CEFX: A novel peptide pool with broad infectious antigen and MHC coverage for use as a positive stimulation control or as a means of eliciting general T-cell responsiveness

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Summary

T-cell activation assays such as ELISPOT or Intracellular Cytokine Staining (ICS) require appropriate positive stimulation controls. Compared with conventional assay controls based on polyclonal stimulation (e.g. PHA/Ionomycin), peptide pools have the advantage of providing a more physiological, TCR-mediated signal. A frequently used control of this type is the so-called 'CEF' pool, containing 23 selected class-I-MHC-presented T-cell stimulating peptides from CMV, EBV, and Influenza A. As a result of frequent and widespread exposure, most if not all individuals have T-cells specific for at least one of these agents. However, the 'CEF pool' only covers the most frequent HLA alleles in European-Caucasoids, but not other ethnic groups, limiting its usefulness. We, therefore, designed an extended peptide pool referred to as 'CEFX pool' with significantly improved coverage of both infectious agents and HLA-alleles (176 peptides) and that contained numerous CD4 T-cell-stimulating peptides. We report here that this novel pool induces stronger responses and identifies more positive responders than the original 'CEF' pool in immune assay formats such as ELISPOT, ICS, and ELISA of stimulated T-cell supernatants. Sub-pools to address CD4 or CD8 T-cell specific responses or devoid of CMV-derived peptides are also available. Due to broader antigen coverage of the 'CEFX pool', we are further exploring its potential use for measuring/monitoring general T-cell responsiveness in clinical situations such as immunodeficiency, immunosuppression, and/or immunosenescence.

CEFX and EFX pools: Background

A logical step towards producing a more universally applicable stimulation control would be to attempt expanding established positive control peptide pools in order to both widen infectious agent coverage and HLA-type coverage. We thus began efforts to create a new positive control peptide pool by selecting 50 peptides from the various JPT control pools [CEF, Extended CEF (extended), CEFT, and CEFT MHC-II CEF]. We next searched publications and databases for CD4 and CD8 T-cell stimulating peptides derived from common infectious organisms. This yielded several hundred such peptides, however, that were further reduced by a subsequent selection step to a more reasonable number while still maintaining maximum coverage of genetic backgrounds. This selection involved using 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) to calculate, for each peptide, the percentage of the population that would theoretically respond based on the presence of the presenting HLA-allele or allele group (population coverage). As a baseline, the population coverage for the existing core pool of 50 peptides was calculated. Subsequently, the coverage gained for each candidate peptide was calculated and that 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 from consideration. The final set contained **176 peptides** derived from 17 organisms (**Table 1**) and was designated the 'CEFX' pool. An alternate pool that excluded all peptides derived from Human herpesvirus 5 (CMV) was also generated ('EFX'-pool). The gain in population MHC coverage of the CEFX and EFX pools compared with the widely used CEFT pool is shown in **Figure 1**.

Figure 1

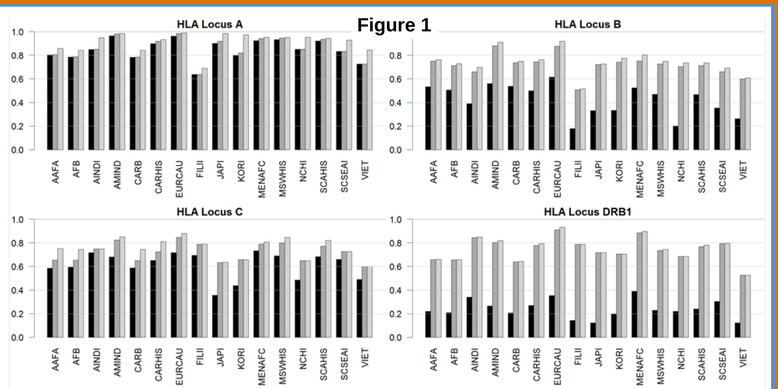

Figure 1: Comparison of population coverage for CEFT Pool versus the CEFX and EFX Ultra SuperStim Pools. Each plot represents one HLA Locus. Coverage is calculated based on HLA allele frequencies for donors of the National Bone Marrow Donor Program. **Black:** CEFT Pool (27 peptides), **grey:** CMV-free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

The CEFX pool includes 176 T-cell stimulating peptides from 17 common infectious agents/vaccine antigens.

Figure 2

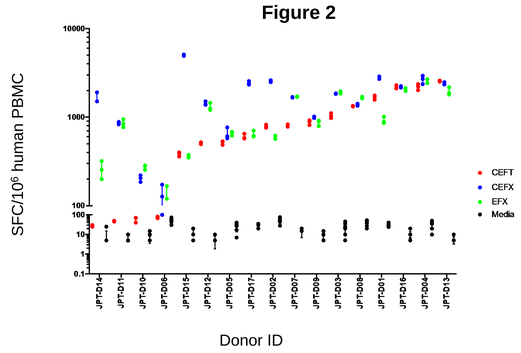

Fig. 2: ELISPOT in 17 healthy individuals, CEFT versus CEFX/EFX (JPT lab). PBMC were isolated from 17 healthy volunteers and cryopreserved within 6 hours of blood collection. 200,000 – 300,000 cells were plated per well in MSIPS 4510 ELISPOT plates (Millipore) and stimulated with either CEFT, CEFX or EFX peptide pools at a final concentration of 0.67 nMol/mL (approx. 1μg/ml) per peptide for 18 hours in a 37ºC, 5% CO_2 incubator. The ELISPOT plate was developed following JPT's standard in-house protocol. The IFN-γ signal is expressed as SFC per million PBMC.

Figure 3

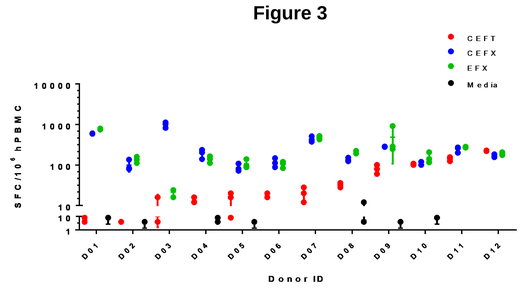

Fig. 3: ELISPOT in 12 healthy individuals, CEFT versus CEFX/EFX (Pfizer lab). PBMC were isolated from 12 normal volunteers and cryopreserved within 6 hours of blood collection. After thawing and counting, 250,000 viable cells/well were seeded on a Mabtech ELISPOT plate and stimulated with either CEFT, CEFX or EFX peptide pools at a final concentration of 4 µg/mL for 20 hours in a 37ºC, 5% CO_2 incubator. The ELISPOT plate was developed following manufacturer's recommended protocol. The IFN-γ signal is expressed as SFC per million PBMC.

Figure 4

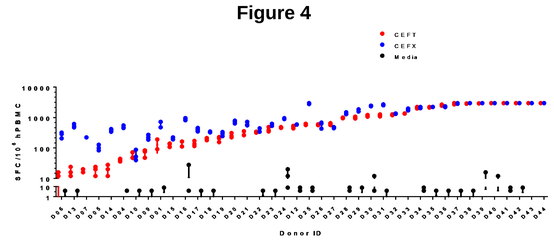

Fig. 4: PBMC were isolated from 40 normal volunteers (low, medium and high CEFT responders) and cryopreserved within 6 hours of blood collection. After thawing and counting, 250,000 viable cells/well were seeded on a Mabtech ELISPOT plate and stimulated with either CEFT or CEFX peptide pools at a final concentration of 4 µg/mL for 20 hours in a 37ºC, 5% CO_2 incubator. Plates were developed as above described in Fig. 3. The IFN-γ signals are expressed as SFC/10^6 PBMC.

Table 1: Microorganism and viruses represented in CEFX and EFX Pools

Coxsackievirus B4, Human adenovirus 5
Human herpesviruses 1, 2, 3, 4, 6
Human papillomavirus, JC polyomavirus,
Measles virus, Rubella virus,
Vaccinia virus, Clostridium tetani,
Influenza A virus, Helicobacter pylori,
Toxoplasma gondii.

Human herpesvirus 5 (HCMV) is contained in the CEFX but not the EFX pool.

Figure 5

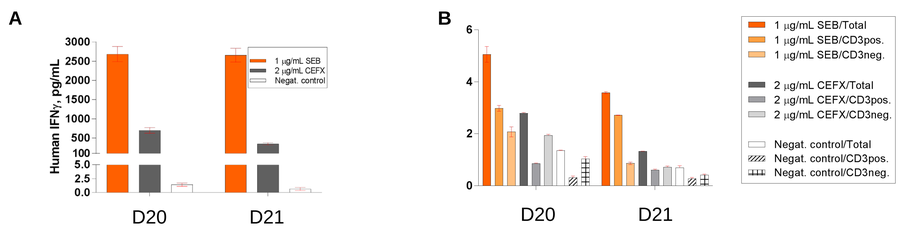

Fig. 5: Intracellular IFN-γ staining after cell stimulation in PepSup Tubes. Fresh whole blood mixed with media (1:1) was incubated for 16h on a 37°C heat block in tightly closed vials with the pre-dissolved CEFX peptide pool in a custom solution ("PepSup"); the final peptide concentration was approx. 2 µg/mL (1.3 nMol/ml) per peptide. Stimulation was performed in triplicate, columns show mean/STD. **A.** IFN-γ in the supernatant was measured by ELISA (ThBMS228HS, ThermoFisher Scientific). **B**. Following addition of BFA (Sigma) at the beginning of the incubation period, intracellular IFN-γ staining was performed (standard in-house protocol) using fluorochrome-labeled anti-CD3-PerCP/Cy5.5, anti-CD8-FITC, and IFN-γ-PE. Cells were acquired on an Accuri C6 Flow-cytometer (Becton Dickinson).

Results

These experiments were conducted to compare JPT's novel peptide pools, CEFX and EFX, with the CEFT pool, which is frequently used as a positive control pool. ELISPOT results from two labs using PBMC from different donors demonstrated that CEFX-induced IFN-γ responses were higher than responses induced with EFX and CEFT pools (**Fig. 2, 3, and 4**). Importantly, CEFX was able to induce IFN-γ ELISPOT responses of higher magnitude in a majority of low to medium CEFT responders (low = >15 to <300, medium= >300-1000 SFC per million PBMC). CEFX-induced IFN-γ responses were equal to or higher than CEFT-induced responses in high CEFT responders (high = >1000 SFC per million PBMC). The CEFX pool also stimulated T-cells in whole blood very effectively, as shown by cytokine measurements in the supernatant (**Fig. 5A**) and flow cytometry (**Fig. 5B**). Flow cytometry additionally confirmed that $CD3^+$ T-cells were the main source of IFN-γ responses whereas $CD3^-$ lymphocytes (NK cells) only made a negligible contribution **(Fig. 5B**) after subtracting the background. Large differences between responses to the CEFX and the EFX pools were observed in a significant proportion of donors, most likely reflecting CMV infection status.

Discussion/Outlook

Because of the many antigens covered by the CEFX pool, individuals without a T-cell response to this pool are exceptions. CEFX is, therefore, an ideal positive stimulation control when used alongside other peptide-based stimulants, for example individual peptides derived from infectious agents or tumors. However, in addition, responses to the CEFX pool may provide a sense of general immune responsiveness with respect to infectious agents. It may, therefore, be useful in its own right to monitor immune responsiveness in individuals under immunosuppression or those with compromised immune systems, such as the HIV infected. Reductions or increases of immune responsiveness over time will be detectable if assays are sufficiently standardized. Standardization of the measurement (read-out) over time is relatively easy when using suitable standards for instrument calibration. However, standardization of the actual assay is more complex, as it requires cells whose responses are reproducible over time (in size and quality) and of which a large enough stock can be kept to cover the duration of a given study. Whereas cell lines of known responsiveness can be used for that purpose, a new product marketed by JPT Peptide Technologies allows the production of large amounts of peptide-specific CD8 T-cells from a buffy coat based on transient transfection (TERS, or T-cell receptor engineered reference samples). If secreted cytokines are used as a readout, the new PepSup format will provide the highest level of standardization. This provides the peptide pools in a custom-solubilized form in ready-to-use tubes, one test per tube, suitable for use with whole blood. Differences between CEFX and EFX pools are due to CMV infection status, since the CEFX pool contains CMV-peptides and the EFX pool does not but is otherwise identical. The EFX pool frequently outperformed the CEFT pool, indicating that non-CMV peptides made a significant contribution to the response.

CEFX/EFX pools outperform the CEFT pool as positive control pools in CMV-infected and CMV-uninfected people.

They might also be used as complex antigens in their own right, such as for longitudinal monitoring of T-cell responsiveness in specific clinical settings (transplantation or aging studies). The ideal formulation for each purpose may differ; the highest level of assay standardization can be achieved by JPT's new PepSup format for use with whole blood and secreted mediators.

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