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Critical Components of Elispot Validation and Sample Analysis in a GLP environment

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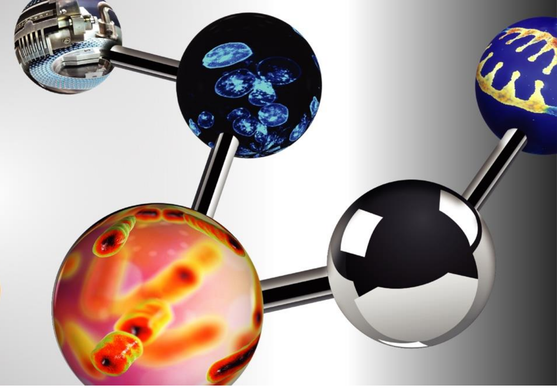

PURPOSE

Background: The Elispot (Enzyme-linked immunosorbent spot) assay provides a powerful tool to monitor the immune system in response to a variety of therapeutic agents. Originally developed to detect secretion of antigen specific antibodies from B-cells, it is now more commonly used to measure T-cell responses. Elispot quantifies antigen specific T-cell reactivity by enumerating spots corresponding to the secretion of INF-g, other cytokines, or secreted molecules such as granzyme B. The utility of Elispot as a sensitive measure of immune function has been recognized for many years. The frequent use of Elispot in clinical trials highlights its importance to a wide variety of fields such as HIV and other infectious diseases, oncology, autoimmunity, gene therapy (GT) and vaccines. More than 400 clinical trials used Elispot as a primary or secondary endpoint (2), consequently the quality of the data stemming from point of collection to statistical analysis is of paramount importance (Figure 1). Here we discuss the critical factors that must be considered for successful Elispot evaluation of immune response in clinical trials.

Guidance: Immune monitoring assays such Elispot and intracellular cytokine staining (ICS) provide unique challenges in regulation as no reference material or gold standard can be utilized, and FDA Bioanalytical Method Validation guidance is not always applicable. Recently, WRIB white papers have begun to address harmonization and validation components (1). Global harmonization efforts for Elispot application include creating optimized protocols and counting guidelines (3,4), as well as targets for precision and linearity (5), and finally response definitions (6). Previously we defined important parameters in the Elispot validation process (7). Sample collection and processing methods for PBMCs, including mechanisms of suppression of T-cell functionality, have been explored in detail by others. Although key to preserving the responsiveness of the PBMCs, this still remains an area often neglected at the validation stage.

Sample Quality: The time from blood collection to processing is a critical factor in achieving high quality Elispot data. Beyond 8 hours, significant numbers of granulocytes become activated which changes their buoyancy such that they will co-localize with PBMCs during density gradient purification. These contaminating granulocytes can inhibit T- cell responsiveness due to hydrogen peroxide release and Arginase activation and will contribute to imprecise PBMC counts and lower spot numbers as well as degraded spot formation (Figure 2), which may result in inaccurate spot counts and possibly false negative results. In the case of multicenter trials, the time to processing can vary, leading to varying degrees of granulocyte contamination. Utilizing CPT collection tubes, which remove the red blood cells and granulocytes within 2 hours at the point of collection, provide an effective way of eliminating granulocytes and their downstream effects on the assay.

Statistical Analysis: Defining response criteria appropriate to the study design is an important part of the validation. For example, in an AAV gene therapy trial evaluating preexisting T-cell responses is a vital component in the assay validation. Utilizing the determined LOD, naïve samples can be screened for potential reactivity to peptide pools corresponding to the AAV vector. Here we describe the evaluation of healthy donors for preexisting reactivity to the vector and discuss analysis of data as it applies to study samples.

METHODS

Twenty lots of PBMCs from healthy donors were purchased to provide an estimate of background reactivity to 2 pools corresponding to an AAV vector. PBMCs were thawed, then plated at 2 x 10^5/well into 96 well plates (Mabtech) containing treatments using serum free media. Serum free medium was chosen as factors in serum can affect reactivity of T-cells and may vary by lot. Treatments run in triplicate were: Pools 1 and 2 containing peptides corresponding to the AAV vector, along with medium (0.2% DMSO), and 2 positive controls (PHA and CEF). After 18 - 24 hours cells were removed and the plate developed according to the manufacturer's instructions. Spot counts were determined using a CTL S6 analyzer.

RESULTS

- Sample collection and handling is an important factor in the quality of Elispot data. Processing blood within 8 hours of collection minimizes granulocyte contamination which can lead to diminished T-cell reactivity and disrupted spot formation (Figure 2). CPT tubes are appropriate if shipping samples, particularly in the case of a multi-site study since they allow granulocyte removal at the collection site.
- Each of the 20 lots of PBMCs were analyzed twice for reactivity to Pools 1 and 2 corresponding to AAV vector. (Tables 1 and 2, Figure 3)
- The LOD (12 spots/well) was calculated by taking 3 fold the median of medium (background) wells.
- Only samples with a mean greater than LOD were assigned a positive response status, when applicable, following distribution-free resampling (DFR) testing. This non-parametric statistical test, specifically developed for Elispot data analysis, permutates spot counts of the treatment wells and the sample wells, resulting in a p-value and response assignment (0 or 1).
- The results of the screening did not find reproducible significant reactivity to Pool 1 or 2.

Figure 1. Industry sponsored studies utilizing Elispot

Figure 2. Well images of typical spots (A) and a sample contaminated with granulocytes (B)

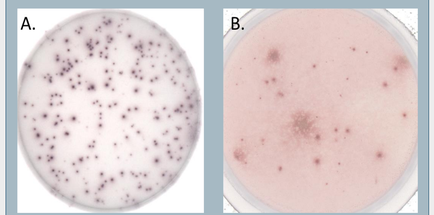

Figure 3. Plate Image showing response of 4 lots of PBMCs

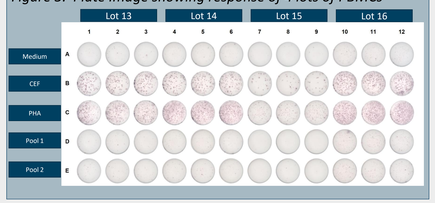

Table 3. Reference Sample treated with CEF pool

Run #	Well 1	Well 2	Well 3	Mean Spots/Well	% CV
1	92	90	111	97.7	12
2	44	48	50	47.3	6
3	60	52	44	52.0	15
4	89	107	102	99.3	9
5	62	57	56	58.3	6
6	87	76	67	76.7	13
7	80	78	79	79.0	1
8	77	79	71	75.7	6
Results (Mean)				73.3	
% CV				26	

Table 1. Results for 20 lots of PBMC treated with Medium or Peptide Pool 1
Note: Results for samples will not be considered when the mean spot number in treatment wells is below the limit of detection (12 spots/well)

PBMC Lot #	Experiment 1				Experiment 2			
	Medium Mean Spots/well	Pool 1 Mean spots/well	P Value	Response	Medium Mean Spots/well	Pool 1 Mean spots per well	P Value	Response
1	4.3	7.0	0.35	0	2.3	5.7	0.2	0
2	1.0	2.0	0.55	0	1.7	3.0	0.50	0
3	7.0	10.3	0.50	0	3.0	6.3	0.10	0
4	6.0	8.3	0.40	0	2.3	3.0	0.55	0
5	4.0	7.3	0.20	0	7.3	7.0	0.80	0
6	4.7	8.7	0.20	0	3.3	7.0	0.20	0
7	2.7	5.7	0.20	0	3.0	8.0	0.10	0
8	1.3	3.0	0.40	0	2.7	6.0	0.15	0
9	5.3	7.0	0.60	0	6.3	9.7	0.45	0
10	2.0	1.3	0.90	0	8.7	14.0	0.10	0
11	3.0	4.3	0.65	0	6.0	7.0	0.6	0
12	7.3	8.0	0.60	0	4.7	4.3	0.85	0
13	6.7	4.0	0.9	0	4.7	4.3	0.85	0
14	3.3	4.3	0.6	0	3.3	6.3	0.35	0
15	2.3	0.0	1.0	0	1.3	0.0	1.0	0
16	14.0	11.3	0.95	0	12.7	16.0	0.40	0
17	2.7	2.3	0.85	0	2.7	5.3	0.45	0
18	4.7	4.7	0.80	0	2.3	5.7	0.20	0
19	3.3	2.7	0.75	0	1.3	3.7	0.10	0
20	7.3	4.7	1.0	0	5.3	8.7	0.35	0

Table 2. Results for 20 lots of PBMC treated with Medium or Peptide Pool 2

PBMC Lot #	Experiment 1				Experiment 2			
	Medium Mean Spots/well	Pool 2 Mean spots/well	P Value	Response	Medium Mean Spots/well	Pool 2 mean spots/well	P Value	Response
1	4.3	9.7	0.30	0	2.3	9.7	0.05	1
2	1.0	2.3	0.30	0	1.7	4.0	0.20	0
3	7.0	9.3	0.50	0	3.0	3.7	0.60	0
4	6.0	7.7	0.70	0	2.3	2.3	0.65	0
5	4.0	5.0	0.55	0	7.3	8.7	0.80	0
6	4.7	8.7	0.20	0	3.3	5.3	0.60	0
7	2.7	6.3	0.15	0	3.0	10.3	0.05	1
8	1.3	7.3	0.05	1	2.7	6.3	0.15	0
9	5.3	6.0	0.60	0	6.3	9.0	0.45	0
10	2.0	2.0	0.85	0	8.7	8.7	0.85	0
11	3.0	3.0	0.90	0	6.0	11.7	0.10	0
12	7.3	8.7	0.60	0	4.7	5.0	0.75	0
13	6.7	8.0	0.70	0	4.7	3.7	0.90	0
14	3.3	6.3	0.30	0	3.3	2.3	0.85	0
15	2.3	1.7	1.0	0	1.3	2.0	0.45	0
16	14.0	18.7	0.35	0	12.7	13.0	0.85	0
17	2.7	5.0	0.30	0	2.7	7.7	0.15	0
18	4.7	7.7	0.30	0	2.3	3.3	0.55	0
19	3.3	3.7	0.70	0	1.3	2.0	0.75	0
20	7.3	6.0	1.0	0	5.3	6.3	0.7	0

CONCLUSIONS

- Elispot provides a sensitive and functional assay to assess immune function. Antigen specific T-cell responses, most commonly measured by INF-γ secretion, yield valuable information during clinical studies for decision making such as dose and formulation in the early stages of development, along with confirming cellular immune response to vaccines, as well as screening for unwanted activity against treatment vector. With recent advances in mRNA vaccines, understanding the durability of the cellular immune response compared to other modalities, will enhance understanding of mechanisms of new vaccine technologies.
- Accurate Elispot results depend on high quality PBMC samples, free of granulocyte contamination, with sample collection and handling a critical component of validation and subsequent sample analysis. This is particularly important in multi-center studies where shipping and processing times may be variable. To avoid inhibitory effects of granulocyte contamination, an 8 hour window from collection to processing is recommended, alternatively CPT tubes can be utilized for shipping overnight.
- Including a reference sample is an important step to ensure consistent results through the full duration of the study. It provides a valuable control at all steps of the assay and data analysis process (Table 3). Running a reference sample each day samples are analyzed provides trending data that is expected by regulatory bodies.
- A T-cell response to gene therapy vectors can result in reduced efficacy or safety issues, such as organ damage. Some subjects may have pre-existing response to the vector. Measuring response of naïve PBMC samples as part of the validation plan provides information on what may be expected in study samples. In this study 20 lots were screened for reactivity to 2 peptide pools corresponding to an AAV vector. For subjects with mean spot counts above the LOD, utilizing a DFR test, we found no significant response to the AAV vector. For sample analysis, this same strategy will be followed. Samples will be evaluated for preexisting reactivity (pre-dose) with subject samples grouped by donor. Each sample will be evaluated utilizing the DFR test comparing the treatment wells to medium control. If the P value is significant, a positive response will be recorded.
- The challenges of measuring a complex immune biomarker in the bioanalytical environment can be addressed by a comprehensive validation and bioanalytical study plan, which includes clear guidance for sample collection, validation components, and appropriate statistical testing carried out within the framework of a GLP environment.

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