Chapter 1

Unique Strengths of ELISPOT for T Cell Diagnostics

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Abstract

The T cell system plays an essential role in infections, allergic reactions, tumor and transplant rejection, as well as autoimmune diseases. It does so by the selective engagement of different antigen-specific effector cell lineages that differentially secrete cytokines and other effector molecules. These T cell subsets may or may not have cytolytic activity, can preferentially migrate to different tissues, and display variable capabilities to expand clonally. The quest of T cell immune diagnostics is to understand which specific effector function and T cell lineage is associated with a given clinical outcome, be it positive or adverse. No single assay can measure all of the relevant parameters. In this chapter, we review the unique contributions that ELISPOT assays can make toward understanding T cell-mediated immunity. ELISPOT assays have an unsurpassed sensitivity in detecting low frequency antigen-specific T cells that secrete effector molecules, including granzyme and perforin. They provide robust, highly reproducible data – even by first time users. Because ELISPOT assays require roughly tenfold less cell material than flow cytometry, ELISPOT is ideally suited for all measurements requiring parallel testing under multiple conditions. These include defining (a) T cell reactivity to individual peptides of extensive libraries, thereby establishing the fine–specificity of the response, and determinant mapping; (b) reactivity to different concentrations of the antigen in serial dilutions to measure the avidity of the T cell response; or (c) different secretory products released by T cells which define their respective effector lineage/functions. Further, because T cells survive ELISPOT assays unaffected, they can be retested for the acquisition of additional information in follow-up assays. These strengths of ELISPOT assays the weaknesses of flow cytometry-based measurements. Thus, the two assays systems compliment each other in the quest to understand T cell-mediated immunity in vivo.

Key words: ELISPOT, Flow cytometry, Intracellular cytokine staining, Tetramers, Pentamers, Multimers, Cytokine bead array, Luminex, ELISA, T cell-mediated immunity, Cellular immune response, Immune monitoring, T cell affinity, T cell avidity, Determinant mapping, Epitope mapping, High-throughput T cell testing, Multiplexing, Cytokines, Frequency measurements, Single cell analysis

1. Introduction

The ultimate goal of T cell diagnostics is to reliably and reproducibly measure those T cells which are mediators of clinical correlates of interest; for example, the specific T cell type that mediates protection

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against a certain infection, or causes transplant rejection, autoimmune disease, allergy, etc. Until recently, efforts to identify such T cells had been misled by a dichotomous concept of T cell effector functions being either Th1 (type 1) or Th2 (type 2). Thus, it was assumed that the measurements of IFN-γ producing T cells by IFN-γ ELISPOT assays would detect all pro-inflammatory T cells, including CD8 T cells that mediate cytotoxicity. As such, IFN-γ ELISPOT assays have been widely used to measure, e.g., the HIV-specific "cellular immune response."

The danger of equating CD8 T cell-mediated immunity with IFN-γ measurements was recently brought to the spotlight by a high profile HIV vaccine trial in which induction of HIV-specific IFN-γ producing T cells was detected without the induction of protective immunity (1). While a central role for T cells in controlling HIV infection has been abundantly documented, measurements of IFN-γ or other cytokines (that also had been assessed in that trial) failed to identify the protective T cell class. While we now know that T cells can differentiate into a multitude of effector lineages, each exerting unique effector functions, we still do not know which of these functions are of particular relevance for a specific condition, such as the control of HIV or other viruses. For HIV, it is tempting to speculate that the cytolytic potential of CD8 T cells rather than their cytokine production capacity is critical for controlling the virus. Cytotoxic activity of CD8 cells, however, is not necessarily associated with IFN-γ secretion. We have recently shown that immunizations with different adjuvants can induce CD8 T cells that produce IFN-γ and other cytokines (TNF-α, IL-2, and IL-17) and mediate delayed type hypersensitivity (DTH) but are noncytolytic, while immunizations with other adjuvants can induce CD8 T cells that are highly cytolytic, but do not produce IFN-γ or other cytokines (TNF-α, IL-2, and IL-17) and do not mediate DTH (2). The measurement of IFN-γ production by antigen-specific T cells does not permit to conclude whether cytolytic T cells had been induced, that, if induced, might have mediated protective immunity against HIV, and it should not matter which assay platform is utilized for the measurement of IFN-y production by T cells. It would be utterly wrong to conclude.

That the ELISPOT assay itself is unsuitable for detecting clinical correlates of HIV protection (3). The correct conclusion is that IFN-γ measurement per se (irrespective of the method used for detection) is not sufficient to reveal the protective T cell class in HIV because apparently T cell functions other than IFN-γ production are essential for controlling HIV. Measurement of cytolytic activity might have provided the sought after information which could have been done with granzyme B or perforin ELISPOT assays (4–6). Furthermore, TNF-related apoptosis-inducing ligand (TRAIL) ELISPOT assays could have revealed whether the HIV antigen-specific CD8 T cells are "helped," functional effector cells (7).

These measurements could be done only by ELISPOT assays measuring the actual secretion of these molecules.

As we start to understand more and more about the complexity of the T cell system (and we are apparently still at the beginning of the learning curve (8)), we also learn to appreciate the importance to account for this complexity in T cell diagnostics. Isolated observations within any such complex system are likely to trigger fundamentally wrong conclusions. This generally applicable wisdom has been captured in the ancient Indian metaphor "The blind men and the elephant" (see Fig. 1). As long as we do not know what the critical effector functions are for a certain clinical condition, it should be wise to attempt to measure multiple facets of T cell immunity: their production of various cytokines, cytolytic, proliferative, and migrational properties, including their abilities to control virus (1, 9).

Various assays are needed for the comprehensive measurement of different T cell functions. In flow cytometry-based measurements, cells need to be "poisoned" (Golgi inhibitors) and "killed" (permeabilized) for the detection of secretory products, as such, flow-based measurements tell us more about physical phenotypes

Fig. 1. The danger of relying on single parameter measurements. Inspired by the ancient Indian parable of "The blind men and the elephant." Graphic artist: Gabor Pesthy.

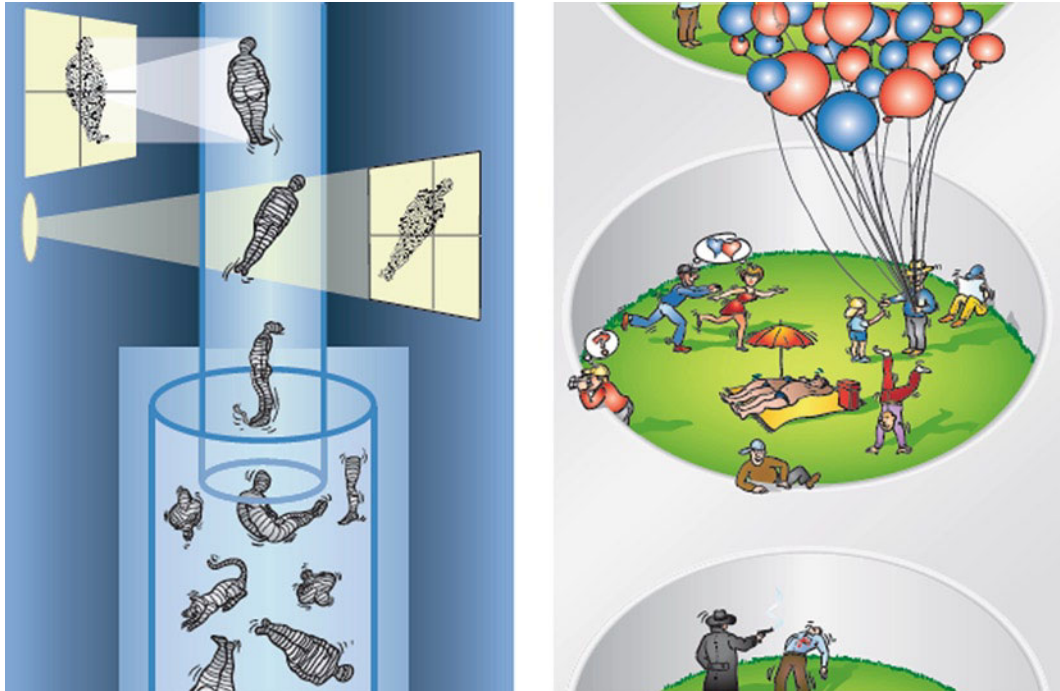

Fig. 2. Measuring T cell functions by flow cytometry vs. ELISPOT. *Left panel*: For the detection of secretory products by ICS, the cells need to be "poisoned" first with Golgi inhibitors to prevent secretion, then permeabilized and fixed/"mummified." The subsequent standard flow cytometric analysis does not make the distinction whether the analyte is indeed bound for secretion and thus is biologically active, or is retained in/on the cell. *Right panel*: In contrast, ELISPOT measures the actual secretory activity of pharmacologically untreated, living cells. The cells survive ELISPOT assays unharmed, and can be retested, phenotyped, expanded, cloned, or cryopreserved. Graphic artist: Gabor Pesthy.

of cells than their biological function (see Fig. 2). ELISPOT does not allow examining of cell surface or introcytoplasmic markers, or sorting of cells based on physical characteristics, however, unlike flow cytometry, it enables single cell measurements of the actual secretion of bioactive molecules. Cell surface marker positive cell populations can be readily obtained and tested in ELISPOT, should it be important to define the cell surface phenotype of the analyte secreting T cell.

Not only the choice of "*what*" to measure is critical, the "*how*" is equally important. Antigen-specific T cells normally occur in low frequencies (1/100,000–1,000,000) in the test material, typically peripheral blood, and detecting them can be a major challenge. Because of the low frequency of antigen-specific T cells, and because of the need to measure their function in complex assay systems, particular consideration needs to be given to the reliability and reproducibility of T cell measurements. Finally,

feasibility issues are also critical when selecting an approach for T cell immune monitoring, such as the number of cells needed and labor and cost involved in the procedure of data analysis. Among the approaches available for T cell immune monitoring, this chapter focuses on the ELISPOT technique's unique contributions to T cell diagnostics.

2. Materials

1. PBMC: Cryopreserved PBMC, high resolution HLA-typed, characterized for peptide and protein antigen reactivity.
2. CTL-CryoABC™ Kit: PBMC freezing medium for loss-free cryopreservation of PBMC without the component of serum.
3. CTL-AntiAggregate™ Wash 20×: PBMC thawing solution with anti-DNAse without the need of serum supplement.
4. CTL-Test™ Medium. ELISPOT assay medium, optimized for low background and high signal without the need to supplement with serum.
5. CEF – MHC Class I Control Peptide Pool "Plus".
6. CMV – MHC Class I Control Peptide Pool.
7. EBV – MHC Class I Control Peptide Pool.
8. CEFT – MHC class II Control Peptide Pool "Plus".
9. ImmunoSpot® Analyzer.
10. PBMC Reference Sample QC set.
11. Practical suggestions for standardized ELISPOT work can be found in Notes 1–17.

3. Methods

3.1. Unique Strengths of ELISPOT

3.1.1. ELISPOT Measures the Functionality of Single Cells via Their Secretory Activities

ELISPOT is the only technique that allows for the quantification of the actual secretory activity of individual cells. Intracellular cytokine staining (ICS) detects, as the name tells, intracellular analyte. The detection of actually secreted vs. intra cellular analyte can be critical for understanding functional properties of T cells. For example, a cytokine which is posttranslationally regulated will be detected upon de novo synthesis by ICS, or by measuring mRNA, but it will not exert biological effects unless it is actually secreted.

Similarly, some highly relevant molecules are stored in granules of T cells – perforin and granzyme being prime examples. The specific release of these molecules upon antigen activation permits to selectively detect antigen-specific cytolytic CD8 effector cells by granzyme B or perforin ELISPOT assays (4–7). In contrast, by ICS all effector memory cells stain positive irrespective of their antigen-specificity, i.e., up to 20% of all CD8 T cells will be positive.

Furthermore, several cell surface molecules important for T cell diagnostics become bioactive only after being cleaved and released from the cells – TNF family members, including TRAIL, fall in this category. ELISPOT detects only the functionally-relevant released molecules upon specific antigen activation. Flow cytometry measures the cell surface molecules, thus, leading to false positive results concerning functional information (7). Therefore, one needs to be thoughtful when interpreting what has been measured by flow cytometry: is it functionally relevant information, or is it a phenotype that possibly bears no functional significance. In all of the above situations, ELISPOT allows the investigator to detect the secreted, bioactive analytes.

3.1.2. ELISPOT Provides High Content Information on Analyte Secretion at Single Cell Resolution

With the advanced platform that recently have become available for ELISPOT data analysis, scientists now can gain information on the quantity and kinetics of analyte secretion as reflected by the size and density of the spots (see Chapter 11 and 13 on this topic). Such information can provide critical insights for T cell diagnostics beyond the frequency measurements. For example, T cells that have been activated recently in vivo, show increased per cell IFN-γ productivity, i.e., produce larger and denser IFN-γ spots (10). This observation made in the context of vaccinations might help to distinguish between long-term T cell memory and ongoing T cell activity. This distinction is especially important for the T cell diagnostic of autoimmune diseases, allergies, or chronic infections, including hepatitis and tuberculosis. Under conditions of immune suppression, T cells show a decreased per cell IFN-γ productivity rate (11). High avidity T cells produce significantly more cytokine than low avidity T cells (12). Per cell productivity information cannot be obtained by supernatant-based measurements, including ELISAs or cytokine bead arrays (CBA/Luminex). The latter assays measure only the net amount of analyte produced, without revealing how many cells produced it, and at what rate.

3.1.3. ELISPOT Is the Most Sensitive Technique for Single Cell Functional Analysis

In systematic comparisons with ELISPOT, ICS was found to be less sensitive with a detection limit around 0.02% (13). In typical ELISPOT assays, 400,000 PBMC are tested per well, in which case the detection limit is 0.00025% (1 analyte producing cell in 400,000 bystander cells) (12). ELISPOT per se is inherently without a detection limit. In regular 96-well plates, the numbers of PBMC plated and spots detected are linear in the range from

100,000 to 800,000 PBMC per well (14). Thus, by plating one million PBMC per well, the lower detection limit of ELISPOT assays can be readily extended to 0.0001%. ELISPOT assays can be performed in larger than 96-well plate format, like in 6-well plates with ten million cells per well, lowering the detection limit to 0.00001%. Practically, the number of cells available for testing is the only limiting factor when it comes to configuring ELISPOT assays for ultra low frequency measurements (but keep in mind, T cells survive ELISPOT assay intact and can be retested in a secondary ELISPOT or any other assays).

Further, when compared to measurements of soluble analyte in supernatant, e.g., by ELISA, CBA or Luminex, ELISPOT has been shown to outperform the latter by far in sensitivity (12). There are two main reasons for this. First, in ELISPOT assays, the analyte is captured around the secreting cell before it is diluted into the supernatant, degraded or captured by receptors of bystander cells. Supernatant-based assays, in contrast, need to detect the analyte after dilution, absorption, and degradation has occurred. Second, unlike in supernatant-based assays that measure net analyte produced by all cells, in ELISPOT assays the secretory activity of individual cells is detected. Due to this quantitative nature of the ELISPOT measurements, even a moderate increase in the numbers of secreting cells becomes detectable, and can provide a statistically highly significant result identifying a T cell response (see Chapters 13–15).

The ability to reliably detect rare antigen-specific T cells is at the very core of immune diagnostic. T cells each express a unique T cell receptor (TCR) which is specific for a single antigen. In order to be able to recognize the universe of antigens, the T cell system relies on an astronomical number (~10^{12}) of various T cell specificities. Subsequently, the frequencies of T cells recognizing individual antigens are very low. While the frequency of antigen-specific effector T cells can transiently rise to as high as 1:100 after acute infections, it typically settles in the range of under 1:10,000 (0.01%) in chronic infections, or after the antigen is cleared (1, 2, 5–7, 10, 11, 14, 15). This frequency is at the lower detection limit of flow cytometry-based techniques, such as ICS, but is well within the linear detection range of standard ELISPOT measurements.

3.1.4. ELISPOT Is Most Economic in Sample Utilization

In ELISPOT assays, every single cell plated is being measured – no cells are lost, as for example, in the tubing of the flow cytometer. While for flow cytometry typically one million PBMC are stained per assay condition, for ELISPOT assays one tenth that number is required (100,000 PBMC per well). Furthermore, ELISPOT assays can be performed with even fewer cells. PVDF plates have become available in the 384-well format, permitting to downscale the cell numbers 1:4, thus 25,000 PBMC per well. Recently, we published a study in which ELISPOT assays were done with a

single drop of blood obtained from the tail vein of mice: the cells obtained from each drop of blood were tested for medium background control and antigen-induced production of IFN-γ and IL-17 in a dual color ELISPOT assay (15). Moreover, when antigen-presenting cells (APC) are provided as a monolayer, even single T cells can be studied in ELISPOT assays (12). Similarly, ELISPOT assays are well suited to run functional tests on the few T cells obtained by needle biopsy.

The economic utilization of cells in ELISPOT compared with flow cytometry-based techniques is critical when either the numbers of cells available are limiting (which is the case with essentially any clinical trial, in particular for pediatric studies or with immune suppressed test subjects) or when several antigens or assay conditions need to be tested for determinant mapping, for measurements of functional affinity, or multiplexing (see below).

PBMC can be efficiently frozen without loss of function when tested in ELISPOT assays (16). For valuable samples, it is wise to freeze them in aliquots so that data can be independently reproduced, or the range of measurements/analytes extended. Freezing away aliquots, however, cuts down on the cell material available for each test, which can make PBMC limiting even from healthy donors. Here again, the efficient cell utilization of ELISPOT assays is of major advantage.

3.1.5. T Cells Survive ELISPOT Assays, Intact, and Can Be Further Utilized

In ELISPOT assays, PBMC are cultured with antigen and remain otherwise untreated. While the cells are typically discarded after an initial incubation period (the optimal duration of which is different for different analytes, Fig. 3), they can be transferred to regular tissue culture plates for later testing. In one such example, we utilized only 11 million PBMC from subjects with type 1 diabetes to study their T cell reactivity to 70 individual peptides first ex vivo, and then again after 12 days of antigen-driven in vitro expansion while measuring IFN-γ and IL-4 in a dual color assay at both time points (17). In Parallel, on day 12 ELISPOT testing was done with the cells transferred from the day 0 ELISPOT assay. We found that the results of the secondary ELISPOT testing were identical for such cells rescued from a primary ELISPOT testing, and PBMC that have been cultured in regular tissue culture plates in parallel (without initially performing an ELISPOT assay on them), further confirming that the T cells survived the primary ELISPOT assay unharmed for further utilization. While we retested them in ELISPOT, they could have been tested by flow cytometry, grown into T cell lines, or frozen down for further characterization at a later time. This “recycling” strategy can be very useful when one works with valuable clinical samples. It cannot be applied to assays in which the primary testing is done

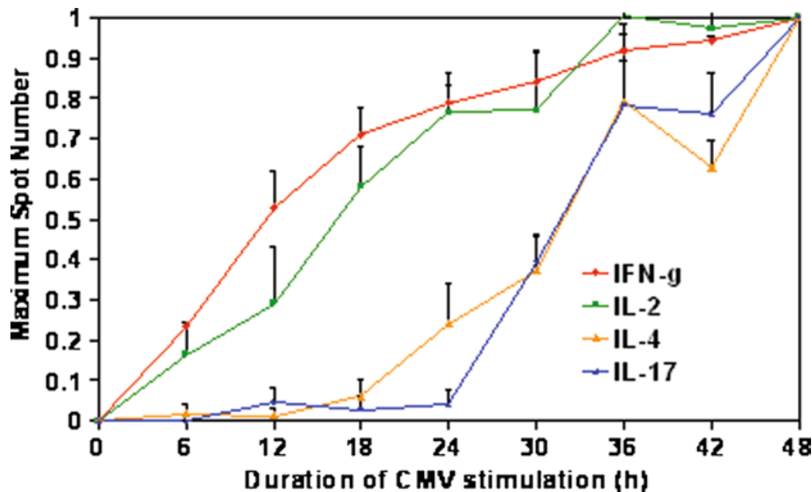

Fig. 3. The different time course of cytomegalovirus (CMV)-induced cytokine production. PBMC were plated with (or without, not show since negative) heat inactivated CMV into IL-2, -4, -5, -17, or IFN-γ coated PVDF plates, and cultured in an incubator for the time period specified before the respective detection antibodies were added, and the analyte was visualized. Since the maximal numbers of spots differed for each cytokine, the maximal number was set as one. Note, the different cytokines each have very different secretion kinetics, which needs to be accounted for when measuring these cytokines.

by flow cytometry, because in such cases, the cells need to be killed (fixed, permealized) for analysis.

Occasionally, the frequency of antigen-specific T cells is very low ex vivo, even below the detection limit of standard 96-well ELISPOT assays, where normally 100,000–500,000 PBMC are plated per well. This has been seen with some cancer vaccines or after immunizations with protein antigens. In such situations, scientists frequently rely on in vitro T cell expansion strategies: the PBMCs are first cultured with antigen plus T cell growth factors for a longer time period (typically 1–2 weeks) in the attempt to detect the antigen-reactive T cells following this expansion. However, frequencies measured after expansion do not necessarily match up with ex vivo frequencies, (17) because different T cell populations do not have uniform expansion potential. Thus, when tested after expansion, the ex vivo measurement is clouded by the proliferative capacity of the T cells. The expansion strategy is advisable only if no ex vivo signal can be obtained via an ex vivo ELISPOT assay. The two approaches can be elegantly combined, however. The fact that the T cells can be harvested without loss after an initial ex vivo ELISPOT assay makes it feasible to test a sample first ex vivo and then again, after expansion. Thus, the PBMC can be first tested in an ELISPOT assay in a 6-well membrane plate at ten million PBMC per well. After the 24-h incubation of an ex vivo ELISPOT assay, the cells can be transferred into 6-well tissue culture plates for further expansion, and after 14 days

of cell culture, can be retested in ELISPOT and/or other assays. In this way, the chances of obtaining direct ex vivo frequency measurements are maximized while still maintaining the option of learning about the frequencies after expansion via retesting. Moreover, by comparing the ex vivo frequencies with the frequencies after expansion, one can learn about the proliferative potential of the antigen-specific T cells, assessing an additional important parameter of T cell-mediated immunity, which one-time measurements by ELISPOT or flow cytometry cannot provide.

3.1.6. ELISPOT Is an Ideal Technique for High-Throughput Testing and Screening

A combination of qualities makes the ELISPOT assay the primary choice for high-throughput testing, e.g., for screening of PBMC for reactivity to a multitude of antigens/peptides (i.e., determinant mapping) or establishing antigen dose–response curves (i.e., T cell avidity measurements), or for testing a high number of donor samples (in CTL's GLP lab, we test up to 300 PBMC samples per day), or for multiplexing by ELISPOT. One important quality that enables high-throughput testing by ELISPOT is the efficient cell utilization in this assay. An example was provided above (17) where only 11 million PBMC were used to test T cell reactivity to 70 individual peptide pools, measuring two cytokines, in that case even testing the cells repeatedly. Second, the simplicity of the assay favors high-throughput testing – the cells and reagents can all be handled in 96-well format, all being pipetted in batches. (The afore mentioned experiments were performed by one single student within a few days). Third, ELISPOT data analysis, including spot recognition and gating, can all be done in a fully automated and walk-away fashion (see Chapter 13 dedicated to this issue in this volume). For the above example of testing 70 peptides individually for two cytokines per test subject, the ImmunoSpot Analyzer requires less than 2 min. These 2 min include the fully automated process of acquiring the images from the wells, analyzing them for two colors, feeding the counts to a database while also saving raw and counted images for audit trails, and automatically preparing the publication-ready graph with the results. By flow cytometry, it would take many hours of intense manual work of highly experienced personal to accomplish the same. Finally, the low cost of ELISPOT assays relative to flow cytometric measurements has also contributed to it being the method of choice for high-throughput testing and screening.

3.1.7. ELISPOT Is the Ideal Technique for Determinant Mapping

T cells recognize peptide fragments of antigens presented on MHC molecules. MHC molecules are polymorphic (there are hundreds of alleles for each locus in the human population), whereby each allele has a unique antigen-peptide binding pattern. Moreover, MHC molecules are polygenic (T cells use several class I and class II gene products as restriction elements). As a consequence, antigenic peptide recognition by T cells in different individuals is highly individualized, being dictated by MHC polymorphism/polygenism,

and other yet poorly understood rules of antigen processing and repertoire selection. This diversity is an insurmountable hurdle for comprehensive tetramer analysis.

The peptides of an antigen that are recognized in the context of an MHC molecule are called determinants (or epitopes). Due to its high-throughput capability, ELISPOT is ideally suited for determinant (epitope) mapping, whereby extensive libraries of overlapping peptides are screened (18). The validity of the ELISPOT approach for determinant mapping was first validated on inbred mice using model antigens, such as hen egg-white lysozyme (HEL) or ovalbumin (OVA), whose determinant recognition in the context of different MHC haplotypes had been well established (19). Since then, screening large peptide libraries has become a standard method for testing the fine specificity of T cell responses and has been applied to many fields of T cell diagnostics.

Here, we would like to give an illustration of the feasibility of high-throughput determinant mapping by ELISPOT – and why ELISPOT is the only technique currently available that can realistically accomplish this. The assumed task is the detection of T cell responses to an entire pathogen's proteome using a library of overlapping peptides. For HIV, for example, a total of 410 peptides of 18 amino acid length, overlapping by 10 amino acids, are sufficient to cover the entire HIV proteome. Testing of these 410 peptides on, e.g., ten donors by ELISPOT requires a simple blood draw of about 40 ml from each individual (41 million PBMC if the PBMC are tested at 100,000 cells/well) or 10 ml of blood if the test is done in the corresponding 384-well format. The plating of the cells and developing the plates can be done by a single experienced scientist (assuming the peptides had been pre-aliquoted) – and it would not even fill his/her work day. The fully automated scanning, analysis and graphing time would be 10 min per test subject, thus less than 2 h for all ten subjects. The entire test could be easily done by a single investigator in 3 days, as a part time effort. If the mapping would be done by ICS, about 400 ml blood would be needed from each donor, and the analysis time alone would take days for the ten test subjects. Supernatant measurements by ELISAs or CBA/Luminex are high-throughput assays; however, these techniques are not sensitive enough to detect the peptide-induced production of cytokine by the low frequency T cells.

3.1.8. ELISPOT Is the Ideal Technique for Measurements of Functional T Cell Avidity

Typically, in functional T cell assays, antigens/peptides are tested at a single dose. This pragmatic approach misses important information about the T cell's affinity/avidity for antigen. (Avidity is the appropriate term, since during T cell activation multiple TCRs bind to multiple MHC-peptide ligands on the APC, whereby the off-rate contributes more to T cell activation than the on-rate.) In practical terms, T cell avidity can be readily measured by titrating

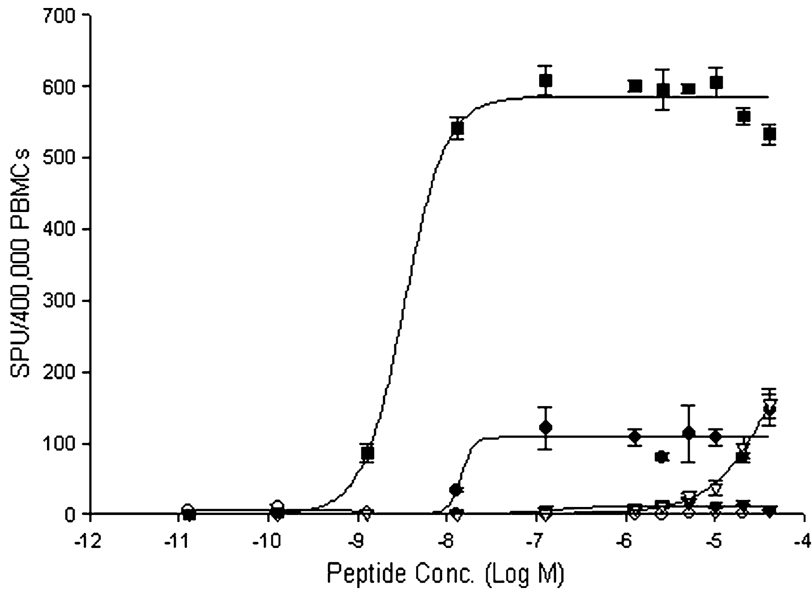

Fig. 4. The different functional avidities of antigen-reactive T cells. PBMC of an HLA-A2 positive subject were plated with different concentrations of individual A-2 restricted CEF peptides, as specified by the different symbols. A standard 24 h IFN-γ ELISPOT assay was performed. Note how far apart the maximum stimulatory concentrations of the different peptides are.

the peptide dose while measuring T cell activation (12). Figure 4 provides an example of the dose–response curves obtained when PBMC are tested for reactivity to different doses of peptides. Some peptides activate T cells only at relatively high concentration (in the 1–10 μg/ml range), other peptide can cause full-blown T cell activation at concentrations as low as 1 pg/ml. High avidity T cells will be stimulated by trace amounts of antigen on APC in vivo, and are likely to exert effector functions. In contrast, the high peptide concentrations that can lead to the stimulation of low avidity T cells in vitro may not be reached in vivo – such T cells might be "ignorant" of the antigen in vivo.

These considerations are of particular relevance for studies of autoimmunity and tumor immunity. We showed, using the example of myelin basic protein (MBP), that T cells in wild-type mice require 10,000-fold higher antigen doses to become activated, relative to T cells in MBP gene defective "shiverer" mice (20). In the wild-type mice, MBP is a "self-antigen" that causes negative selection of the high avidity MBP-specific T cell repertoire; whereas in the MBP deficient mice it is a foreign antigen encountering an unselected T cell repertoire. Due to negative selection, most tumor antigens (that are self-antigens) are recognized by low avidity T cells. Thus, when immunizing with such antigens, there is the danger of loading APC with a higher concentration of the antigen/peptide than that which is present on the tumor cell. This would

result in the induction of low avidity antigen-specific T cells. Such T cells would be detected ex vivo when a high concentration of the antigen is used for their activation. The data would truthfully show the induction of a tumor antigen-specific T cell response, but, will not reveal whether those T cells could also recognize lower concentrations of the peptide on the tumor cells, i.e., whether they could function as effector cells. Measurements of T cell avidity by titrating the peptide in the recall assay will add an extra dimension to these tests providing important information toward the latter.

T cell avidity measurements require functional assays that are highly efficient in cell utilization to permit testing of antigen in serial dilution while at the same time being sensitive enough to detect low frequency T cells. Among T cell assays, ELISPOT is the only technique that readily fulfills these requirements.

3.1.9. ELISPOT Is Readily Standardized and Validated for Immune Monitoring

Ever since T cell assays have been around, they have been surrounded by the stigma of being an art form that only few can successfully perform after a high level of specialization. Also there has been a perception that data from such assays are hard to reproduce. Indeed, the magnitude of this problem has been recently highlighted by a multicenter assay harmonization attempt (21). The same PBMC were tested in different laboratories for reactivity to the same antigen, yet the frequency measurements were more than 3,000% apart. It remains unclear to what extent this alarming variation resulted from the different level of expertise and training by the participants, the variations of protocols and reagents that were permitted to be used, subjective analysis of the data, or whether such variations are inherent to complex biological assays. Are T cell assays really so complex and their results so hard to reproduce?

The authors of this chapter helped provide evidence that ELISPOT assays can produce very reproducible data among different laboratories, even in the hands of first time users, if all assay parameters are standardized and the data analysis is performed with scientifically validated principles (14). Expertise and GLP structure were found to be not critical, only the adherence to an optimized protocol that eliminates the variables in the ELISPOT assay, and importantly the utilization of an automated, scientifically validated algorithm for user-independent analysis of the test results. Note, the same PBMC tested in this study, along with reagents, are available from CTL to anyone who wishes to reproduce this claim. The finding in this study is also particularly encouraging for anyone who would like to get started with ELISPOT.

Alerted by the high level of variation caused by the subjectivity of flow cytometry data analysis – which is still done manually – the iSBTc/SITC recently announced an "ICS Gating Panel" which invites scientists experienced in ICS to develop a gating harmonization strategy. While the field is struggling to come up with a software that is capable of automated, objective analysis of flow

cytometry data, this goal has been accomplished for ELISPOT with the ImmunoSpot platform. Scientifically validated and statistical-based analysis is used by ImmunoSpot® analyzers to define spot recognition parameters and to set gates automatically, making sure that the results are objective and user independent, hence ELISPOT data become reproducible between laboratories (see Chapter 13). Thus, as the first among T cell assays, ELISPOT has transited from an "art" form into an exact science – a technique that provides solid, reproducible measurements.

3.1.10. ELISPOT Is Well Suited for Multiplexing

Because T cells occur in many different effector classes, and because most of the time we do not know which of the effector functions are relevant, it is important to measure as many parameters as we can (see Fig. 1). Bead-based multiple analyte measurements in supernatants (CBA/Luminex) seem to be one of the ways to proceed in these efforts. However, being supernatant-based, they are most of the time not sensitive enough to reliably detect antigen-specific T cell activities that occur at low frequencies. Multiparameter flow cytometry is also an option for such measurements. However, anything more than four colors is presently an art form – even "high art" – such measurements can be reliably performed and reproduced by few researchers. Moreover, by the very nature of the measurements, flow cytometry excels in defining phenotypes of cells, not their functions. For many key functions however, such as antigen-specific killing, we have no reliable corresponding phenotypes.

Dual color ELISPOT assay has been established since a decade (22). Cytokine combinations have been defined that, when measured in the double color format, provide the same spot count for each color as the corresponding analytes measured in parallel in single color ELISPOT assay (23). Also, cytokine coexpression can be studied by dual color ELISPOT, detecting coexpressing cells with the same frequency as measured by ICS (22). Double Color ELISPOT, therefore is well suited for detecting polyfunctional T cells that coexpress cytokines. Fully-automated double color analysis software largely facilitates such studies.

Double color ELISPOT analysis can be done via the classical enzymatic approach using precipitating red and blue substrates, or by fluorescent detection (fluorospot). Both approaches provide equal sensitivity in the detection of two analytes simultaneously, and coproducers. Fluorospot becomes indispensable, however, when it comes to detecting more than two analytes. Fully-automated instrumentation and software for up to 8-color multiplexing via fluorospot analysis is already available from CTL. We believe that reliable, readily applicable and standardized 8-color fluorospot analysis will be sooner realized than 8 parameter flow cytometry with the ELISPOT-based approach having the additional advantage

of high sensitivity for the detection of low frequency cells, economy with cells, high-throughput capacity, and being a functional assay that measures biologically relevant secreted analyte.

It also should be noted that "multiplexing" T cell measurements by ELISPOT, can be readily done by running multiple single- or double-color assays in parallel, or in succession. Since ELISPOT requires only 100,000 PBMC as a standard sample size, with one million PBMC, that a standard flow cytometry sample requires, one can obtain 10 single- or ten double color ELISPOT measurements, detecting 10 or 20 analytes, respectively – a target that is hard to match by multiparameter flow cytometry. One can further increase the number of analytes measured in ELISPOT assays by testing cells in succession. For example, granzyme and perforin are released within 4 h after antigen stimulation while the production of IL-4, IL-5, or IL-17 requires a longer activation period. Thus, the cells can be tested in a granzyme/perforin assay first, and then transferred into an IL-4/5 assay, doubling the number of analytes measured with one sample of 100,000 PBMC. One can also easily combine ELISPOT assays with proliferation assays. Because the cells can be retrieved from the ELISPOT assay unaffected, they can be transferred afterward into a proliferation assay or used for measuring other functions or for identifying phenotypes. Cells treated with Golgi inhibitors, permeabilized and fixed, in contrast, will no longer provide functional information.

3.2. Concluding Remarks

Clearly, reliable measurements of several key T cell functions will be required for a better understanding of these cells' roles in diverse immune processes, and for mediating different clinical outcomes. These parameters include the type of cytokine, chemokine, and other mediators T cells produce, their cytolytic activity, migratory properties, proliferative potential, and their functional avidity. It will take the thoughtful utilization and combination of several different techniques to assess these functions. ELISPOT will continue to be the technique of choice for screening, measurements of effector functions mediated by secretory products, fine specificity, and avidity. Flow cytometry will continue to be indispensable for multiparameter phenotypic analysis. Neither of the two, however, will obviate the need for a new generation of killer assays, or migration assays. Each of these techniques excels in providing a specific type of information – and does not permit interpretations beyond what actually is being measured. Interpreting only one type of read-out inherently goes with the danger of being one of the "blind men studying the elephant." The sum of the information gained, however, can help reveal the true nature of the "beast" studied. When used to its full potential, ELISPOT will continue to make major contributions to this quest.

4. Notes

In the following, we provide some practical suggestions for ELISPOT work:

1. Blood draw: The use of heparin as anticoagulant is recommended.
2. Blood storage/shipping: Never chill blood or the PBMC! Keep at room temperature. Store in dark. If shipping in winter, add warm packs to keep at ambient temperature. Do not use cold media for Ficoll gradient separation or washing – it is better to prewarm media in a water bath to 37°C. If handled in this way, PBMC can be stored/shipped for 24 h without significant loss of CD4 or CD8 cell function (more than 90% of reactivity being retained after 24 h).
3. Media: Do not use untested serum for testing or even for washing or freezing – even brief exposure to a mitogenic or suppressive serum can ruin an assay (14). It is best to use special serum-free media that have been developed specifically for ELISPOT work for freezing, thawing, washing, and testing. Such media are available from CTL (14). Do not use PBS or similar minimal buffers for washing cells – it can ruin your assay!
4. Freezing and thawing: PBMC can be frozen without any loss in function, i.e., the frequencies of antigen-induced CD4 or CD8 cells producing IFN-γ, IL-2, IL-4, IL-5, and IL-17 are identical in freshly isolated PBMC, and after freeze–thawing (16). To achieve this result, specific protocols that are available from CTL need to be followed. One of the key factors for success is that the freezing medium and the cells need to be at room temperature when mixed, and not chilled on ice, as commonly recommended (16). Also for thawing, the cells need to be warmed up to 37°C and warm washing media needs to be added, slowly, to avoid osmotic lysis of cells. Use pretested (ideally) serum-free media for processing the cells. Detailed protocols are available from CTL.
5. Final storage temperature: After rate-controlled freezing of cells in a –80°C freezer (e.g., using Mr. Frosty cryo-freezing container, Nalgene), or sealed, plastic wrapped Styrofoam racks, transfer them to liquid nitrogen within 48 h – do not store them at –80°C after freezing, or for short- or long-term storage – they will gradually lose functionality. Also for shipping, use dry ice only for overnight shipping – ship in vapor nitrogen containers.
6. Resting of PBMC: For work with freeze–thawed PBMC, the notion has been put forth that a resting period (keeping the cells in a tissue culture incubator overnight before recounting

and plating them for the assay) would increase the spot counts without increasing the medium background, thus resulting in a higher signal to noise ratio. We have tested this many times – different members of the lab on different PBMC samples – and we could not verify a real benefit of resting. We occasionally observed a <10% increase which can be explained by the depletion of apoptotic cells in a not well-cryopreserved PBMC samples. Resting may be more beneficial if the freezing conditions have not been optimized. We recommend verifying whether resting indeed improves your results because resting prolongs the assay, adds labor, and leads to loss of precious cells materials.

7. Cell counting: Once reagents are standardized, cell counting introduces the largest variability into ELIPSOT assays. Trypan blue exclusion is not ideal for cell counting because apoptotic cells are still alive when the counting occurs: they will be counted as live cells, but they will be dead by the time the assay is performed. Ideally, dyes should be used for counting which permit the distinction between live, dead and apoptotic cells under a UV microscope, or by an automated reader (CTL's latest UV readers have three color live/dead/apoptotic cell counting functionality in addition to ELISPOT data analysis) or by flow cytometry.
8. *Counting apoptotic cells* is not only important for establishing the correct number of viable cells, but it is also an excellent indicator of the overall quality of the PBMC sample. If the cells were damaged during shipment, or freeze–thawing, it will become evident by an increase in the numbers of apoptotic cells. In contrast, dead cells frequently lyse, so they either cannot be detected or become indirectly evident by cell clumping caused by free strands of DNA that are released. In those cases, including a DNAse into the washing solution improves cell recovery.
9. Membranes: The use of PVDF plates is recommended – T cell ELISPOT assays started to perform robustly only after we introduced these plates (24).
10. *Prewetting* of membranes with ethanol. While recommended by some reagent manufactures, prewetting with ethanol is only required for monoclonal antibodies that are low in hydrophobicity (the PVDF membrane is hydrophobic). The antibodies offered by the different vendors specific for different analytes largely vary in their hydrophobicity. At CTL, we prefer to use antibodies that are hydrophobic, so they have robust performance in ELISPOT assays without prewetting. Since prewetting can cause severe membrane leakage, and prewetting adds six additional steps to your assay (prewetting itself followed by five washing steps), it is recommended to test whether prewetting indeed improves the performance of the assay in question.

11. Cell numbers plated: Three to five hundred thousand cells per well is a good starting point if the frequency of antigen-specific T cells is unknown. However, because of the linearity between cell numbers plated and spot counts in the 100,000 to 800,000 cells/well range, one can readily make adjustments as needed. Since cells of clinical samples are precious, valuable results could be obtained by testing 4–5 times more conditions using PBMC at 100,000 cells per well. On the other hand, if frequencies are low, reliable measurements may require increasing the cell number, and the number of replicates (see below).
12. Adding APC: PBMC contain abundant APC that are capable of stimulating T cells. Macrophages, B cells, and dendritic cells (DC) are similarly stimulatory – while the activation on DC is faster, and the per cell cytokine production is increased compared to B cell or macrophages, by the end of the 24-h activation period of a standard ELISPOT assay, these differences disappear (25). For a standard ELISPOT assay, adding DC as APC may not improve the results, and because of the substantial additional effort involved, it is recommended to verify whether adding DC indeed improves the results of a particular assay.
13. Serum-free media: Serum is a limited, unique biological product. The only serum which is suited for ELISPOT assays is that which has been thoroughly tested, i.e., supports the maximal induction of T cells while it does not induce an elevation of the background. The different sera used in laboratories are a prime source of substantial interlaboratory variation of ELISPOT results (14). For standardized ELISPOT testing, CTL has been the first to develop a complete serum-free media platform to freeze, wash, and test human PBMCs while achieving optimal results in ELISPOT assays. Serum-free media developed by CTL perform equally or better for ELISPOT assays than the best sera selected for T cell work (14).
14. Numbers of replicates: Because ELISPOT lends itself to high-throughput testing, and because it is efficient with cell utilization, generally assay conditions are run in triplicates. If response levels are unknown, triplicates are a good practice. If frequencies are high, single measurements are sufficient. If high accuracy is desired for frequency measurements, one should plate cells in serial dilution. Spot counts are linear between 800,000 and 100,000 PBMC per well (14). (If results are not linear in this range, the analyte is most likely not T cell derived (26).) If spot counts indicate a borderline positive result, retesting at higher cell number, up to one million per well, and/or increasing the number of replicates, can lead to more definitive conclusion.
15. ELISPOT counting and audit trails: T cell-derived spots follow, at the population level, log normal distributions. This notion

allows automatic identification (with 99% confidence) of the lower and upper "gates" for the spots that are produced by T cells, thus properly identifying cell clusters and excluding background spots. Such analysis is done automatically by the ImmunoSpot® software (see Chapter 13).

16. Evaluating ELISPOT data: The spot counts per se need to be a solid starting point (see Note 14). It is a questionable practice to determine the number of antigen-specific spots by subtracting the number of background spots from the number of spots found after addition of the antigen. With minor limitations, the T-test is suited for statistical evaluation of ELISPOT data (see Chapters 13–15).

17. PBMC reference samples: CTL offers PBMC that are high resolution HLA-typed, and their T cell reactivity to various viral peptides and protein antigens characterized with ELISPOT assays. The cytokine profile of these responses and their functional avidity is also defined. Since such PBMC have predefined reactivity types and levels, they are well suited (a) for newly establishing ELISPOT assays in a laboratory, (b) for expanding the range of analytes in a laboratory (e.g., selecting PBMC that display an antigen-specific IL-17 T cell response), (c) for testing the ELISPOT proficiency of a new lab member, (d) for being used as a reference standard in GLP or exploratory research settings to assess inter assay variations within a laboratory, or to compare interlaboratory variations, and (e) for developing assay variants with increased performance (e.g., to test whether the inclusion of co-stimulatory antibodies, resting, in vitro expansion strategies, or addition of DC enhance the assay).

18. Measuring cytokine coexpression or switching: In ELISPOT assays, the analyte is continuously captured around the secreting cell during the assay's entire duration, thereby providing an integral of analyte produced over time. Even if the secretion kinetics of different analytes is asynchronous, which frequently is the case (see Fig. 3), multicolor ELISPOT assays of several days duration will detect each analyte. Assays that rely on killing the cells at a certain time point, like mRNA or ICS measurements, provide information about that time point only. For example, since IL-17 production by T cells does not even start by 48 h after antigen stimulation, while IL-2 production is finished by 48 h, ICS or mRNA measurements done at 24 h would miss IL-17, and measurements done at 48 h would fail to detect IL-2. If ICS or mRNA assays were done at both times, IL-2 and IL-17 would be detected, but one could not tell whether T cells switch from IL-2 production to IL-17 or whether different cell lineages produce the two cytokines: even if the cells would switch, they would appear as IL-2 single positive if killed early on, and IL-17 single positive

if killed at the later timepoint. In a double color ELISPOT assay of 3-day duration, cells that do not switch will appear IL-2 or IL-17 single positive, while cells that switch will appear IL-2/IL-17 double positive. Because Golgi inhibitors are highly toxic to the cells, they cannot be used for measurements over such extended periods of time to reveal this information by ICS.

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