CEFX/EFX Ultra SuperStim Pools: Superior positive controls for T-cell assays with broad MHC-allele and antigen coverage

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The development of successful vaccines and other immunotherapies requires efficient immune monitoring techniques. Typically, antigen-specific T cell assays detect secreted cytokines (ELISA and ELISPOT) or intracellular cytokines (flow- cytometry). Pools of commonly recognized viral peptides are often used as positive controls in these assays, because they tend to induce responses in a majority of individuals. However, a considerable number of individuals respond only weakly or not at all to these pools, because they have not been exposed to the infectious agents covered, or, they do not have the required HLA-type to present one or more of the selected peptides. In some situations, individuals may not respond to such peptides despite previous exposure and the matching HLA-type, which is probably related to their specific T-cell receptor repertoire and epitope dominance hierarchies (1,2). In order to address this problem, we have developed an improved positive control pool that covers a wider range of target species and provides broader coverage of populations with different ethnic backgrounds than any other available peptide pool to date.

Introduction

T-cell stimulation-dependent T-cell assays require positive controls to confirm that the tested cells were able to respond to an adequate stimulus in principle. One frequently used positive control is a panel of 23 MHC class I presented viral peptides called 'CEF pool' (3). This pool consists of known epitopes from Cytomegalovirus (CMV, HHV-5), Epstein–Barr virus (EBV, HHV-4), and Influenza A virus (Flu). The prevalence of infection with each of these viruses in adults exceeds 50-80%, depending on the virus and the studied population. As a result, there is a high degree of probability that most individuals will have T-cells specific for at least one of these viruses. Modified versions of the CEF pool contain the original 23 peptides plus 9 additional peptides from the same viruses, referred to as 'Extended CEF pool' (32 peptides) or the original 23 peptides plus 4 additional stimulating peptides from *Clostridium tetani*, referred to as 'CEFT pool' (27 peptides). These extended versions are widely accepted and now more frequently used than the original CEF pool. However, the huge diversity of MHC in humans, the human leukocyte antigen (HLA for short), adds another layer of complexity. The original CEF pool was designed to cover the most frequent HLA alleles found in European-Caucasoids. However, in other ethnic groups different HLA-alleles/allele groups dominate. Therefore, the existing positive control pools often do not work as well in other ethnic groups as they do in European-Caucasoids. Here we describe the design of a novel, enhanced positive control-pool with a strongly improved coverage of infectious agents and HLA-alleles.

Methods

The abundance of known stimulating peptides and their presenting HLA-alleles prompted the extension of existing positive control pools in terms of the number of infectious agents targeted and HLA-types covered in order to increase the probability of a positive T-cell response. Together, the 50 peptides already used in various JPT control pools (CEF, CEF (extended), CEFT, and CEFT MHC-II) formed the core pool to be extended. As a first step, we searched publications and publicly available epitope repositories for relevant epitopes covering a wide range or relevant organisms and HLA-types (4). This search yielded several hundred stimulating peptides of interest. The subsequent peptide selection was carried out with the aim of covering a majority of ethnic backgrounds. Information published by the US National Bone Marrow Donor Program on the HLA allele frequencies of 5.7 million individuals

divided into 16 ethnic groups (5) was used to calculate, for each peptide, the percentage of the population that would theoretically be able to respond to this peptide based on the presence of the presenting HLA-allele or allele group (population coverage).

Table 1: Organism coverage of CEFX and EFX Pools

Coxsackievirus B4, Human adenovirus 5, Human herpesvirus 1, Human herpesvirus 2, Human herpesvirus 3, Human herpesvirus 4, Human herpesvirus 6, Human papillomavirus, JC polyomavirus, Measles virus, Rubella virus, Vaccinia virus, Clostridium tetani, Influenza A virus, Helicobacter pylori, and Toxoplasma gondii
Human herpesvirus 5 (HCMV) not contained in EFX

As a baseline, the population coverage for the existing core pool was calculated. Subsequently, the coverage gained with each candidate peptide to be added was calculated and the peptide was included in the final pool if the increase in coverage was >5% (across all ethnic groups). Peptides predicted to be chemically unstable were excluded. The final pool encompassed 176 peptides derived from 17 organisms (**Table 1**). Apart from serving as a positive assay control, it might be of interest to compare the responses induced by such a comprehensive positive control pool between different individuals (or groups of individuals) to assess T-cell immunity in a more general way. For this purpose, we have designed two new pools, one including and one excluding peptides from Human herpesvirus 5 (CMV). The pools are referred to as CEFX Ultra SuperStim Pool (including CMV peptides) or EFX Ultra SuperStim Pool (excluding CMV peptides). CMV often leads to very large T-cell responses that could make a fair comparison between CMV-infected and uninfected individuals difficult if the CEFX Ultra SuperStim Pool were used. Please see **Table 1** for the species coverage of the new pools. The gain in population MHC coverage of the CEFX/EFX pools compared to the currently most widely used pool, the CEFT pool, is shown in **Figure 1**.

Results

PBMC were used in an IFN-γ ELISPOT assay and stimulated with the standard positive control pools, CEFT, and the new pools, CEFX and EFX Ultra SuperStim Pools. A significantly higher number of spots was observed with CEFX and EFX Ultra

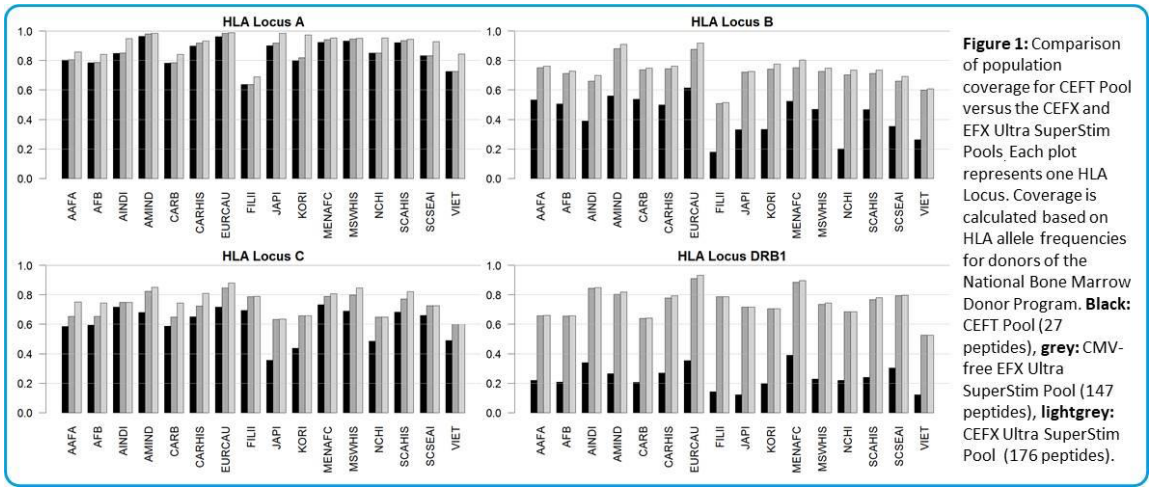

Figure 1: Comparison of population coverage for CEFT Pool versus the CEFX and EFX Ultra SuperStim Pools. Each plot represents one HLA Locus. Coverage is calculated based on HLA allele frequencies for donors of the National Bone Marrow Donor Program. **Black:** CEFT Pool (27 peptides), **grey:** CMV-free EFX Ultra SuperStim Pool (147 peptides), **lightgrey:** CEFX Ultra SuperStim Pool (176 peptides).

SuperStim Pools compared to CEFT, translating into a 50% response increase in the example in Fig. 2. In order to obtain representative estimates for the expected (average) response increase, a larger series of tests is currently being carried out.

Discussion & Conclusions

Two new control pools, CEFX and EFX Ultra SuperStim Pools, were created based on a library of known epitopes from multiple species with a high level of endemic infection. The selection was based on observed or predicted HLA allele associations as well as reported HLA allele frequencies for a wide range of ethnic backgrounds. Theoretical organism coverage and population HLA coverage of the new positive control pools are superior to those of the most popular, conventional positive control pools in current use, for example the CEFT pool. Our new CEFX and EFX Ultra SuperStim Pools, therefore, have a much higher probability of causing a positive response in a given individual than conventional pools and should, therefore, be preferred in most situations.

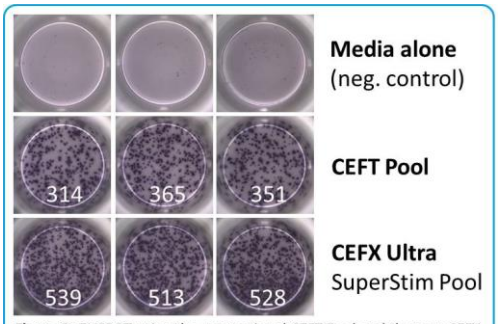

Figure 2: ELISPOT using the conventional CEFT Pool and the new CEFX Ultra SuperStim Pool. A standard ELISPOT (300,000 cells/well, 18h stimulation, 37°C standard incubator) was performed in order to compare the result with the two peptide pools. Cells were incubated with 0.7 nMol/ml per peptide.

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