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Vaccine applications of flow cytometry

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Abstract

Highly effective vaccines have yet to be identified for many widespread infectious diseases including HIV, tuberculosis and malaria. Many vaccine candidates for these diseases have been designed to induce both cellular and humoral immunity, and measurement of the induced cellular immune response and antibody response is critical for monitoring immunogenicity. The flow cytometric intracellular cytokine staining assay is one of the primary assays for enumerating vaccine-induced T cells in vaccine clinical trials. The assay is flexible, allowing for measurement of various cytokines or functions and phenotyping markers, and the assay can be validated. Changes in other cell types such as innate immune cells are monitored by flow cytometric phenotyping assays. Cell sorting of vaccine-induced T cells and B cells is used to allow genomic and transcriptional analysis of these cells. Thus, flow cytometric methods are commonly used in trials testing the next generation of vaccines.

Keywords

Flow cytometry; vaccine; T cell; immunogenicity; intracellular cytokine staining

1. Introduction

HIV, tuberculosis, and malaria are the three infectious diseases with the greatest global impact and prophylactic vaccines are the best hope for control. However, many challenges remain in the development of such vaccines, in part due to the nature of these pathogens. Unlike most licensed vaccines for other pathogens, for which a humoral response is proposed to be the key mediator of efficacy, there is likely also a requirement for a dominant cellular immune response. Hence, many candidate vaccines have been designed to induce both CD4+ and CD8+ pathogen-specific T cells. As a consequence, assays to quantify these T cells are required to assess immunogenicity. Although the IFN- γ ELISpot assay was historically the primary measure of immunogenicity in candidate vaccine clinical trials, the flow cytometric intracellular cytokine staining (ICS) assay is now used more commonly due to its ability to measure multiple cytokines and to determine the type of T-cell response.

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A key challenge in the field of HIV vaccine research has been identifying a correlate for vaccine efficacy. This has been compounded by the lack of success in most efficacy trials of early candidate vaccines and a lack of effective natural immune responses on which to focus vaccine design and screening efforts. To broaden the likelihood of identifying a correlate, flow cytometry is used to capture multiple characteristics of the induced response as discussed in more detail in the sections that follow. For T cells, flow cytometry provides information about polyfunctionality, proliferative capacity, phenotype and activation status. It can characterize details of the innate immune response such as dendritic cell phenotype and NK cell function and phenotype. Flow cytometry is also used to characterize B cells and new methods allow for identification and sorting of antigen-specific B cells. New anti-HIV broadly neutralizing monoclonal antibodies have been obtained using this method and similar methods are in use in HIV vaccine recipients. Finally, vaccine-induced T cells are sorted for subsequent transcriptional analyses. While these assays typically profile blood due to ease of sampling, they can also be used for examination of mucosal tissue samples. Table 1 summarizes the major uses of flow cytometry in vaccine clinical trials. Our laboratory focuses on HIV vaccine research and thus the focus of this report is on the use of flow cytometry in HIV vaccine clinical trials.

2. Intracellular cytokine staining to measure vaccine-induced T-cell immunogenicity

Quantification of the antigen-specific CD4⁺ and CD8⁺ T cells induced in response to the proteins encoded in a candidate HIV vaccine is an important measure of cellular immunogenicity. There are limited methods for identification of antigen-specific T cells. Two common methods, ELISpot and ICS, are dependent on the secretion or production of a cytokine in response to short-term ex vivo stimulation with antigen (such as HIV proteins or pools of overlapping peptides for an HIV protein). For ICS, multiple cytokines (or chemokines) can be measured, and the choice of cytokines to include can be tailored depending on the anticipated response to the particular vaccine candidate, within the constraints of availability of staining reagents that work well in the flow assay. IFN- γ , IL-2 and TNF- α are commonly measured and work well for identification of both CD4⁺ and CD8⁺ T cells. Within our HIV Vaccine Trials Network (HVTN) Laboratory Program, we use a combined measure of cells producing IFN- γ and/or IL-2 as a simple metric for quantification of responding T cells.

Both the IFN- γ ELISpot and the ICS assay have proven to be useful as a means for measurement of T-cell immunogenicity and there are numerous examples in the published literature, including recent publications from our group [1-5]. However, there is concern whether such measurements will correlate with vaccine efficacy. This concern was highlighted after the Step study was terminated in 2007 due to lack of efficacy and potential increased HIV acquisition risk in vaccine recipients [6]. This vaccine was highly immunogenic as measured by ICS [7], yet was not effective. Among male vaccine recipients who did not become HIV infected, 73% had CD8⁺ T-cell responses and 40% had CD4⁺ T-cell responses to Gag, Pol, or Nef peptides. There was no decrease in infection rates in vaccine versus placebo recipients, despite robust T-cell responses as measured by ICS, indicating that the robust T-cell responses measured in the trial did not correlate with efficacy. Therefore, there is interest in further characterizing the T-cell response in order to identify the features or qualities that may correlate with efficacy for other vaccine candidates. The ICS assay can include multiple measurements that further characterize the quality of the response as described below.

2.1 Measurement of other functions in the ICS assay

In addition to production of cytokines or chemokines, other functions can be assessed in the ICS assay. This includes assessment of cytotoxic potential by measurement of degranulation and granule contents and assessment of helper capacity by measurement of the expression of CD40 ligand (CD40L, also known as CD154).

Degranulation may indicate cytotoxic potential since an effector mechanism for cytotoxic T cells is release of cytotoxic proteins such as perforin and granzymes from granules. Degranulation is measured by the expression of CD107a on the cell surface. CD107a is normally expressed only in internal granular membranes, and is transiently expressed on the cell surface during degranulation. A “co-culture” of the cells with fluorescently-labeled anti-CD107a is used to label the cells that are stimulated and degranulate during the in vitro stimulation of the ICS assay [8].

However, measurement of CD107a does not identify the contents of the granules that degranulate. Perforin and several serine proteases (granzymes) contained in these granules are particularly relevant for cytotoxic function through the proposed mechanisms of pore formation in the target cell membrane by perforin that allows entry of the granzymes that induce apoptosis [9]. Perforin and several granzymes (commonly granzymes A and B) can be measured by intracellular staining [10, 11], but there is concern about the accuracy of such measurements in the context of the ex vivo-stimulated ICS assay. During the ex vivo stimulation, antigen-stimulated cells will quickly degranulate and thus lose their intracellular content for these cytotoxic proteins, thereby rendering them difficult to detect. This is especially notable for perforin, but recent work has shown that perforin is re-synthesized during the typical 5- or 6-hour stimulation and can be detected using a particular anti-perforin antibody reagent [12]. In fact, this newly-synthesized perforin may play a role in cytotoxicity independent of cytotoxic granules [13]. The detection of the granzymes following ex vivo stimulation does not seem to be as problematic as for perforin, although this has not been well-studied and likely will be affected by different rates of regeneration for different granzymes and may additionally be affected due to differences by cell type.

CD40L is expressed on the surface of activated CD4⁺ T cells and is one component of the helper interaction with B cells and CD8⁺ T cells. Therefore, it may be a marker for detection of cells that can provide helper function. CD40L can be detected either by co-culture, similar to CD107a, or by intracellular staining [14]. We find expression of CD40L to be the most sensitive indicator of activation of CD4⁺ T cells as a consequence of the antigen-specific stimulation in the ICS assay, at least in the context of HIV vaccines including recombinant Env protein. In such situations, virtually all cytokine-producing CD4⁺ T cells also co-express CD40L, and some of the CD40L-expressing cells did not produce any of the cytokines included in the assay.

Thus there is considerable flexibility in the ICS assay and due to continuing advances in the technology and increased commercial availability of reagents, the ICS assay can be customized to include the cytokines or functions of most relevance for the vaccine candidate. We have recently developed an ICS assay that includes cytokines and functions most useful for characterizing a CD4⁺ T-cell response (IL-4, IL-17, CD40L) in addition to cytokines relevant for both CD4⁺ and CD8⁺ T cells (IFN- γ , IL-2, TNF- α). An example of the staining profile for this panel for CD4⁺ and CD8⁺ T cells in response to polyclonal stimulation with staphylococcal enterotoxin B (SEB) is shown in Figure 1.

2.2 T-cell polyfunctionality

Due to the multi-parameter nature of flow cytometry, multiple cytokines, chemokines and the functions referred to above can be examined simultaneously in the ICS assay. This

enables multi-functional characterization at the single-cell level, and a “polyfunctionality” type of analysis is often performed. These analyses describe the response based on the number of functions per cell. Analysis and display of any type of multi-parameter data are challenging. To simplify the results, polyfunctionality is often reported as the proportion of the responding cells producing one function, two functions, three functions, etc., without specifying the particular function(s) and this is referred to as the “degree of functionality”. This is often shown as a pie chart [15], where slices of the pie indicate the average proportion of responding cells that produce the indicated number of functions. This is shown in Figure 2 for a comparison of responses to CMV and SEB for the four most prevalent functions in our new ICS panel (IFN- γ , IL-2, TNF- α and CD40L). We prefer an alternate display that uses box plots so that data for all samples can be shown rather than simply a summary statistic. A published example is shown in the left graphs of McElrath et al [7], Figure 2.

The proportion of each individual functional combination, sometimes referred to as a “flavor”, can be further discerned. As expected, the number of these combinations increases exponentially as the number of functions measured increases. For three functions, there are seven possible combinations, not including the one combination that is negative for all functions. For four functions, there are 15 combinations, and for five, there are 31. These more detailed functional subsets can be summarized in a pie chart as individual slices or can be shown as pie arcs (Figure 2). The individual subset percentages can also be shown as bar graphs (Figure 2) where the percentage refers to percent of all responding cells. Alternatively, we prefer to show these as box plots where each functional sub-classification is shown on a scale normalized to 100% for each degree of polyfunctionality (see McElrath et al [7], Figure 2, or Spearman et al [2], Figure 3).

3. Validation of the ICS assay

When the ICS assay is used in the context of clinical trials in humans, it needs documentation that characterizes the expected performance of the assay, and optimally, it should also be validated. Validation is generally required for assays used to document efficacy in a clinical trial. For vaccine candidates that proceed to large phase efficacy testing, efficacy will likely be determined through clinical endpoints such as HIV acquisition or HIV viral load rather than laboratory measurement of immune response. However, ICS assays are often used to assess a primary objective of immunogenicity in Phase I and II trials, and results of such trials are used to determine the fate of vaccine candidates. Therefore, ICS assays play a critical role in the clinical developmental pathway for these candidates and validation of these assays is often warranted.

The eight validation parameters as described in the International Conference on Harmonization (ICH) Q2(R1) document (<http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html>) are listed in Table 2. Even if an assay is not to be fully validated, it is still beneficial to characterize it for at least some of the validation parameters. Though it is beyond the scope of this report to describe in detail all of these parameters and how they relate to the ICS assay, some that we consider especially important to characterize when feasible are highlighted here: accuracy, specificity and precision.

3.1 Accuracy may be summarized as how closely the assay reports the true value for a measure

If feasible to assess, this would be a critical parameter to document for any assay. However, for the ICS assay (and for most flow cytometric assays) there is no means of determining what the true value is. As an alternative, the accuracy of an assay can be assessed by

comparing it to a reference assay. This is also problematic since there generally are no reference assays for the types of flow cytometric measures used to evaluate vaccines. Since over time we have developed different versions of the primary ICS assay we use for examination of clinical trial samples, our approach has been to use the current version of our ICS assay as the reference assay for the next version. To enable use of HIV-uninfected samples for the validation experiment, we generally use responses to the CMV pp65 peptide pool rather than responses to HIV peptide pools. Note that potentially there are many measurements from the ICS assay that could be compared. To simplify the number of cytokine subsets, we restrict analysis to marginal responses for the three cytokines included in most of the versions of our ICS assays: IFN- γ , IL-2, and TNF- α . The marginal response refers to cells producing one of the cytokines whether or not the other cytokines are co-produced. Figure 3 shows an example of the results from an experiment comparing a new assay with our current reference assay. CD4+ and CD8+ T-cell responses were well correlated, but the CD4 responses were slightly lower for the new assay (as indicated by the slope <1).

3.2 Specificity refers to the ability to assess or distinguish the analyte, in this case a cytokine-producing antigen-specific T cell, among the mixture of components expected to be present in a peripheral blood mononuclear cell (PBMC) sample, such as other T cells and other cell types

These other cells may contribute to non-specific background, so the experimental procedure assesses how often the assay detects a response when one should not be present (i.e., the false-positive rate). In order to assess this, a method for determining the positivity of a response must be developed. This positivity method is also necessary when the assay is used for its intended purpose, assessing immunogenicity. This enables reporting of the proportion of vaccine recipients with a detectable response.

Positivity can be calculated by several mathematical methods, and is most appropriately determined in reference to an unstimulated negative control. A common approach is to require a stimulated response to be at least 3-fold (or 4-fold) above the control, and often also includes a threshold (e.g., at least 0.05% of CD4+ or CD8+ T cells). Another method is to use the Fisher's exact test to compare the number of cytokine-producing cells between the antigen-stimulated and unstimulated samples [16]. The p-value associated with this comparison is used to assign positivity, and the threshold for this p-value can be chosen empirically. An additional threshold for the T-cell response may also be included.

The optimal procedure is to perform an experiment in which samples with known responses are compared to samples known not to have a response. In this way, the method can be optimized to reduce the false positive responses while maintaining sensitivity for detecting responses among the positive samples. A limitation of this approach is that there is not a method to verify which samples are expected to be positive. Our approach is to evaluate PBMC samples from HIV-infected and HIV-uninfected individuals; we obtained leukapheresis samples from 50 HIV-uninfected and 20 chronic HIV-infected individuals for this purpose. We are confident in our ability to assess the false positivity since samples from HIV-uninfected individuals are unlikely to contain many HIV cross-reactive cells. However, we are not confident in assessing the sensitivity of the method since we cannot verify which of the HIV-infected samples will have responses to the HIV proteins examined. Since we have repeatedly tested these samples, we can choose a small number of the samples from HIV-infected individuals (eight or more) that show responses as detected in prior ELISpot or ICS assays. The sample size for the HIV-uninfected sample group is more critical, and we generally include at least 30 to robustly determine the false-positivity rate. This experimental procedure can be used to determine the positivity thresholds, but must be

repeated (optimally with a different set of samples) to then validate the specificity of the assay.

Positivity can be examined for individual cytokines or for combinations of cytokines. As noted above, our HVTN program uses a combined measure of cells producing IFN- γ and/or IL-2 as a primary measure of immunogenicity. Simplifying to a single metric provides advantages in terms of multiplicity adjustments. Because our newer ICS assays include a larger variety of cytokines and functions, we are developing an alternate approach where positivity is assessed separately for the marginal response for each cytokine or function of interest. This has an advantage over assessing positivity for a combined response since each individual cytokine may have different background and sensitivity is higher for those cytokines with lower background. Overall positivity for several cytokines/functions can still be determined, but it is calculated based on whether any of the marginal responses is positive. Although we have not yet formally tested this, it is likely that the advantage of increased sensitivity outweighs the disadvantage of multiplicity adjustment using this approach.

3.3 Precision characterizes the amount of variation in the assay and has three subcategories

Repeatability refers to intra-assay precision, such as replicates. Intermediate precision refers to intra-laboratory variations such as between days, operators or instruments.

Reproducibility refers to inter-laboratory variations. The third category is rarely considered since it is more common for testing to be performed in a central laboratory. Variability in the ICS assay tends to increase as the magnitude of the response decreases [16, 17]. The acceptable level of variability relates to the limit of quantitation (LOQ, Table 2); all responses above this limit must have coefficients of variation (CVs) for repeated measurements below this threshold (we use a CV of 30%). It is optimal in all validation experiments to assess a similar type of response as will be assessed by the assay when employed for a clinical trial. Thus, it would be optimal to assess HIV-specific responses in HIV vaccine recipients. Since leukapheresis samples from known responders are often not available for this use, we substitute HIV-specific responses in HIV-infected individuals, or CMV-specific responses in HIV-uninfected individuals as alternate antigen-specific responses. Another alternative is to use a polyclonal response, such as to SEB, although this is generally of much higher magnitude than the expected vaccine-induced responses.

4. Proliferation

The ICS assay provides information about several important cell functions. The proliferative ability of a cell is another function that can be measured using flow cytometry. This is likely a beneficial function for vaccine-induced T cells for the short-term expansion of these cells and for the long-term maintenance of memory T cells. The traditional non-flow cytometric method for examination of cell proliferation uses ^3H -thymidine incorporation into newly synthesized DNA. A comparable flow cytometric method uses bromodeoxyuridine (BrdU) incorporation and detection by fluorescently-labeled anti-BrdU antibodies. An alternate method to detect DNA synthesis uses incorporation of ethynyl-deoxyuridine (EdU) and “click” chemistry [18]. In addition, antibodies to Ki-67, a nuclear antigen only expressed in dividing cells, is an alternate method for identifying proliferating cells on the flow cytometer (and is also useful for detection of in vivo-activated T cells, see below).

Perhaps the most commonly used method for assessing proliferation is through dyes that allow for discrimination of cell generations. Therefore, unlike the cell cycle-specific dyes described above that enumerate the total frequency of cells in division, these cell generation dyes also report the number of times cells divide and allow for calculation of the frequency

of the starting population that divided. Although there are now multiple types of such dyes, carboxyfluorescein succinimidyl ester (CFSE) is the prototype and is still commonly used. Cells are initially labeled with CFSE, cultured *in vitro* for several days with a stimulant, and the CFSE dye is progressively diluted among the proliferating cells. Several issues are of particular relevance when using this assay to measure vaccine-induced T cells. The frequency of these cells is typically low, and consequently the background in the assay must be low. Also, similar to the ICS assay, a method for determining positivity of the assay is needed. Finally, there are several methods for reporting CFSE data.

Background in the CFSE assay, especially among CD4⁺ T cells, can be higher than observed in the ICS assay. We and others have found that the use of human serum, rather than bovine, has a major impact on lowering this background. In addition, we have found that different lots of human serum have differing effects on background and therefore we screen several lots and then obtain a large supply of a lot demonstrating low background. Because there are likely many undefined factors that may affect this background, we have developed a detailed SOP and require strict adherence to it for analysis of vaccine trial samples in order to enhance reproducibility in the performance of the assay. An important component of the procedure is the cell staining with CFSE. Note that CFSE can be toxic to cells and the appropriate titer to use should be chosen not only based on staining intensity but also on potential toxicity that is only detectable after several days in culture.

Often data are reported as the percentage of CD4⁺ or CD8⁺ T cells that are low for CFSE, but this over-represents the starting frequency of the proliferating cells since the cell number doubles with each cell cycle, so a sample with a single cell that undergoes eight rounds of division (i.e., 256 granddaughter cells) would appear more proliferative than a sample where fifty cells each divided twice. In order to derive a more quantitative measure, it is optimal to calculate the precursor frequency based on the number of cell divisions each of the CFSE-low cells have undergone [19]. One issue with this procedure is that the typical cell division peaks, as observed for the positive control, are not usually observed for antigen-specific cells. Our approach is to determine the position of the cell division gates on the positive control and then apply those same gates to the negative control and the antigen-specific samples.

As for the ICS assay, validation experiments are useful to characterize the performance of the assay. Specificity experiments are used to determine the positivity method and/or to determine the false positive rate when applying a positivity method. As described for ICS, samples not expected to have responses are compared with samples that may have responses. Our first approach used samples from HIV-uninfected and HIV-infected individuals. However, unlike the cytokine-producing cells in the ICS assay, there were few proliferating T cells in the samples from the HIV-infected individuals in our cohort. Even when we tested PBMC from HIV-infected long-term non-progressors, we mainly detected proliferating CD8⁺ T cells, but few proliferating CD4⁺ T cells. This allowed us to develop positivity thresholds only for CD8⁺ T cells. For developing a positivity method for CD4⁺ T cells, we used PBMC samples from a clinical trial of a candidate vaccine known to induce a CD4⁺ T-cell response by ICS. Vaccine recipients post-vaccination were compared to a combination of placebo recipients and vaccine recipients at baseline before vaccination. As for the ICS assay, we used a Fisher's exact test for the comparison between the calculated precursor cells for the antigen-stimulated and non-stimulated cells for each sample and empirically chose the positivity threshold to maintain the false-positive rate below 5%.

The variability of the CFSE assay was generally higher than the ICS assay. For higher magnitude responses as measured to the anti-CD3/CD28 positive control, intra-assay replicates had the lowest variability (generally under 15% CV), and the variability for the

same sample examined over three days was somewhat higher, but remained under 30%. For lower magnitude responses to CMV, the variability was higher, although mainly below 30%. Interestingly, as the CFSE assay has been used over several months, the CV for the same experimental control sample in response to CMV is higher than the inter-day CV observed over the shorter-term performance of the validation testing (37% for CD4+ and 30% for CD8+). This emphasizes the need for “post-validation” monitoring in order to characterize the actual performance of an assay over longer periods of time and also for monitoring trends in assay performance. An experimental control sample (e.g., a cryopreserved leukapheresis sample) examined in each experiment is the optimal type of control for continual monitoring of assay performance.

5. Phenotyping

5.1 Phenotyping information in combination with functional ability can be obtained in the ICS assay

Polychromatic flow cytometry allows for the simultaneous measurement of multiple functional markers as well as markers that define phenotypic characteristics of the responding cells. Often, memory classification markers are included in the ICS assay. Several such markers have been described, but CCR7, CD45RA (or alternatively CD45RO), CD27 and CD28 are most commonly used [20]. There are a few practical considerations when including memory markers in an ICS assay. One is the performance of the particular fluorescent-conjugated monoclonal antibody in an assay that requires fixation and permeabilization. We have observed that some reagents are less effective when the cells are fixed and permeabilized. In some cases, this is true even when the cells are stained prior to the fixation/permeabilization. In those cases, a different fix and permeabilization method can be tested. Sometimes, an alternate marker may need to be examined. For example, we have found CD45RO to be problematic when using some permeabilization methods and use CD45RA instead.

5.2 Phenotyping analyses of PBMC without a mechanism to identify antigen-specific cells such as ICS or MHC-class I/II tetramer staining is often referred to bulk phenotyping

In the context of examination of the adaptive immune response to vaccination, bulk phenotyping may not reveal many changes because it is anticipated that the antigen-specific T-cell response will be a small fraction of total T cells. Markers of activation can be included and this may serve as an alternate method for identification of vaccine-induced T cells, however, these cells are not necessarily specific for the vaccine due to bystander activation and due to a background level of activation that will vary between individuals and likely between geographic locations due to differing exposure to pathogens. CD38 and HLA-DR are two markers often used in combination to identify activated cells. Recently, Ki67 in combination with BcL-2 has been used in studies where multiple blood draws allowed for a description of the kinetics of responses to vaccination with yellow fever, vaccinia and candidate HIV vaccines [1, 21]. Thus, activation markers may be useful for describing the acute vaccine response, but will likely not identify the memory response.

Unlike the study of the adaptive immune response, bulk phenotyping may be very useful in describing changes among innate immune cells that occur within hours to days after vaccination. This may be an important measure when evaluating different adjuvant formulations and when searching for early measures that predict the later adaptive response. In one study examining responses to yellow fever vaccination, expression of the activation marker CD86 was measured on several types of antigen presenting cells at days 0, 1, 3, 7 and 21 (Supplementary Figure 1 in reference [22]). These included myeloid and plasmacytoid dendritic cells, monocytes and CD16+ inflammatory monocytes. Expression

was increased at days 3 and 7, although these changes were not predictive of the CD8⁺ T-cell response. Within our HVTN program, we are designing clinical trials that include early blood draws to enable enumeration of various innate cell types. For these studies, whole blood is stained and counting beads are included so that absolute cell concentrations can be calculated. Preliminary results from one recombinant adenovirus serotype 5 (rAd5) trial detect changes in several immune cell types as early as 1 day post vaccination (data not shown).

6. Sorting antigen-specific T and B cells for genomic/transcriptional analysis

6.1 A new technology has enabled cloning and further functional analyses of antibodies from individual B cells specific for the HIV Envelope

The Env-specific B cells are identified and sorted as single cells using fluorescently-labeled gp140 oligomers that bind to the B cells. One study sorted cells from six HIV-infected individuals who had broadly neutralizing antibodies in serum [23]. This study found that the neutralizing activities were due to multiple clonal responses directed against several gp120 epitopes. Another research group used a similar sorting procedure with structurally-engineered Env probes designed to isolate B cells with specificity to the Env CD4 binding site [24]. Three antibodies (VRC01, VRC02, VRC03) were identified that had broad neutralizing activity.

An alternate method for identification and isolation of antigen-specific B cells is to search for the plasmablasts that transiently appear in circulation shortly after infection or vaccination [25]. These can be identified by surface staining as CD19⁺CD20⁺CD27^{br}CD38^{br}. Both of these methods are now being used to isolate antigen-specific B cells from vaccine recipients, and to clone the antibodies encoded by individual B cells [26, 27].

6.2 Systems biology is now used more commonly to examine immune responses to infection and vaccination [22, 28]

A common approach is to measure changes in gene expression in response to vaccination. This can be performed in whole blood or in PBMC, either directly ex vivo or following ex vivo stimulation with antigen-specific stimulation. In these bulk analyses, it is not possible to determine the cell type-specific gene expression. Flow cytometry can be used to increase the specificity of these studies by first sorting cell types of interest. In our HVTN program, in addition to enumerating various innate cell types of interest soon after vaccination, we are sorting several of these cell types and then measuring gene expression using gene array platforms.

Cells of the T-cell adaptive immune response, in particular the vaccine-induced T cells, can also be more specifically examined by sorting. Although ICS is the most common method for identification of vaccine-induced T cells, this is not optimal for sorting and subsequent transcriptional analysis due to the requirement for fixation. Alternate methods for identification of antigen-specific T cells include MHC-class I or II tetramer staining, or staining for markers that are up-regulated on the T cells following ex vivo culture with a specific antigen. Tetramer staining works well for CD8⁺ T cells, but is limited by the availability of appropriate epitope/MHC tetramers. Up-regulation of CD137 has been used to sort both CD4⁺ and CD8⁺ T cells [29]. Expression of CD40L can be used to sort CD4⁺ T cells [14]. Individual T cells or varying numbers of specific T cells can be sorted, and phenotyping markers can additionally be used to sort different classes of cells such as based upon memory marker expression or other parameters of interest.

7. Examination of tissue samples other than blood

Blood is the easiest tissue to sample and is therefore most commonly collected in vaccine trials. However, for most pathogens, it is protection at mucosal sites that must be induced by vaccination. Therefore, it would be optimal to assess the ability of vaccines to induce immune responses at relevant mucosal surfaces. It is possible to obtain samples for flow cytometric analysis from selected mucosal sites. Cervical cytobrush in women and semen in men provide minimally invasive access to mucosal cells in the genital tract. For cytobrush samples, we generally recover less than 100,000 CD3+ T cells and therefore only perform a single phenotyping assay, although T cells can be expanded for functional assays [30, 31]. For semen, the T-cell yield is variable, and for some samples we can successfully perform functional assays (ICS or IFN- γ ELISpot) to look for vaccine-induced T cells (unpublished). In the gastrointestinal tract, endothelial biopsies can be obtained from the rectum through anoscopy or from the sigmoid colon through sigmoidoscopy. Methods have been established to process these samples for analysis by flow cytometry for vaccine clinical trials [32].

8. Conclusions

Flow cytometry is commonly used to assess immunogenicity in clinical trials of newer vaccines where T cells are expected to be a major component of efficacy. Intracellular cytokine staining (ICS) enumerates vaccine-induced T cells and measures multiple functions. The assay has been standardized and validated and is fully implemented as an endpoint assay in several vaccine trials networks. Other flow-based methods are less standardized but provide alternate information about the immunogenicity of vaccine candidates and this information is potentially useful in the quest to identify a correlate of protection. Although a flow cytometric-based correlate of vaccine efficacy has yet to be identified, this may occur in the near future as several newer vaccine candidates have recently been tested or are entering into larger phase efficacy testing. Results from these trials may reveal a correlate that will help focus the design of the flow cytometric assays that will be used for future evaluation of vaccines.

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Abbreviations

HIV	human immunodeficiency virus
TB	tuberculosis
HVTN	HIV Vaccine Trials Network
ICS	intracellular cytokine staining

Highlights

Flow cytometry is used to monitor next generation HIV, TB and malaria vaccine trials

Intracellular cytokine staining is a primary assay to measure vaccine-induced T cells

Changes in innate immune cells are monitored by phenotyping assays

Systems analyses are performed on sorted vaccine-specific T and B cells

Mucosal tissue samples can be examined using flow cytometry

Figure 1.

Example of the staining profile for a newly developed ICS assay. Cells have been gated as live, CD3+ scatter-gated lymphocytes, and then as CD4+ (top row) and CD8+ T cells (bottom row). Previously cryopreserved PBMC from a healthy adult donor were stimulated with SEB for six hours. Standard methods were used for intracellular staining for the markers shown. The gates and frequencies show the marginal responses for each of the markers.

Figure 2.

Four-function polyfunctionality analysis for CMV and SEB responses. Previously cryopreserved PBMC from 48 healthy adult donors were stimulated with CMV and SEB for six hours and stained for the ICS assay shown in Figure 1. Samples with a positive response for any of the four functions were included in the analysis (n=48 for SEB, n=26 for CD4+ CMV, n=24 for CD8+ CMV). The pie slices indicate the average proportion of the response producing 1, 2, 3, or 4 functions (color key at right). Each arc indicates one of the functions (color key at right). The graphs below the pie charts show the individual functional combinations as a proportion of the total responding cells for CMV (blue) and SEB (red). The boxes show the interquartile range and the median. This analysis was performed using the SPICE software [15] using a threshold of 0.03%. The lower graphs were created using JMP software (SAS Institute, Cary, NC).

Figure 3.

Assessment of accuracy by comparing the newly developed ICS assay with the current ICS assay. For this experiment, the current HVTN assay is considered the reference assay and is shown on the x-axis. The new ICS assay is the assay shown in Figure 1, and is shown on the y-axis. The assays were performed on the same PBMC samples from 30 healthy adult donors. The percentages of CD4+ and CD8+ T cells responding to CMV pp65 peptide pool stimulation are shown in A and to SEB stimulation in B. The left column shows percentage of cells producing IFN- γ and/or IL-2 and the remaining columns show the marginal responses for IFN- γ , IL-2 and TNF- α . A standard least squares line fit was performed and the R^2 and slope are shown in each graph.

Table 1

Flow cytometric assays used in vaccine clinical trials.

Assay	Purpose	Markers	Measure
Assays requiring ex vivo antigen-specific stimulation			
ICS	Cytokines	IFN- γ , IL-2, TNF- α	Immunogenicity, polyfunctionality
	Cytotoxic potential	CD107a, granzymes, perforin	
	B-cell help	CD40L (CD154)	
	Memory profile	CD45RA, CCR7, CD27, CD28	Memory profile of vaccine-induced T cells
CFSE proliferation	Proliferation	CFSE	Immunogenicity
Assays performed directly ex vivo			
Phenotyping	Activation markers	HLA-DR, CD38, Ki-67, BcL-2	Kinetics of immune response
	Innate cell markers	Multiple markers for NK, DC, monocytes	Innate cell changes at early time points
	Plasmablasts (transiently in circulation at 1 to 2 weeks)	CD3, CD19, CD20, CD27, CD38	Plasmablast frequency
	Cell types of interest	Various markers	Changes in bulk cell populations
Cell sorting for further analyses			
Cell sorting for genomic/transcriptional analysis	Antigen-specific T-cell isolation	CD137 ^a , CD40L ^a , MHC class I or class II tetramers	Gene expression
	Cell types of interest	E.g., innate cell types	Cell type-specific gene expression
	Antigen-specific B-cell isolation	Plasmablast staining, antigen labeling	Antibody cloning

^a requires ex vivo antigen-specific stimulation

Table 2

Assessment of validation parameters for the ICS assay.

Parameter	Short definition ^a	Experimental plan ^b
Accuracy	Since comparison to the true value is not possible, alternate is comparison to reference assay	Compare responses to CMV and SEB for new and reference assays
Precision ^c	Variability of repeated measurements	
Repeatability	Variability of intra-assay replicates	CMV responses to three PBMC samples measured in triplicate (repeatability) performed by three technicians on one day and by one technician over three days
Intermediate Precision	Within-laboratory variations such as inter-day, inter-operator	
Specificity	Ability to unequivocally detect an analyte (e.g., antigen-specific T cell) amongst a mixture of other components	Evaluate false-positive response rate to HIV peptide pools in PBMC from HIV-uninfected individuals
Limit of Detection (LOD)	Lowest amount that can be detected	LOD varies between samples since background is variable; no definitive experiment
Limit of Quantitation (LOQ)	Lowest amount that can be quantified with suitable precision and accuracy	Assess CV of replicates at each dilution of linearity experiment; dilution at which CV remains below the pre-determined threshold is lower limit ^d
Linearity	Proportionality of test results to amount of analyte	Create serial dilutions of CMV-stimulated PBMC into unstimulated autologous PBMC
Range	Range over which assay has acceptable precision, accuracy and linearity	Defined based on linearity and quantitation limit; typically upper end of range is not defined for ICS
Robustness	Ability of assay to remain unaffected by various method parameters (sometimes undefined) expected to vary during normal usage	Selected specific variables may be assessed (e.g., lots of peptides and antibodies, incubation times)

^a Refer to the ICH Q2(R1) document for the full definition (<http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html>)

^b Example of experimental plan used for our validation of the ICS assay for use in HIV vaccine trials

^c Reproducibility is a third precision parameter referring to precision between laboratories and only needs to be assessed if the assay will be performed in more than one laboratory.

^d Quantitation limit varies depending on the type of precision examined. The plan described here evaluates intra-assay variability. Experiments to assess inter-day and inter-operator variability for the dilution series can be used to evaluate the limit for those types of precision.