PepSup™: a highly standardized, peptide-based ex-vivo stimulation assay for antigen-specific whole blood profiling

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Introduction – the problem

The main goal of monitoring the immune system following an immunotherapeutic intervention is the identification of intervention-induced changes, for example, **an increase or decrease of the frequency of antigen-specific T-cells** or **cytokine production**. Immunological assays for monitoring antigen-specific T-cell responses like **Elispot**, **ICS**, or **MHC-multimer staining**, are complex and prone to significant variation at all assay stages (*e.g.* sample acquisition, storage, shipment, processing, and analysis). As a result, these assays are generally **poorly standardized**.

However, additional variation occurs as a result of variable interpretation of and adherence to experimental protocols, and, last but not least, the inherent variability of the methods used to read samples, *e.g.* flow-cytometry (intracellular-cytokine staining, ICS) or Elispot. Multiple efforts have been made to standardize immune monitoring over the last two decades, but not all significant sources of variation have been removed.

Materials & Methods

We have now developed a simple, peptide-based whole-blood stimulation protocol that provides maximum standardization:

1. Peptides used to stimulate whole blood are synthesized in a **highly standardized** fashion and **pre-aliquoted** into reaction tubes, one test per tube. This eliminates antigenic variation that would typically be expected with antigenic lysates. Peptides can be optimized to stimulate CD4 and CD8 T-cells at the same time very effectively, which is an advantage over recombinant proteins.

2. Anticoagulated whole blood and sterile, complete, CO_2-free media is added to the peptide containing tubes, which are subsequently sealed.

3. Tubes are then **incubated** in a standard, **programmable heat-block**. Following the desired incubation time of 16 hours, for example, or as required otherwise, incubation is stopped by cooling samples to 4ºC.

4. The cells are then **pelleted in a micro-centrifuge** and supernatants carefully aspirated and immediately frozen at -20ºC until analysis.

Results

Our initial experiments used peptide pools from a variety of human viruses and bacteria for *in vitro* stimulation. SEB was used as positive control, peptide solvent alone (DMSO) as negative control. The Meso Quickplex SQ120 platform (Mesoscale Discovery) was utilized to measure, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-12p70, IFN-γ and TNF-α in the supernatants (V-Plex Proinflammatory Panel 1 Human).*[1]

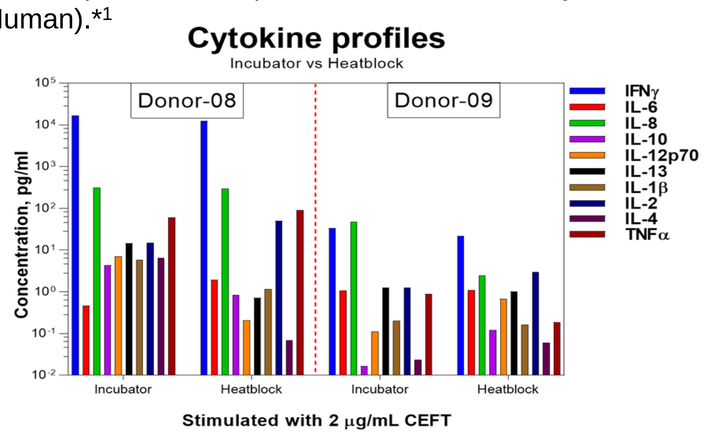


Fig. 1: Examples for incubations in a standard CO_2 incubator versus a dry heat block with CO_2-free media (16h, 37° C). All antigens used in duplicate and at 2ug/mL per peptide final concentration. Bars show means of two biological replicates.

The PepSup Assay was initially established for standard CO_2 incubator conditions using RPMI 1640 medium and then further optimized to be used in a dry heat block with a special, CO_2-independent media as shown in Fig.1.

Also, peptide solutions kept in ready-to-use PepSup™ Tubes (stored at -80 ° C) were compared to freshly made-up peptide solutions with respect to the efficiency of T-cell stimulation (Fig. 2 and 3, respectively).

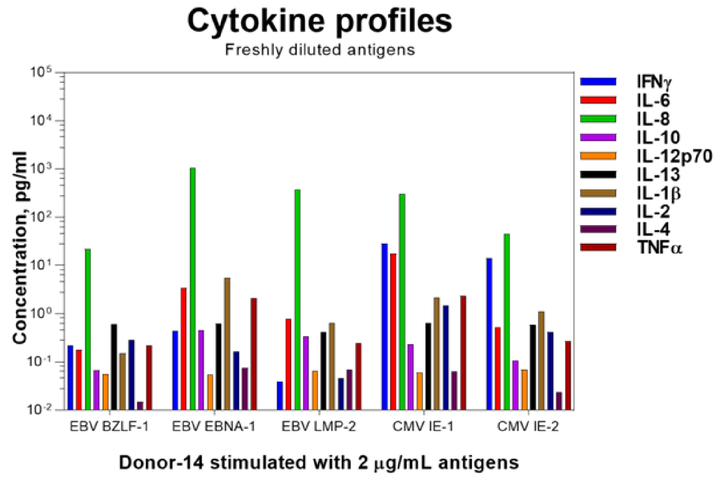


Fig. 2: Stimulation with freshly dissolved peptide pools. Incubation conditions: heat block - 16h, 37° C, no CO_2, closed vials. All antigens used in duplicate and at 2ug/mL per peptide. (means of two biological replicates are shown).

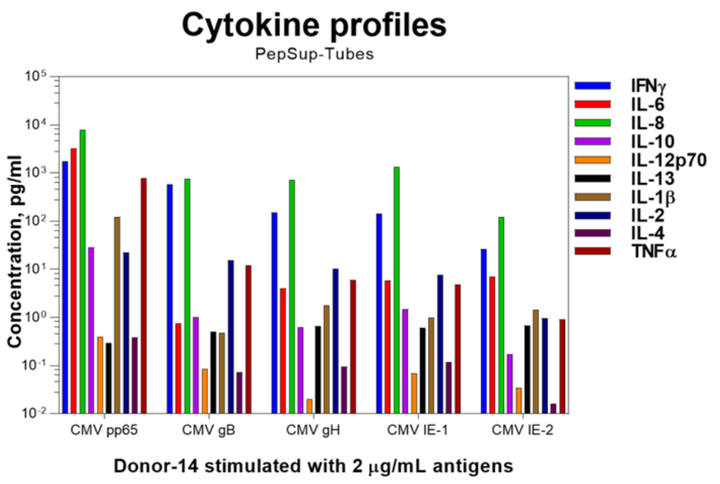


Fig. 3: Stimulation with a variety of pre-dissolved antigens (frozen in DMSO). Incubation conditions: heat block - 16h, 37° C, no CO_2, closed vials. All antigens used in duplicate and at 2ug/mL per peptide (means of two biological replicates are shown).

The PepSup Assay can be easily combined with all other commonly used methods to determine cytokine levels after stimulation, *e.g.* with ELISA or ICS.

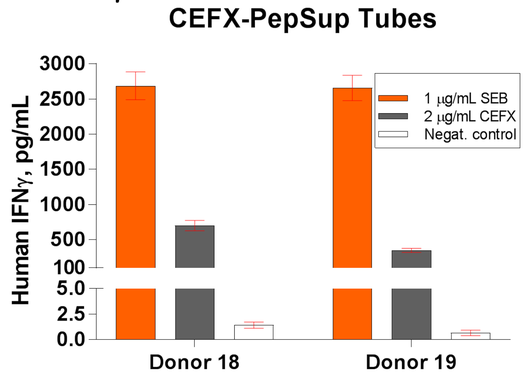


Fig. 4: Absolute IFNγ amounts measured with a high sensitivity ELISA assay after cell stimulation in PepSup Tubes. Incubation conditions: heat block - 16h, 37° C, no CO_2, closed vials. A new JPT peptide antigen pool, **CEFX,** was used in the PepSup Tubes. The stimulation was performed in triplicate and at 2 ug/mL per peptide (means of the biological replicates are shown).

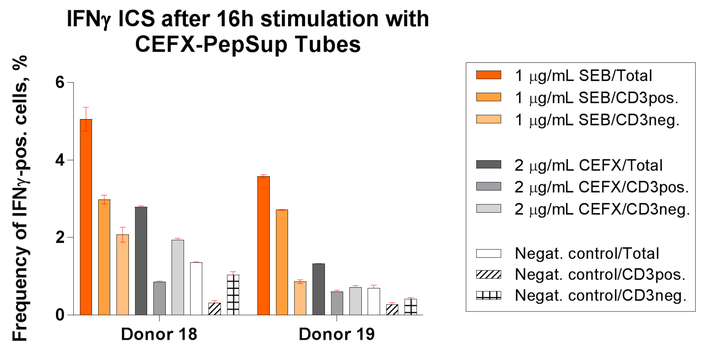


Fig. 5: Intracellular IFNγ staining after cell stimulation in PepSup Tubes. Incubation/stimulation conditions: same as in Fig.4 (parallel experiments). A new JPT peptide antigen pool, **CEFX,** was highly effective in stimulating CD8 and also CD4 T cells. IFNγ-pos. Activation of NK-cells (CD3-neg.) was also identified, however, these cells displayed much lower production of IFNγ. The antigen pools were used in duplicate and at 2ug/mL per peptide (means of two biological replicates are shown).

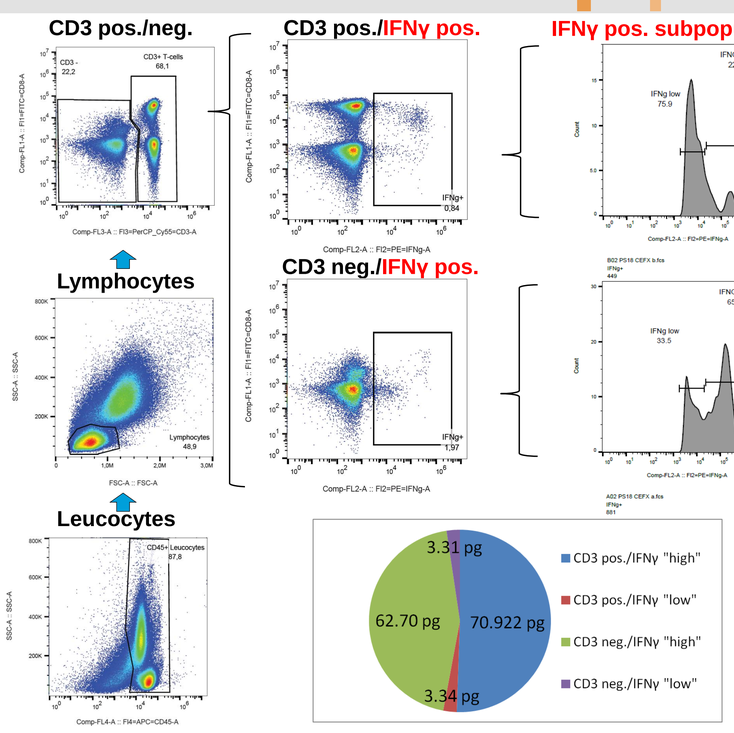
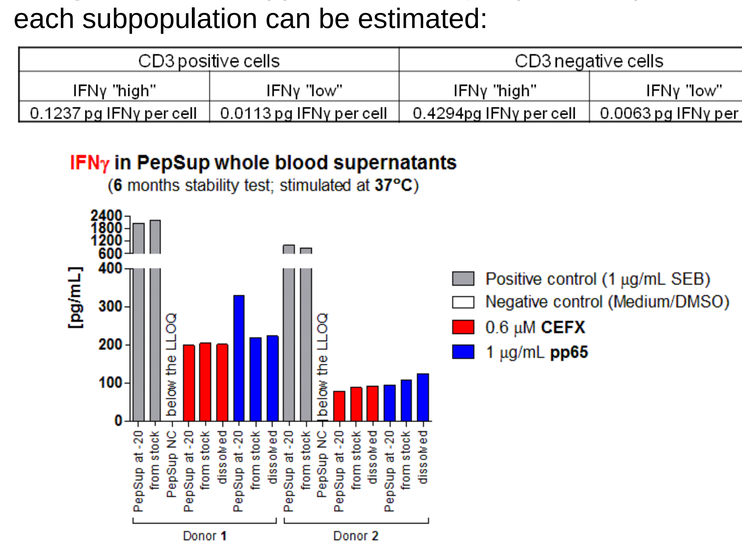


Fig. 6: Distribution of IFNγ expression by distinct lymphocyte subpopulations upon stimulation with 2ug/mL CEFX in PepSup Tubes (Donor 18). The absolute IFNγ amounts were calculated using quantitative ELISA (Fig.4).

Using cell counts, approximate IFNγ expression per cell in each subpopulation can be estimated:

CD3 positive cells		CD3 negative cells	
IFNγ "high"	IFNγ "low"	IFNγ "high"	IFNγ "low"
0.1237 pg IFNγ per cell	0.0113 pg IFNγ per cell	0.4294pg IFNγ per cell	0.0063 pg IFNγ per cell



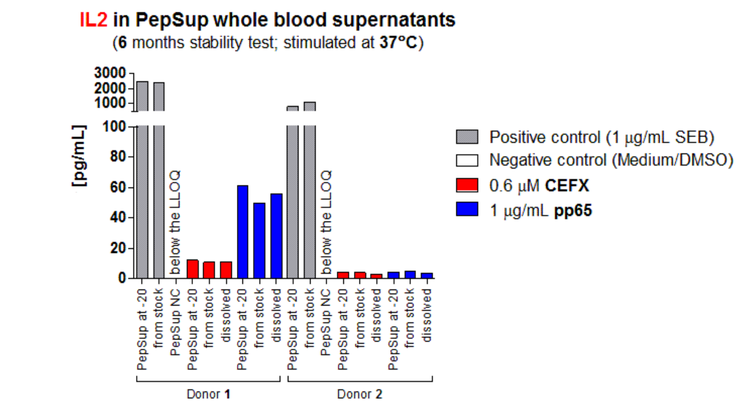


Fig. 7: PepSup Kit stability test after 6 months storage. A comparison of freshly prepared and stored PepSup Tubes (-80° C, 6 months) showed no differences in the stimulation result, indicating that the ready-to-use reagents are stable. Additional stability testing (12 and 18 months) is ongoing.

Summary/Outlook

Our new, highly standardized assay has potential to be used in immune monitoring after or during tumour immunotherapy or other clinical settings (infection, autoimmunity, immunosenescence, etc.). Its level of standardization is considerably higher than for currently used monitoring tools.

In combination with JPT's new CEFX pool (176 peptides covering 17 organisms), the new test format provides a novel, broad test of recall antigen responsiveness in a simple format that will be tested in various populations in the near future.

Contact

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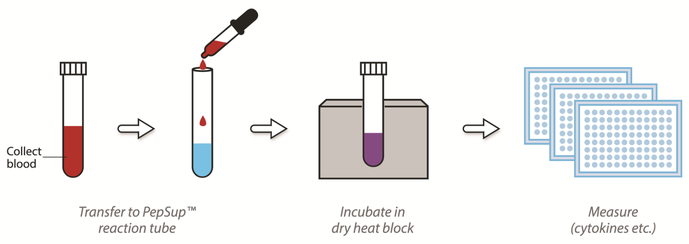
The PepSup workflow in a Nutshell



*[1] we deliberately selected a mix of cytokines originating mainly from T-cells (in particular IL-2, IL-4, IFN-g, TNF) but also from other cells (e.g. IL-6, IL8, IL-12-p70, IL-13) in order to be able to assess non-specific activation in addition to T-cell activation.

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