Qualification and Use of Peptide Libraries for Clinical Trial Immunomonitoring

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Understanding cellular immune responses, and in particular T cell responses, is critical for understanding the pathogenesis of disease and for vaccine development. Interrogating T cell responses with libraries of overlapping peptides has become a standard practice. A large component of the laboratory budget can be spent on peptides and here we share some common practices for assessing the quality and specificity of peptides for use in T cell assays.

Introduction

Synthetic peptides are used for the stimulation of peripheral blood mononuclear cells (PBMC) or cells derived from mucosal and other tissues in ELISPOT, intracellular cytokine staining (ICS) and other immunological assays to assess vaccine induced or other immunological responses (1-6). The synthetic peptides are designed to precisely match the vaccine insert sequence, the pathogen or tumor of interest or can be specifically designed to capture variant sequences (7). Synthetic peptides are typically ordered as individual peptides of 9-20 amino acids (AA) in length with the most common format of 15 AA (15-mers) with overlapping sequences of 11 AA in length. They are synthesized in bulk quantities by manufacturers specializing in custom peptide synthesis. The peptides are synthesized to a desired purity and specification; at least 70% purity for research purposes and 90% purity for clinical trial applications. Each manufacturer provides information on the biochemical characterization of the peptides by stand-alone Mass Spectrometry (MS), High Performance Liquid Chromatography (HPLC) or HPLC-MS combinations. In addition, peptide content determination by amino acid analysis (AAA) provides the basis for accurate determination of final peptide concentrations.

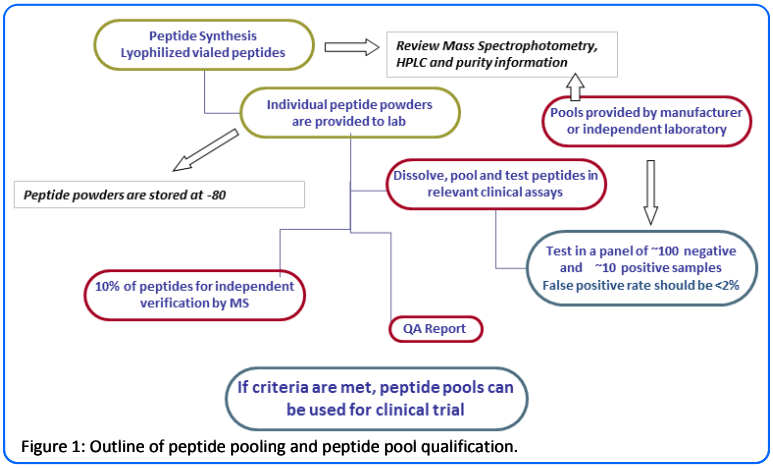


Figure 1: Outline of peptide pooling and peptide pool qualification.

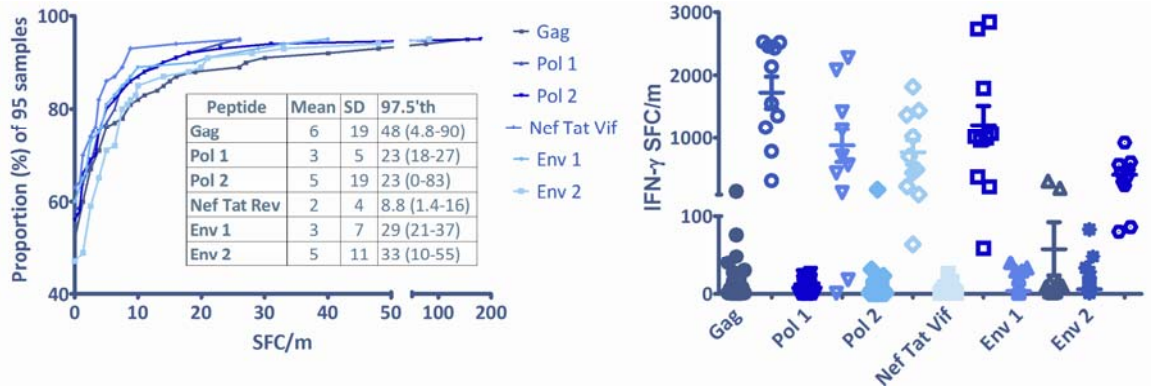
This report outlines the recommendations for defining the biological quality of peptides and for pooling individual peptides into pools of desired size for use in cellular immune response assays (8-10). False positive signals may arise from 1) impurities or contaminations of the peptides, 2) authentic biological stimulation as a result of cross-reactive T cell receptor or HLA degeneracy, 3) competition because of too many peptides per pool or peptides at a high concentration, or 4) sub-optimal length of peptides (3, 10-12). To assess whether each individual peptide in a peptide pool is capable of stimulating T cell responses would require testing of hundreds of PBMC samples to get coverage of epitopes across multiple HLA alleles. This combined with the complexity of assessing epitope specific T cell responses in sensitive and specific T cell assays would make this task virtually impossible. However, the presence of individual peptides in peptide mixtures of limited complexity can be determined by validated, high-performance LC-MS methods. In addition, definition and tracking of "marker peptides" with unique HPLC retention times and molecular weights within such pools allow GLP-compliant preparation of mixtures containing even hundreds of peptides. The lyophilized (powdered) synthetic peptides are typically dissolved in dimethyl sulfoxide (DMSO) and either stored in individual aliquots or pooled and further aliquoted. The peptide pools are then tested in ELISPOT, ICS, CFSE or other appropriate clinical immunology assays using PBMC from donors with no anticipated response to the peptide pools of interest to assess the false positive response rate and PBMC from donors who would be expected to respond to the peptide pools. In practice, it is not always easy to obtain appropriate positive and negative responders, for example in endemic malaria countries, there may be low T cell responses in exposed individuals making it difficult to separate true positives from negatives. We share here an example of the biological qualification of a set of HIV peptide pools that were synthesized and pooled by JPT Peptide Technologies.

Materials & Methods

A plan for qualifying peptides and peptide pools is outlined in **Figure 1**. Approximately 700 15-mer peptides with an 11-mer overlap were made to 90% purity representing HIV-1 Gag, Pol, Nef, Tat, Vif and Env peptides (**Figure 2 inset**). The peptides were tested against 95 HIV-seronegative and 10 HIV-seropositive frozen PBMC samples using an Interferon-gamma (IFN-γ) ELISPOT assay in a GCLP-accredited laboratory (13, 14). The PBMC from HIV seronegative individuals were representative of the populations that would be tested in vaccine clinical trials; 38 from North America, 36 from South Africa and 21 from East Africa. The PBMC viability was > 97% overall and all individuals had a PHA response. The results are expressed as background-subtracted spot forming cells / million (SFC/m) PBMC. A positive response is defined as the number of SFC/m and had to satisfy the following criteria: 1) Average number of background-subtracted spots >38 SFC/m; 2) Mean count must be >4 times mean background; 3) Mean background must be <50 SFC/m PBMC.

Results

Figure 2 left panel shows the cumulative distribution of the ELISPOT responses with the inserted table showing the basic statistics for each peptide pool. **Figure 2** right panel shows the



Peptide	Mean	SD	97.5'th
Gag	6	19	48 (4.8-90)
Pol 1	3	5	23 (18-27)
Pol 2	5	19	23 (0-83)
Nef Tat Rev	2	4	8.8 (1.4-16)
Env 1	3	7	29 (21-37)
Env 2	5	11	33 (10-55)

Figure 2: Left, cumulative distribution of ELISPOT responses in the 95 samples from HIV seronegative individuals. Table, mean and standard deviation (SD) for each peptide pool indicated as SFC/m PBMC with 97.5 percentile (80% confidence interval). Right, IFN-γ ELISPOT responses for PBMC from 95 HIV seronegative (closed symbols) and 10 HIV seropositive samples (open symbols) for each of the HIV peptide pools.

IFN-γ ELISPOT responses for PBMC from 95 HIV seronegative and 10 HIV seropositive samples for each of the HIV peptide pools. None of the seronegative samples were positive by the pre-defined
criteria and therefore this allowed the determination of an ELISPOT positivity cut-off for the clinical trial which was set at 38 SFC/m. So far out of 69 baseline samples from the clinical trial there has been only 1 false positive response to Env P1 (false positive rate = 1.4%).

Discussion & Conclusions

Before starting a clinical trial, it is very important to characterize the peptide pools that will be used to ascertain whether there is stimulation of T cell function where T cell responses would not be expected by use of PBMC samples from subjects who have not been exposed to the pathogen under study. Likewise the ability to stimulate T cell responses where a response is expected should be demonstrated by use of PBMC samples from subjects who have been exposed and infected with the pathogen under study. The results of this particular study showed that high quality peptides induced minimal false positive responses in an IFN-γ ELISPOT either during the peptide qualification or in samples collected prior to vaccination.

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