important factor that also needs to be taken into account for the successful detection and measurement of T-cell immunity in response to immunization is the timing of when each assay is performed. For each particular T-cell assay, the best time point for assessing the immune response must be chosen carefully as choosing the wrong time point may deliver false negative results [15], as the cells being assayed might not yet have developed or may already have disappeared from the peripheral blood into another lymphoid compartment. Whereas antibodies are generally present in the peripheral blood for a long period of time postimmunization and are thereby easily measured, T-cell responses are more ethereal and even at the time of maximum response the frequency of antigen-specific T cells in the peripheral blood may be at the limits of detection. This requires the use of sensitive methods

for their detection. However, with increasing operator skill and additional detection channels, flow cytometry techniques such as intracellular cytokine staining offer additional advantages, particularly the ability to extensively phenotype the responding cells. This provides the opportunity to correlate the clinical response in vaccine trials with each of the many T-cell parameters measured, thereby enabling identification of one or more parameters that best predict a successful clinical outcome. Thus, multiparameter flow cytometry assays offer the best future prospects for identifying *in vitro* correlates of T-cell vaccine efficacy to facilitate T-cell vaccine development.

References

Papers of special note have been highlighted as:

- of interest
- of considerable interest

- 1•. Seder RA, Hill AV. Vaccines against intracellular infections requiring cellular immunity. *Nature*. 2000; 406(6797):793–798. Overview of the need for specific T-cell vaccines. [PubMed: 10963610]
- 2•. Mallone R, Mannering SI, Brooks-Worrell BM, et al. T-Cell Workshop Committee, Immunology of Diabetes Society. Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. *Clin Exp Immunol*. 2011; 163(1):33–49. One of several international attempts to standardize human T-cell assays. [PubMed: 20939860]
3. Costantini A, Mancini S, Giuliodoro S, et al. Effects of cryopreservation on lymphocyte immunophenotype and function. *J Immunol Methods*. 2003; 278(1–2):145–155. [PubMed: 12957403]
4. Goodell V, dela Rosa C, Slota M, MacLeod B, Disis ML. Sensitivity and specificity of tritiated thymidine incorporation and ELISPOT assays in identifying antigen specific T cell immune responses. *BMC Immunol*. 2007; 8:21. [PubMed: 17850666]
5. Lyons AB. Analysing cell division *in vivo* and *in vitro* using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods*. 2000; 243(1–2):147–154. [PubMed: 10986412]
6. de Souza MS, Ratto-Kim S, Chuenarom W, et al. Ministry of Public Health–Thai AIDS Vaccine Evaluation Group Collaborators. The Thai phase III trial (RV144) vaccine regimen induces T cell responses that preferentially target epitopes within the V2 region of HIV-1 envelope. *J Immunol*. 2012; 188(10):5166–5176. [PubMed: 22529301]
7. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. *J Immunol Methods*. 1994; 171(1):131–137. [PubMed: 8176234]
8. Last'ovicka J, Budinský V, Spísek R, Bartunková J. Assessment of lymphocyte proliferation: CFSE kills dividing cells and modulates expression of activation markers. *Cell Immunol*. 2009; 256(1–2): 79–85. [PubMed: 19233349]
9. Leif RC, Stein JH, Zucker RM. A short history of the initial application of anti-5-BrdU to the detection and measurement of S phase. *Cytometry A*. 2004; 58(1):45–52. [PubMed: 14994219]
10. Shedlock DJ, Talbott KT, Morrow MP, et al. Ki-67 staining for determination of rhesus macaque T cell proliferative responses ex vivo. *Cytometry A*. 2010; 77(3):275–284. [PubMed: 20104580]
11. Stubbe M, Vanderheyde N, Goldman M, Marchant A. Antigen-specific central memory CD4⁺ T lymphocytes produce multiple cytokines and proliferate *in vivo* in humans. *J Immunol*. 2006; 177(11):8185–8190. [PubMed: 17114495]
12. Leroux-Roels G, Van Hecke E, Michiels W, Voet P, Hauser P, Pêtre J. Correlation between *in vivo* humoral and *in vitro* cellular immune responses following immunization with hepatitis B surface antigen (HBsAg) vaccines. *Vaccine*. 1994; 12(9):812–818. [PubMed: 7975860]
13. Heeg K, Reimann J, Kabelitz D, Hardt C, Wagner H. A rapid colorimetric assay for the determination of IL-2-producing helper T cell frequencies. *J Immunol Methods*. 1985; 77(2):237–246. [PubMed: 3156935]

14. Kaplan DR, Griffith R, Braciale VL, Braciale TJ. Influenza virus-specific human cytotoxic T cell clones: heterogeneity in antigenic specificity and restriction by class II MHC products. *Cell Immunol.* 1984; 88(1):193–206. [PubMed: 6236884]
- 15••. Han Q, Bagheri N, Bradshaw EM, Hafler DA, Lauffenburger DA, Love JC. Polyfunctional responses by human T cells result from sequential release of cytokines. *Proc Natl Acad Sci USA.* 2012; 109(5):1607–1612. Demonstrates the importance of temporal changes in T-cell cytokine production. [PubMed: 22160692]
16. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol.* 1989; 7:145–173. [PubMed: 2523712]
17. Yamane H, Paul WE. Memory CD4⁺ T cells: fate determination, positive feedback and plasticity. *Cell Mol Life Sci.* 2012; 69(10):1577–1583. [PubMed: 22481436]
18. Petrovsky N, Harrison LC. Cytokine-based human whole blood assay for the detection of antigen-reactive T cells. *J Immunol Methods.* 1995; 186(1):37–46. [PubMed: 7561146]
19. Carson RT, Desai DD, Vignali KM, Vignali DA. Immunoregulation of Th cells by naturally processed peptide antagonists. *J Immunol.* 1999; 162(1):1–4. [PubMed: 9886362]
20. Flagella M, Bui S, Zheng Z, et al. A multiplex branched DNA assay for parallel quantitative gene expression profiling. *Anal Biochem.* 2006; 352(1):50–60. [PubMed: 16545767]
21. Zheng Z, Luo Y, McMaster GK. Sensitive and quantitative measurement of gene expression directly from a small amount of whole blood. *Clin Chem.* 2006; 52(7):1294–1302. [PubMed: 16709626]
22. Bomert M, Köllisch G, Roponen M, et al. Analytical performance of a multiplexed, bead-based cytokine detection system in small volume samples. *Clin Chem Lab Med.* 2011; 49(10):1691–1693. [PubMed: 21663461]
23. Chowdhury F, Williams A, Johnson P. Validation and comparison of two multiplex technologies, Luminex and Mesoscale Discovery, for human cytokine profiling. *J Immunol Methods.* 2009; 340(1):55–64. [PubMed: 18983846]
24. Elshal MF, McCoy JP. Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. *Methods.* 2006; 38(4):317–323. [PubMed: 16481199]
25. Khan SS, Smith MS, Reda D, Suffredini AF, McCoy JP Jr. Multiplex bead array assays for detection of soluble cytokines: comparisons of sensitivity and quantitative values among kits from multiple manufacturers. *Cytometry B Clin Cytom.* 2004; 61(1):35–39. [PubMed: 15351980]
26. Richens JL, Urbanowicz RA, Metcalf R, Corne J, O'Shea P, Fairclough L. Quantitative validation and comparison of multiplex cytokine kits. *J Biomol Screen.* 2010; 15(5):562–568. [PubMed: 20176857]
27. Defawe OD, Fong Y, Vasilyeva E, et al. Optimization and qualification of a multiplex bead array to assess cytokine and chemokine production by vaccine-specific cells. *J Immunol Methods.* 2012; 382(1–2):117–128. [PubMed: 22626638]
28. Lalor MK, Floyd S, Gorak-Stolinska P, et al. BCG vaccination induces different cytokine profiles following infant BCG vaccination in the UK and Malawi. *J Infect Dis.* 2011; 204(7):1075–1085. [PubMed: 21881123]
29. Shebl FM, Pinto LA, García-Piñeres A, et al. Comparison of mRNA and protein measures of cytokines following vaccination with human papillomavirus-16 L1 virus-like particles. *Cancer Epidemiol Biomarkers Prev.* 2010; 19(4):978–981. [PubMed: 20332253]
30. Dabitaio D, Margolick JB, Lopez J, Bream JH. Multiplex measurement of proinflammatory cytokines in human serum: comparison of the Meso Scale Discovery electrochemiluminescence assay and the Cytometric Bead Array. *J Immunol Methods.* 2011; 372(1–2):71–77. [PubMed: 21781970]
31. Lehmann PV, Zhang W. Unique strengths of ELISPOT for T cell diagnostics. *Methods Mol Biol.* 2012; 792:3–23. [PubMed: 21956497]
32. De Rosa SC, Lu FX, Yu J, et al. Vaccination in humans generates broad T cell cytokine responses. *J Immunol.* 2004; 173(9):5372–5380. [PubMed: 15494483]
33. Waldrop SL, Pitcher CJ, Peterson DM, Maino VC, Picker LJ. Determination of antigen-specific memory/effector CD4⁺ T cell frequencies by flow cytometry: evidence for a novel, antigen-

- specific homeostatic mechanism in HIV-associated immunodeficiency. *J Clin Invest.* 1997; 99(7): 1739–1750. [PubMed: 9120019]
34. Lovelace P, Maecker HT. Multiparameter intracellular cytokine staining. *Methods Mol Biol.* 2011; 699:165–178. [PubMed: 21116983]
 35. Maecker HT, Rinfret A, D'Souza P, et al. Standardization of cytokine flow cytometry assays. *BMC Immunol.* 2005; 6:13. [PubMed: 15978127]
 36. Karlsson AC, Martin JN, Younger SR, et al. Comparison of the ELISPOT and cytokine flow cytometry assays for the enumeration of antigen-specific T cells. *J Immunol Methods.* 2003; 283(1–2):141–153. [PubMed: 14659906]
 37. Maecker HT, Hassler J, Payne JK, et al. Precision and linearity targets for validation of an IFN γ ELISPOT, cytokine flow cytometry, and tetramer assay using CMV peptides. *BMC Immunol.* 2008; 9:9. [PubMed: 18366814]
 38. Assenmacher M, Löhning M, Radbruch A. Detection and isolation of cytokine secreting cells using the cytometric cytokine secretion assay. *Curr Protoc Immunol.* 2002; Chapter 6(Unit 6.27)
 39. Campbell JD, Foerster A, Lasmanowicz V, et al. Rapid detection, enrichment and propagation of specific T cell subsets based on cytokine secretion. *Clin Exp Immunol.* 2011; 163(1):1–10. [PubMed: 20964638]
 40. Sojka DK, Fowell DJ. Regulatory T cells inhibit acute IFN- γ synthesis without blocking T-helper cell type 1 (Th1) differentiation via a compartmentalized requirement for IL-10. *Proc Natl Acad Sci USA.* 2011; 108(45):18336–18341. [PubMed: 22025707]
 41. Versteegen JM, Logtenberg T, Ballieux RE. Enumeration of IFN- γ -producing human lymphocytes by spot-ELISA. A method to detect lymphokine-producing lymphocytes at the single-cell level. *J Immunol Methods.* 1988; 111(1):25–29. [PubMed: 3134488]
 42. Zhang W, Caspell R, Karulin AY, et al. ELISPOT assays provide reproducible results among different laboratories for T-cell immune monitoring – even in hands of ELISPOT-inexperienced investigators. *J Immunotoxicol.* 2009; 6(4):227–234. [PubMed: 19908941]
 43. Hill PC, Brookes RH, Fox A, et al. Longitudinal assessment of an ELISPOT test for *Mycobacterium tuberculosis* infection. *PLoS Med.* 2007; 4(6):e192. [PubMed: 17564487]
 44. Kester KE, Cummings JF, Ockenhouse CF, et al. RTS,S Malaria Vaccine Evaluation Group. Phase 2a trial of 0, 1, and 3 month and 0, 7, and 28 day immunization schedules of malaria vaccine RTS,S/AS02 in malaria-naïve adults at the Walter Reed Army Institute of Research. *Vaccine.* 2008; 26(18):2191–2202. [PubMed: 18387719]
 45. Solares CA, Tuohy VK. ELISPOT determination of interferon- γ T-cell frequencies in patients with autoimmune sensorineural hearing loss. *Methods Mol Biol.* 2005; 302:253–260. [PubMed: 15937357]
 46. Gulley JL, Arlen PM, Madan RA, et al. Immunologic and prognostic factors associated with overall survival employing a poxviral-based PSA vaccine in metastatic castrate-resistant prostate cancer. *Cancer Immunol Immunother.* 2010; 59(5):663–674. [PubMed: 19890632]
 47. Kirkwood JM, Lee S, Moschos SJ, et al. Immunogenicity and antitumor effects of vaccination with peptide vaccine⁺/granulocyte-monocyte colony-stimulating factor and/or IFN- α 2b in advanced metastatic melanoma: Eastern Cooperative Oncology Group Phase II Trial E1696. *Clin Cancer Res.* 2009; 15(4):1443–1451. [PubMed: 19228745]
 48. Smith JG, Liu X, Kaufhold RM, Clair J, Caulfield MJ. Development and validation of a γ interferon ELISPOT assay for quantitation of cellular immune responses to varicella-zoster virus. *Clin Diagn Lab Immunol.* 2001; 8(5):871–879. [PubMed: 11527795]
 49. Reece WH, Pinder M, Gothard PK, et al. A CD4⁺ T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural *Plasmodium falciparum* infection and disease. *Nat Med.* 2004; 10(4):406–410. [PubMed: 15034567]
 50. Yang SH, Lee CG, Park SH, et al. Correlation of antiviral T-cell responses with suppression of viral rebound in chronic hepatitis B carriers: a proof-of-concept study. *Gene Ther.* 2006; 13(14): 1110–1117. [PubMed: 16525482]
 51. Calarota SA, Folli A, Maserati R, et al. HIV-1-specific T cell precursors with high proliferative capacity correlate with low viremia and high CD4 counts in untreated individuals. *J Immunol.* 2008; 180(9):5907–5915. [PubMed: 18424710]

52. Goonetilleke N, Moore S, Dally L, et al. Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8⁺ T-cell epitopes. *J Virol*. 2006; 80(10):4717–4728. [PubMed: 16641265]
53. Ahlborg N, Axelsson B. Dual- and triple-color fluorospot. *Methods Mol Biol*. 2012; 792:77–85. [PubMed: 21956502]
54. Gazagne A, Claret E, Wijdenes J, et al. A Fluorospot assay to detect single T lymphocytes simultaneously producing multiple cytokines. *J Immunol Methods*. 2003; 283(1–2):91–98. [PubMed: 14659902]
55. Harari A, Petitpierre S, Vallerian F, Pantaleo G. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood*. 2004; 103(3):966–972. [PubMed: 12958069]
56. Janetzki S, Panageas KS, Ben-Porat L, et al. Elispot Proficiency Panel of the CVC Immune Assay Working Group. Results and harmonization guidelines from two large-scale international Elispot proficiency panels conducted by the Cancer Vaccine Consortium (CVC/SVI). *Cancer Immunol Immunother*. 2008; 57(3):303–315. [PubMed: 17721781]
57. Moodie Z, Price L, Gouttefangeas C, et al. Response definition criteria for ELISPOT assays revisited. *Cancer Immunol Immunother*. 2010; 59(10):1489–1501. [PubMed: 20549207]
58. Altman JD, Moss PA, Goulder PJ, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science*. 1996; 274(5284):94–96. Development of tetramers to analyze antigen-specific T-cell populations. [PubMed: 8810254]
59. Wagar LE, Rosella L, Crowcroft N, et al. Humoral and cell-mediated immunity to pandemic H1N1 influenza in a Canadian cohort one year post-pandemic: implications for vaccination. *PLoS One*. 2011; 6(11):e28063. [PubMed: 22132212]
60. Aslam A, Chapel H, Ogg G. Direct ex-vivo evaluation of pneumococcal specific T-cells in healthy adults. *PLoS One*. 2011; 6(10):e25367. [PubMed: 22039412]
61. Schmidt J, Neumann-Haefelin C, Altay T, et al. Immunodominance of HLA-A2-restricted hepatitis C virus-specific CD8⁺ T cell responses is linked to naive-precursor frequency. *J Virol*. 2011; 85(10):5232–5236. [PubMed: 21367907]
62. Li Pira G, Ivaldi F, Moretti P, Manca F. High throughput T epitope mapping and vaccine development. *J Biomed Biotechnol*. 2010; 2010:325720. [PubMed: 20617148]
63. Wu C, Zanker D, Valkenburg S, et al. Systematic identification of immunodominant CD8⁺ T-cell responses to influenza A virus in HLA-A2 individuals. *Proc Natl Acad Sci USA*. 2011; 108(22):9178–9183. [PubMed: 21562214]
64. Ru Z, Xiao W, Pajot A, et al. Development of a humanized HLA-A2.1/DP4 transgenic mouse model and the use of this model to map HLA-DP4-restricted epitopes of HBV envelope protein. *PLoS One*. 2012; 7(3):e32247. [PubMed: 22403638]
65. Taneja V, David CS. HLA transgenic mice as humanized mouse models of disease and immunity. *J Clin Invest*. 1998; 101(5):921–926. [PubMed: 9486959]
66. Migueles SA, Osborne CM, Royce C, et al. Lytic granule loading of CD8⁺ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity*. 2008; 29(6):1009–1021. Demonstrates the importance of antigen-specific cytotoxic T lymphocytes as a correlate for efficacy in long-term HIV nonprogressors patients. [PubMed: 19062316]
67. Wonderlich J, Shearer G, Livingstone A, Brooks A. Induction and measurement of cytotoxic T lymphocyte activity. *Curr Protoc Immunol*. 2006; Chapter 3(Unit 3.11)
68. Krähenbühl O, Rey C, Jenne D, et al. Characterization of granzymes A and B isolated from granules of cloned human cytotoxic T lymphocytes. *J Immunol*. 1988; 141(10):3471–3477. [PubMed: 3263427]
69. Lowin B, Hahne M, Mattmann C, Tschopp J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature*. 1994; 370(6491):650–652. [PubMed: 7520535]
70. Masson D, Tschopp J. Isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes. *J Biol Chem*. 1985; 260(16):9069–9072. [PubMed: 3874868]

71. Brunner KT, Mauel J, Cerottini JC, Chapuis B. Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells *in vitro*; inhibition by isoantibody and by drugs. *Immunology*. 1968; 14(2):181–196. [PubMed: 4966657]
72. Zaritskaya L, Shurin MR, Sayers TJ, Malyguine AM. New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Rev Vaccines*. 2010; 9(6):601–616. [PubMed: 20518716]
73. Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. *J Immunol Methods*. 1983; 64(3):313–320. [PubMed: 6199426]
74. Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods*. 1988; 115(1):61–69. [PubMed: 3192948]
75. Cui J, Bystryń JC. An improved europium release assay for complement-mediated cytolysis. *J Immunol Methods*. 1992; 147(1):13–19. [PubMed: 1541836]
76. Bachy M, Bonnin-Rivalland A, Tilliet V, Trannoy E. β galactosidase release as an alternative to chromium release in cytotoxic T-cell assays. *J Immunol Methods*. 1999; 230(1–2):37–46. [PubMed: 10594352]
77. Memon SA, Petrak D, Moreno MB, Zacharchuk CM. A simple assay for examining the effect of transiently expressed genes on programmed cell death. *J Immunol Methods*. 1995; 180(1):15–24. [PubMed: 7897244]
78. Gupta P, Tayal R, Durgapal H, Rath S, Acharya SK, Panda SK. Development of highly sensitive Bicistronic vector based non-radioactive antigen-specific cytotoxicity assay. *J Immunol Methods*. 2009; 349(1–2):28–37. [PubMed: 19682994]
79. Shafer-Weaver K, Sayers T, Strobl S, et al. The Granzyme B ELISPOT assay: an alternative to the 51Cr-release assay for monitoring cell-mediated cytotoxicity. *J Transl Med*. 2003; 1(1):14. [PubMed: 14697097]
80. Zuber B, Levitsky V, Jönsson G, et al. Detection of human perforin by ELISpot and ELISA: *ex vivo* identification of virus-specific cells. *J Immunol Methods*. 2005; 302(1–2):13–25. [PubMed: 16005014]
81. Malyguine AM, Strobl SL, Shurin MR. Immunological monitoring of the tumor immunoenvironment for clinical trials. *Cancer Immunol Immunother*. 2012; 61(2):239–247. [PubMed: 22080408]
82. Darmon AJ, Nicholson DW, Bleackley RC. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature*. 1995; 377(6548):446–448. [PubMed: 7566124]
83. Sarin A, Haddad EK, Henkart PA. Caspase dependence of target cell damage induced by cytotoxic lymphocytes. *J Immunol*. 1998; 161(6):2810–2816. [PubMed: 9743340]
84. He L, Hakimi J, Salha D, Miron I, Dunn P, Radvanyi L. A sensitive flow cytometry-based cytotoxic T-lymphocyte assay through detection of cleaved caspase 3 in target cells. *J Immunol Methods*. 2005; 304(1–2):43–59. [PubMed: 16076473]
85. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med*. 1995; 182(5):1545–1556. [PubMed: 7595224]
86. van Engeland M, Ramaekers FC, Schutte B, Reutelingsperger CP. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. *Cytometry*. 1996; 24(2):131–139. [PubMed: 8725662]
87. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods*. 1995; 184(1):39–51. [PubMed: 7622868]
88. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods*. 2004; 294(1–2):15–22. [PubMed: 15604012]
89. Rubio V, Stuge TB, Singh N, et al. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. *Nat Med*. 2003; 9(11):1377–1382. [PubMed: 14528297]
90. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8⁺ T cells by a flow cytometric assay for degranulation. *J Immunol Methods*. 2003; 281(1–2):65–78. [PubMed: 14580882]

91. Burkett MW, Shafer-Weaver KA, Strobl S, Baseler M, Malyguine A. A novel flow cytometric assay for evaluating cell-mediated cytotoxicity. *J Immunother.* 2005; 28(4):396–402. [PubMed: 16000959]
92. Harty JT, Badovinac VP. Shaping and reshaping CD8⁺ T-cell memory. *Nat Rev Immunol.* 2008; 8(2):107–119. [PubMed: 18219309]
93. Schmidt NW, Butler NS, Harty JT. Plasmodium-host interactions directly influence the threshold of memory CD8 T cells required for protective immunity. *J Immunol.* 2011; 186(10):5873–5884. [PubMed: 21460205]
- 94•. Newell EW, Sigal N, Bendall SC, Nolan GP, Davis MM. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8⁺ T cell phenotypes. *Immunity.* 2012; 36(1):142–152. Elegant analysis of the complexity of memory T-cell phenotypes using the new technology of cytometry by time-of-flight. [PubMed: 22265676]
95. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 1999; 401(6754):708–712. [PubMed: 10537110]
96. Berard M, Tough DF. Qualitative differences between naïve and memory T cells. *Immunology.* 2002; 106(2):127–138. [PubMed: 12047742]
97. Pepper M, Jenkins MK. Origins of CD4⁽⁺⁾ effector and central memory T cells. *Nat Immunol.* 2011; 12(6):467–471. [PubMed: 21739668]
98. Sallusto F, Lanzavecchia A. Heterogeneity of CD4⁺ memory T cells: functional modules for tailored immunity. *Eur J Immunol.* 2009; 39(8):2076–2082. [PubMed: 19672903]
99. Hansen SG, Ford JC, Lewis MS, et al. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. *Nature.* 2011; 473(7348):523–527. [PubMed: 21562493]
100. Wherry EJ, Teichgräber V, Becker TC, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol.* 2003; 4(3):225–234. [PubMed: 12563257]
101. Buchbinder SP, Mehrotra DV, Duerr A, et al. Step Study Protocol Team. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet.* 2008; 372(9653):1881–1893. [PubMed: 19012954]
102. Haynes BF, Gilbert PB, McElrath MJ, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med.* 2012; 366(14):1275–1286. [PubMed: 22475592]
103. Abate G, Eslick J, Newman FK, et al. Flow-cytometric detection of vaccinia-induced memory effector CD4⁽⁺⁾, CD8⁽⁺⁾, and γ Δ TCR⁽⁺⁾ T cells capable of antigen-specific expansion and effector functions. *J Infect Dis.* 2005; 192(8):1362–1371. [PubMed: 16170753]
104. Chattopadhyay PK, Yu J, Roederer M. A live-cell assay to detect antigen-specific CD4⁺ T cells with diverse cytokine profiles. *Nat Med.* 2005; 11(10):1113–1117. [PubMed: 16186817]
- 105•. Bendall SC, Simonds EF, Qiu P, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science.* 2011; 332(6030):687–696. Technology of single cell mass cytometry as applied to the analysis of immune cell populations. [PubMed: 21551058]
106. Downey JS, Attaf M, Moyle G, Gazzard B, Gotch F, Imami N. T-cell signalling in antiretroviral-treated, aviraemic HIV-1-positive individuals is present in a raised state of basal activation that contributes to T-cell hyporesponsiveness. *AIDS.* 2011; 25(16):1981–1986. [PubMed: 21811141]
107. Puronen CE, Thompson WL, Imamichi H, et al. Decreased interleukin 7 responsiveness of T lymphocytes in patients with idiopathic CD4 lymphopenia. *J Infect Dis.* 2012; 205(9):1382–1390. [PubMed: 22454463]
108. Huebner RE, Schein MF, Hall CA, Barnes SA. Delayed-type hypersensitivity anergy in human immunodeficiency virus-infected persons screened for infection with *Mycobacterium tuberculosis*. *Clin Infect Dis.* 1994; 19(1):26–32. [PubMed: 7948554]
109. Nagafuchi S, Kashiwagi S, Hayashi J, Katsuta H, Ikematsu H, Harada M. A delayed type hypersensitivity (DTH) skin reaction to hepatitis B surface antigen (HBsAg) and intradermal hepatitis B vaccination. *Fukuoka Igaku Zasshi.* 2004; 95(12):305–313. [PubMed: 15732372]
110. Koff WC. HIV vaccine development: challenges and opportunities towards solving the HIV vaccine-neutralizing antibody problem. *Vaccine.* 2012; 30(29):4310–4315. [PubMed: 22100891]

111. Middleton JR. Staphylococcus aureus antigens and challenges in vaccine development. *Expert Rev Vaccines*. 2008; 7(6):805–815. [PubMed: 18665778]
112. Proctor RA. Challenges for a universal Staphylococcus aureus vaccine. *Clin Infect Dis*. 2012; 54(8):1179–1186. [PubMed: 22354924]
- 113••. Maecker HT, McCoy JP, Nussenblatt R. Standardizing immunophenotyping for the Human Immunology Project. *Nat Rev Immunol*. 2012; 12(3):191–200. Current state of standardization of human immunophenotyping and discusses a number of improvements to decrease variability in clinical trials. [PubMed: 22343568]
- 114••. Janetzki S, Britten CM, Kalos M, et al. 'MIATA'-minimal information about T cell assays. *Immunity*. 2009; 31(4):527–528. Proposes a minimum dataset for reporting of T-cell assay data. [PubMed: 19833080]

Key issues

- There is a need for better correlates of vaccine-induced T-cell immunity.
- Human clinical trials require robust and user-friendly T-cell assays.
- Cytokine ELISPOT assays are sensitive and reproducible but labor intensive.
- Flow cytometry-based assays are ideal for immune phenotyping and functional analysis.
- New assays particularly cytometry by time-of-flight offer unequalled multiparameter capability.
- Extensive validation is required if assays are to be used for regulatory purposes.

Figure 1. Assays available for measuring the T-cell response to vaccines

The success of a T-cell vaccine requires the induction of specific T-cell memory against the cognate vaccine antigen. A variety of assays are currently available for evaluating the function, immune phenotype and frequency of T-cell responses including: tetramers to quantify total memory T cells, proliferation assays to measure T-cell activation, cytokine-based assays to measure functionality and the profile of the vaccine response and immunophenotyping via flow cytometry-based methods to integrate many of the aforementioned assays. Finally, T-cell effector function assays are critical to show the vaccine-induced T cells are able to kill antigen-expressing target cells.

BrdU: 5-bromo-2'-deoxyuridine; CBA: Cytometric bead array; CFSE: Carboxyfluorescein succinimidyl ester; CyTOF: Cytometry by time-of-flight; ELISPOT: Enzyme-linked immunosorbent spot; ICS: Intracellular cytokine staining.

Figure 2. Examples of assays used to assess T-cell responses in a human immunization trial
(A) PBMCs were collected from a clinical trial subject 4 weeks after a first, second or third immunization with hepatitis B surface antigen (HBs) combined with Advax™, a novel polysaccharide adjuvant. PBMCs were stained with CFSE and cultured in presence of saline control (left column) or HBs antigen (right column) for 5 days then washed and stained with anti-CD4 monoclonal antibody and analyzed by FACS Diva. Increased CD4 T-cell proliferation to HBs is observed 4 weeks after the third immunization as measured by reduction in CFSE intensity (red circle, bottom right figure). **(B)** Antigen-specific T cells can be identified by stimulating PBMCs with the relevant peptide overnight and performing intracellular cytokine staining for IFN- γ , IL-2 and/or TNF- α . Here, influenza-specific CD4⁺ T cells have been identified using intracellular cytokine staining for IFN- γ and IL-2 following stimulation with relevant vaccine peptide and compared with unstimulated cells. **(C)** Human antigen-specific T cells can also be detected by cytokine enzyme-linked immunosorbent spot (ELISPOT). Here, fresh or thawed cryopreserved human PBMCs were stimulated in an IFN- γ capture plate with either mitogen mix (pokeweed mitogen and phytohemagglutinin) or inactivated influenza virus (B/Brisbane) or media and then the number of IFN- γ -producing T cells quantified using a cytotoxic T lymphocyte ELISPOT reader. The results confirm that PBMC cryopreservation does not diminish the ability to detect the secretion of IFN- γ production by thawed T cells.
 CFSE: Carboxyfluorescein succinimidyl ester; PBMC: Peripheral blood mononuclear cell.

Table 1

Advantages and disadvantages of available T-cell assays.

Assays	Reproducibility	Sensitivity	High-throughput analyses	Advantages	Disadvantages
<i>Proliferation assays</i>					
³ H-thymidine incorporation	+++	++++	++	Historic. Well validated	Radioisotope Semi-quantitative Cell phenotypes cannot be analyzed
CFSE	++	++	++	Non-radioactive FACS-based Cell phenotyping possible	CFSE is cytotoxic Relatively insensitive Lack of standardization Long term (4–6 days) <i>in vitro</i> stimulation can bias phenotype/function
Ki67	++	++	++	Non-radioactive, non-toxic Does not require cell culture FACS-based Cell phenotyping possible	Cells must have recently proliferated Cells must be fixed Relatively insensitive
<i>Cytokine-based assays</i>					
ELISA	+++	++	+	Simple Historic Well validated	Only one cytokine studied per assay Cannot analyze phenotype of cells producing cytokines Low sensitivity for cytokine detection for low-frequency T cells
CBA	++	++	+++	Multiplexing capability Require only small samples sizes Can be done on lab flow cytometer	Low sensitivity for cytokine detection for low-frequency T cells Cannot analyze phenotype of cells producing cytokines
Luminex	+++	++	+++	Multiplexing capability RNA probing Dedicated platform for analyzing data	Requires dedicated instrumentation and trained personnel Low sensitivity for cytokine detection for low-frequency T cells Cannot analyze phenotype of cells producing cytokines Requires cell culture
ELISPOT	+++	+++	+++	Quantitative Most sensitive technique to detect low-frequency T cells Suitable for cryopreserved cells Does not require cell fixation: cells can be restudied	Absence of phenotyping (subsets, activation markers) Not all activated T cells make the same cytokine Not well suited to multiplexing cytokines
<i>Flow cytometry assays/cell phenotyping</i>					
ICS	+++	+++	++	Allows identification of multiple cytokines at single cell level Cell phenotyping is possible	Cells cannot be sorted for further analysis due to fixation and permeabilization

Assays	Reproducibility	Sensitivity	High-throughput analyses	Advantages	Disadvantages
Tetramer staining	+++	+++	+	Identify antigen-specific T-cell population Can use to enrich for rare cell populations	Limited by peptide choice Human tetramer customization requires individual HLA typing Not well validated in human studies
<i>CTL assays</i>					
Chromium release (⁵¹ Cr)	+	+	+	Historic Directly measures cytotoxic activity	Radioactive No cell phenotyping
CTL/flow cytometry	++	++	++	Allows comprehensive phenotyping of CTLs	Only measures cytotoxic potential, not direct CTL activity

CBA: Cytometric bead array; CFSE: Carboxyfluorescein succinimidyl ester; CTL: Cytotoxic T lymphocyte; ELISPOT: Enzyme-linked immunosorbent spot; ICS: Intracellular cytokine staining.