Application and Advantages of ELISPOT

Differences between ELISPOT assays and other approaches for measuring antigen-specific T cell immunity

Every naïve T cell expresses a unique T cell receptor (TCR) which is specific for a single antigen. In order to be able to recognize potential infinite numbers of microbial antigens and at the same time to distinguish them from self antigens, T cells system relies on an close to infinite number (~10^{12}) of different possible T cell specificities.

Subsequently, the frequencies of T cells recognizing individual antigens are very low. As a result of the clonal expansion associated with infections, the frequency of antigen-specific effector cells can transiently rise to as high as 1:100, but these frequencies typically settle in the range of 1:10,000 for effector memory or memory cells. The frequencies of specific T cells in blood after immunizations with subunit vaccines (proteins/peptides), environmental sensitization and in long term anti-microbial immunity rarely exceed the 1:10,000 thresholds.

Therefore, techniques that measure T cell immunity need to be sensitive enough to reliably detect such rare cells. In addition to measuring the frequencies of antigen specific T cells, which delineates the magnitude of immunity, it is essential to understand the functions of these T cells. The quality of T cell immunity depends on whether theses cells are Th1 or Th2 (Tc1/Tc2) type cells and whether they are capable of direct killing. Sophisticated assays therefore will also provide information on the effector lineage of the antigen-specific T cells.

Optimized ELISPOT assays measure the magnitude and the quality of T cell immunity at single cell resolution by detecting individual events of antigen-specific T cells that engage in secretion of cytokines and effector molecules such as granzyme B and/or perforin. The following is a comparison of the performance and utility of ELISPOT assays with other T cell diagnostic techniques.
Frequency measurements by single cell assays like ELISPOT in comparison to bulk supernatant measurements

ELISPOT measures the frequencies of antigen-specific T cells within freshly isolated cell material such as peripheral blood mononuclear cells (PBMC). The same applies for tetramers/pentamers and intracytoplasmic cytokine staining (ICS), which are single cell assays that count the numbers of antigen specific T cells and thereby providing accurate information about the clonal sizes, which translates into the magnitude of antigen-specific T cell immunity. Defining frequencies of the specific T cells is one of the primary goals of ex vivo T cells diagnostic.

On the other hand cytokine ELISA assays, cytokine bead arrays (CBA) and cytokine protein arrays (CPA) measure the bulk cytokine that the activated T cells release into the antigen-stimulated culture supernatant. These assays have the following fundamental disadvantages relative to ELISPOT.

Sensitivity: Cytokine measurements on culture supernatants are at least 200 times less sensitive in detecting low frequency antigen specific T cells than is ELISPOT. The sensitivity of ELISPOT assays was further increased dramatically by the introduction of PVDF membranes and the use of PVDF membranes has been, along with computerized image analysis, the major step in making ELISPOT the robust, high resolution assay that it is today.

The reason for ELISPOT’s increased sensitivity in comparison with ELISA-type assays is that in ELISPOT assays the cytokine secreting cell directly sits on the membrane and the cytokine is captured around the secreting cell as it is released where the local concentration of the cytokine is high. In ELISPOT assays, the cytokine is captured before it becomes diluted in to the supernatant, captured from the supernatant by (abundant) cytokine receptor bearing bystander cells and before it is degraded by enzymatic cleavage. ELISA, CBA and CPA detect cytokine in the supernatant, after dilution, absorption and degradation has already occurred and thereby explaining the ELISPOT assay’s much higher sensitivity when it comes to detecting low frequency cytokine secreting cells.

T cells specific for recall antigens such as Tetanus Toxoid, Candida, Measles, Mumps, Flu-, Cytomegaly and Epstein Barr Virus typically occur in the low frequency range below 1:10,000 antigen-specific T cells in fresh PBMC. Also T cells that recognize HIV and Hepatitis C peptides are commonly less than 1:10,000 in freshly isolated PBMC. Therefore, a T cell assay’s ability to reliably detect the low frequency antigen specific T cells is critical for that assay’s suitability for ex vivo T cell diagnostic. The ELISPOT assay has in principle no inherent lowest detection limit and can be configured to measure as few as 1 in 100,000,000 cytokine producing cells.

Per cell cytokine productivity: For T cell diagnostic it is critical to know how many antigen-specific T cells are present in the test cell population. In the ELISPOT assay the numbers of spots per well provides this information and in addition the size of the spots reflects how much cytokine the individual T cells produce. Cytokine protein measurements done on culture supernatants (ELISA, CBA, CPA) or cytokine mRNA determinations (RT-PCR) do not account for productivity per individual cell and hence do not permit to distinguish whether few cells produce much cytokine or many cells produce little cytokine. This distinction however can be critical for the interpretation of the test results, as illustrated by three following...
examples.

When PBMC of subjects undergoing acute HIV infection are tested by ELISPOT, typically recall antigens induce IFN gamma spots in numbers comparable to healthy donors, but the spot size can be dramatically reduced to as little as 0.1% of that of the healthy donors. This observation clearly shows that the numbers of the recall antigen specific T cells is largely unchanged in these patients, instead T cell functionality, as expressed in the per cell cytokine productivity, is severely impaired. Direct or indirect bulk cytokine measurements done on supernatants (e.g. with ELISA, CBA, CPA, RT-PCR) only reveal the reduced net cytokine production, and those data are likely to be misinterpreted as reduced antigen-specific T cell frequencies.

The amount of cytokine a T cell produces depends on the extent of T cell stimulation. At a given antigen dose, high avidity T cells produce considerably more cytokine than low avidity cells do. At the same time cytokine productivity is affected by the time period that has elapsed since the T cell’s last antigen encounter (6). CD8 cells produce less cytokine on a per cell basis than CD4 cells (P.V. Lehmann, manuscript submitted). Moreover, in assays that measure per cell productivity, ELISPOT assays and ICS assays alike, it has been shown that the per cell cytokine productivity of T cells is highly variable over a wide range. Therefore, measurements of net cytokine in the supernatants provide rather inaccurate estimates of clonal sizes. Few high avidity CD4 cells can produce more cytokine than hundreds of low avidity CD8 cells. Facilitating ELISPOT assays the accurate clonal sizes along with the per cell productivity can be revealed.

Most cytokines are not only produced by T cells. However, per cell productivity of antigen stimulated T cells is orders of magnitude higher than that of the non-antigen specific bystander cells of the innate immune system (8). Therefore, morphometric analysis or spot size gating of ELISPOT assays is permitting to clearly distinguish between relevant T cell derived cytokine spots and non relevant bystander responses. This allows the to outline the differences in clonal size of T cells that mediate long term immunity versus the short term bystander reactions of the innate immune system, that may or may not have immune diagnostic relevance.

Assays that measure cytokine in bulk culture supernatants can not provide information on the cellular source of the cytokine. In ELISPOT assays very few antigen-specific T cells can be readily detected among thousands of cells of the innate immune system secreting the cytokine with low per cell productivity based on the spot size, with large spots, representing the signal and small spots, representing the background noise. In bulk culture supernatant assays this information is lost because the cytokine contribution of the T cells is diluted by the background activity resulting in the disappearance or flattening of the signal.

Resolution: Because ELISPOT assays detect individual cytokine secreting cells, the data obtained in replicate wells can be subject to stringent statistical analysis. This provides clear-cut results even in low signal to noise scenarios. For example in an assay done in triplicates spots counts in the negative control wells are 2, 3, and 1, while spot counts in the antigen containing wells are 5, 6, and 5. Despite the antigen-induced signal being weak, statistical analysis of the data yields a p value of 0.007 showing that the difference is highly significant, which translates into the conclusion that antigen-specific T cells have
been induced. Because measurements that are done on supernatants of bulk cell populations do not
distinguish between T cell-derived and non-T cell-derived cytokine, they neither have the resolution to
detect such subtle differences in order to identify rare T cells nor do they permit stringent statistical
analysis of replicate wells.

ELISPOT assays need fewer cells, therefore working with multiple replicate wells does not add much labor
compared to testing single wells. The cost of per well determination is a fraction of a CBA or a CPA
assay. ELISPOT assays are typically done in replicate wells and thorough statistical analysis is an integral
part of ELISPOT’s high resolution ex vivo measurement of the frequency of antigen-specific T cells.

This leads to the conclusion that assays that do not measure cytokine production by individual cells but
measure bulk cytokine amounts provide lower resolution and sensitivity relative to single cell resolution
assays. Because antigen-specific T cells typically occur in low frequencies and reasons listed previously,
these bulk cytokine assays have limited value for monitoring antigen-specific T cells directly ex vivo.
However, CBA and CPA are powerful techniques for measuring multiple cytokines simultaneously when
cytokine signals are strong, e.g. after mitogen or polyclonal stimulation. Hence they have their accepted
advocacy in certain questions and applications.

The primary application of ELISPOT assays is monitoring low frequency antigen-specific T cells directly ex
vivo with the option of high throughput screening capability. Therefore, in the following we will focus on
alternative single cell resolution techniques to ELISPOT.

**Characteristics of ELISPOT versus use of Tetramer/Pentamer approaches**

*Tetramers and pentamers* are multimerized MHC molecules. When loaded with a specific peptide, they
represent the T cell receptor (TCR) ligand for peptide-specific T cells. Specific T cells can be stained with
tetramers/pentamers and subsequently detected by flow cytometry. While tetramers are powerful basic
research tools, their value for human T cell diagnostic might be limited, for the subsequent reasons.

*MHC polymorphism and polygenism:* Because the human population is out bred different individuals
expresses typically two different HLA-alleles at each class I and class II locus. Tetramer/pentamer studies are
only meaningful for high resolution HLA-typed individuals with all alleles known. Despite this, for practicality
and feasibility reasons common limited approaches facilitating tetramers/pentamers restrict the study to
individuals that express a common allele such as HLA-A2. However, this shortcut is not reasonable for most
vaccine trials or infectious and immune mediated diseases, because it would exclude all non-HLA-A2 positive
individuals from the study. Even for A-2 positive individuals studied, the immune diagnostic information
gained would be incomplete and highly selective.

Such A-2 positive individuals are likely to be heterozygotic for the HLA-A locus, expressing an additional HLA-
A allele that is equally important for restricting antigen specific CD8 cells. In addition these A-2 positive
individuals will express HLA-B and C molecules, with typically two different allelic products for each locus.
Each of these six alleles can be equally important for restricting antigen specific CD8 cells as the A2 locus.
Comprehensive tetramer/pentamer analysis would therefore require complete HLA-typing of every study
subject and customization of tetramer/pentamers for the respective alleles expressed at each locus of the

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individuals in order to account for the MHC polymorphism and polygenism in surveyed population. The resulting effort and associated costs make this approach unconceivable for a clinical trial or study. But even if undertaken, it would be just the first step leading to an even more challenging issue, the choice of the peptide-antigen.

Multitude of peptide antigens: T cells recognize short peptide sequences of the antigen located in the peptide-binding groove of MHC molecules. Occasionally, there is immune dominance of a peptide, meaning a single short peptide of a complex antigen is prevalently recognized by T cells in the context of an HLA allele. In this case, this single peptide can be selected for the tetramer/pentamer to detect peptide specific T cells. However, in many infections there is no immune dominance and the T cells recognize a multitude of different peptides distributed over the full length of proteins from the infectious agent. Similarly diverse peptide targets are recognized in autoimmune diseases.

In such cases, it would be misleading and prohibitive to select a single or few peptides to obtain a comprehensive assessment of frequencies for antigen-specific T cells.

Since each tetramer/pentamer can be loaded only with a single peptide, comprehensive immune monitoring would not only require that the tetramers are customized to match the HLA-type of each test subject, but in addition each of these HLA molecules would need to be loaded with hundreds of peptides to generate a comprehensive panel of tetramers/pentamers. This too would represent an effort that for all practical reasons is inconceivable.

In ELISPOT assays and ICS this limitation is readily overcome because these assays rely on the HLA molecules expressed by the test cells themselves. In addition extensive peptide libraries can be tested either as peptide pools or individually in high throughput screenings.

An apparent strength of tetramers/pentamers is that the antigen specific cells can be counter stained to identify additional cell surface markers expressed by these cells. However, the usefulness of such information might not be as straightforward as it seems at the first glance. Since class I tetramers/pentamers stain CD8 cells, counterstaining for CD4 and CD8 is merely a control.

Moreover, because cross-linking of the TCR by the tetramer/pentamer represents a non-physiological signal that activates the T cell to die (12), studies of activation markers on specific T cells tell little about the phenotype of these T cells in vivo or about their phenotype following more physiologic activation. This non-physiological activation leading to cell death might also be one of the reasons why tetramers/pentamers were found to be suitable to saturate and count specific cells, but have been found unsuitable to delineate T cell effector functions.

In addition T cells become frequently non-functional in states of chronic immune stimulation, such as infections and autoimmune disease. Such T cells survive and re-circulate in the body, but they display a TCR-zeta chain signaling defect effectively preventing them from becoming activated upon antigen encounter. Such non-functional T cells will be stained by tetramers/pentamers and correctly identified as antigen-specific T cells, but it would be misleading to interpret them as cells contributing to host defense.
The critical information needed is the number of functional T cells and their actual effector function e.g. Th1/Th2 or Tc1/Tc2 respectively. However these questions can not be answered satisfactory by utilizing tetramers/pentamers alone. Functional assays, such as ELISPOT and ICS on the other hand are capable of providing exactly this information.
ELISPOT in comparison to ICS techniques

ELISPOT and ICS both follow similar principles. The antigen specific T cells become activated by antigens presented by the APC present in the sample, unlike with tetramers/pentamers, these APC display all the relevant HLA-molecules of an individual. Moreover, other then with tetramers/pentamers, high numbers of peptides can be tested either in pools or individually and permit systematic coverage of the different antigen determinants presented by the different HLA molecules expressed in an individual or trial group. Complex antigens and even entire microorganisms can be tested, because antigen processing and presentation by the APC generates the peptides for HLA-restricted antigen recognition. Both ELISPOT and ICS are single cell assays providing information on frequencies meaning the magnitude, and effector function translating into the quality of T cell immunity. However there are major differences between them, which define their usefulness for answering different questions.

ICS measures intracellular cytokine, ELISPOT measures the actually released cytokine. Only the latter is biologically relevant, because cytokine production can be post-translationally regulated, meaning cytokine that is synthesized by a cell but not released, will not exert any effector function in vivo.

Several molecules of T cell-diagnostic interest, such as granzyme B and perforin are preformed and stored in granules of all activated T cells, irrespective of their antigen specificity. Therefore, during intracellular staining, all activated T cells will appear positive, but only the fraction of antigen specific T cells will release e.g. granzyme B upon antigen encounter, which can be detected by ELISPOT. In order to detect secreted cytokine with ICS the cells need to be treated with a secretion inhibitor, while in ELISPOT pharmacologically un-manipulated cells are tested.

The cells need to be killed for detection with ICS, while they survive ELISPOT assays unaffected the assay. Therefore cells can be readily propagated from ELISPOT assays for subsequent testing in other platforms, including ICS, or can be frozen for re-testing. If needed, ELISPOT assays permit serial testing of the very same cells in different assay formats. This optional maximization of sample resources can be critical when it comes to clinical trials where the numbers of PBMC available for testing is frequently a limiting factor.

In ICS cells are killed at a supposed ideal time point and the intracellular cytokine content is measured. In ELISPOT assays the cytokine is continuously captured during the entire incubation time around the secreting cell, leaving the characteristic spots behind long after the secretion already stopped and the cells are transferred or washed away. This basic feature makes the ELISPOT assay largely independent of secretion kinetics of individual cells. This is an important feature of ELISPOT, because the kinetics of cytokine secretion by T cells is unsynchronized even in the case when a single T cell clone is tested on a clonal population of APC (6). This principle of non-synchronization applies even more on a potential polyclonal population of effector cells reacting to diverse population of APC.

ICS together with flow cytometric measurements excel in measuring cells that occur in relatively high frequencies at about 1:1,000. Detecting positive cells in lower frequencies than 1:1,000 requires highly trained personnel and the acquisition time needed on the flow cytometer becomes very long and cumbersome for antigen-specific T cells that regularly occur in frequency ranges of 1:10,000 to 1:1,000,000.
The ELISPOT in comparison excels in a frequency range of 1:1000 to 1:300,000 and above. ELISPOT measurements in this low frequency range do not require highly trained staff and the procedure is identical with standard assay protocols used for higher frequency events. The counting process is fast, on modern ImmunoSpot® readers 96 wells are scanned and analyzed within 5 minutes translating to a rate of about 3 seconds per sample.

ELISPOT lends itself more to high throughput testing. The initial phases of both assays are identical when it comes to setting up the activation cultures. Also the subsequent processing of the cells including washing steps, etc. require about the same amount to work. However, the actual data acquisition and analysis is much faster for ELISPOT. In typical ELISPOT assays, spots generated by 300,000 PMBC per well are analyzed from a single image with an acquisition and analysis time of about 3 seconds per 300,000 cells.

In flow cytometry individual events need to be collected one by one and in case of rare events like antigen-specific T cells, the analysis time becomes lengthy. Typically at a fast rate of 1,000 cells/sec it takes 300 seconds to run 300,000 cells in flow cytometry.

The only advantage of ICS over ELISPOT is that the cytokine positive cells can be subject to further phenotypic analysis e.g. to define the CD4 or CD8 phenotype or additional activation marker expression. However, in most cases this advantage might be more perceived than real.

All cytokine expressing T cells are activated and hence will express certain activation markers. Furthermore, the nature of antigen used for activating the T cells defines whether CD4 or CD8 cells become activated, measuring the CD4/CD8 phenotype frequently serves only control purposes. When short peptides are used, between 9 and 11 amino acid long, such peptides can bind only MHC class I molecules and not to Class II molecules, hence will activate only CD8 cells. If protein antigens are used, the natural processing pathways will result exclusively in presentation on MHC Class II molecules, and therefore activate only CD4 cells. When it is critical define whether the cytokine secreting cells are CD4 or CD8 positive, wells that scored positive in the primary ELISPOT assay can be retested subsequently by ICS or in antibody blocking experiments. Alternatively CD4 or CD8 cell depleted samples can be tested in the primary ELISPOT assay.

ICS and ELISPOT permit studies of cytokine coexpression by single cells and have identical sensitivity for the detection of coexpressing cells. The ELISPOT approach permits the measurement of the actual effector function, for example the cytolysis of target cells. In comparison there is no known molecular surface marker on CD8 cells that could be studied by flow cytometry to provide information on the cell’s cytolytic in vivo activity. ELISPOT assays can be done with cryopreserved PBMC without loss of function and provide high inter- and intra-assay reproducibility.

For all the above reasons, ELISPOT assays have become the technology of choice for human ex vivo T cell diagnostic. In recent years they developed into an indispensable tool to study infections such as HIV and Hepatitis C and for clinical trials that aim at monitoring the induction of specific T cell response after vaccinations.