

## INTRODUCTION

Chimera peptide vaccines including assembled B-cell and T-cell epitopes are one approach to induce a humoral as well as a cellular immune response. In this respect, overlapping B-cell and T-cell epitopes with coincident core sequences could benefit a pursued minimal protective epitope subset. Epitope prediction tools can help to forecast immunogenicity. However, the prediction is limited and cannot replace laboratory screenings. Here (Fig.1) we describe the use of peptide microarrays combined with functional T-cell activation assays to discover overlapping B-cell and T-cell epitopes.

We applied this approach to unravel immunogenic epitopes in Epstein-Barr Virus (EBV) Nuclear Antigen-1 (EBNA-1) as a proof of principle. To identify B-cell as well as T-cell epitopes in EBNA-1, we analyzed sera and peripheral blood mononuclear cells (PBMCs) from EBV-positive healthy donors.

## B-CELL RECEPTOR EPITOPES

First, we mapped the humoral immune response and determined infection-elicited antibodies against EBNA-1, via screening sera with PEPPERCHIP® Epstein-Barr Virus Peptide Microarrays. The microarray contains 5,549 linear peptides of the most immunogenic EBV antigens printed in duplicates, and were framed by poliovirus VP1 protein 20-22 and influenza hemagglutinin (HA) derived control peptides. The microarray also includes 257 overlapping EBNA-1 peptides for high-resolution epitope data.

The in-depth analysis of the IgG responses highlighted clearly differential antibody profiles of both donors against EBNA-1. One of the main epitopes of donor 1 was based on peptides with the consensus motif FHPVGEADY (individual peptides RRPFFHPVGEADY, PFFHPVGEADYFE, FHPVGEADYFEYH). In contrast, the serum of donor 2 did not show remarkable antibody responses against the EBNA-1 peptides and particularly with epitope FHPVGEADY.

**Fig. 2. Discovery of B-cell receptor epitopes via peptide microarray.** (A) Scans of the PEPPERCHIP® Epstein-Barr Virus Peptide Microarrays and (B) fluorescence intensity plots. The microarrays were incubated with the sera of two donors at a dilution of 1:150 overnight at 4°C. Detection was done using the secondary goat anti-human IgG (Fc) DyLight680 antibody. Fluorescence readout was performed using an INNOPSIS Imaging System. Red spots=IgG responses. The frame highlights the position of the EBNA-1 peptides. (C) Antibody response profile against EBNA-1. Scanned images were analyzed with the PepSlide® Analyzer software. The intensity plots show the fluorescence intensities of 257 overlapping EBNA-1 peptides.

## T-CELL RECEPTOR EPITOPES

To subsequently discover potential overlapping T-cell epitopes within the identified B-cell epitopes, the three peptides were synthesized and utilized in IFN-γ ELISpot assays as single peptide or peptide minipool together with published immunogenic EBV-peptides (Table 1). The high sensitivity of the ELISpot assay allowed a precise discrimination between T-cell-activating and non-activating peptides and facilitated the identification of the recognized, immunogenic core sequence.

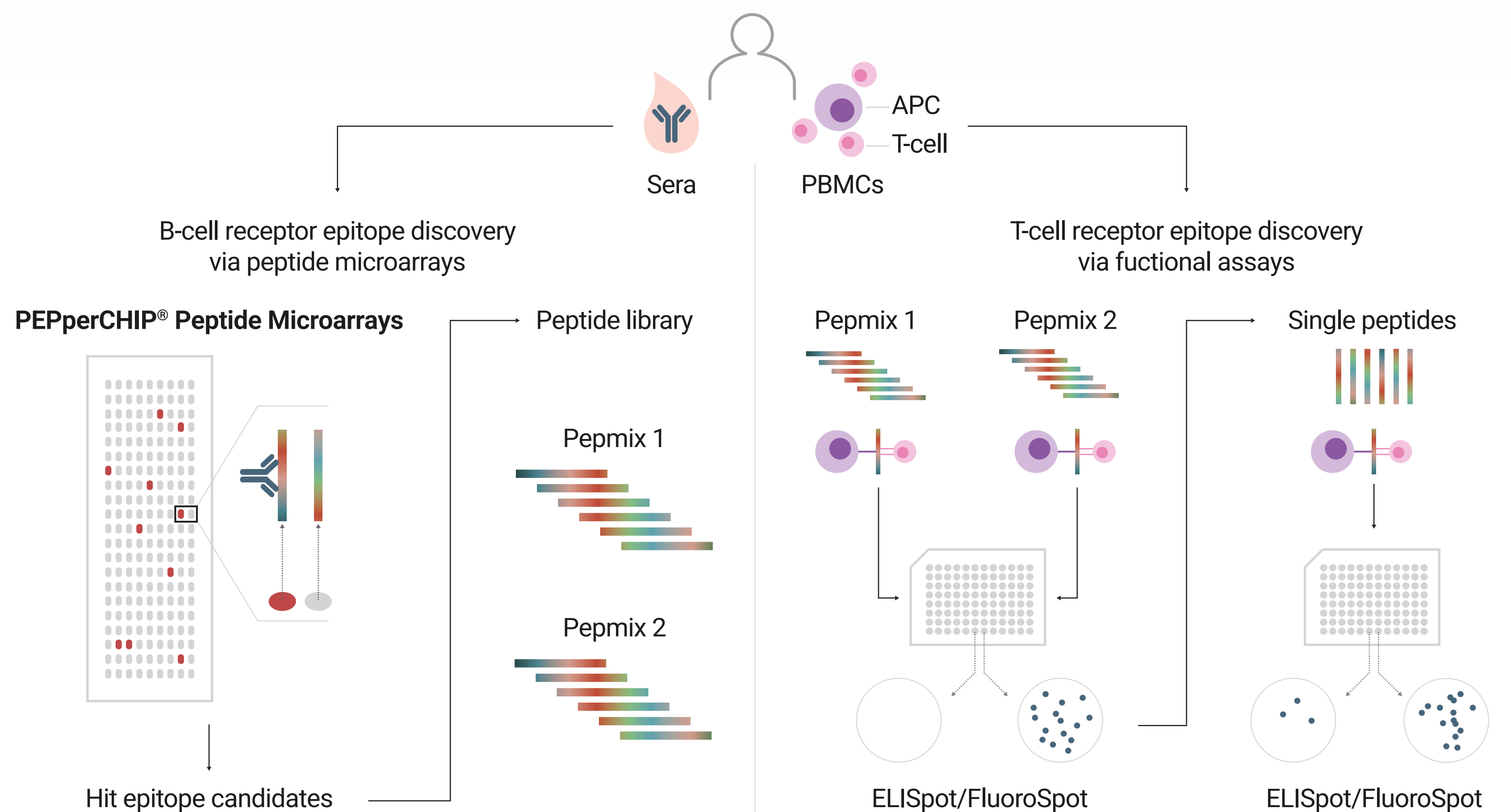
As expected, the positive control EBNA-1 peptide mix induced a pronounced IFN-γ response in both donors. Donor 1 could also be activated with the mini peptide library. Based on the tests with the single peptides, this activation was due to immunogenicity of peptides FHPVGEADYFEYH, HPVGEADYFEY and DYCNVLNKEF. To a minor degree, donor 2 could also be activated with the mini peptide library. Interestingly, this activation was exclusively due to the immunogenicity of peptide DYCNVLNKEF, as none of the EBNA-1 derived peptides induced an IFN-γ response. EBNA-1 peptides FHPVGEADYFEYH and HPVGEADYFEY induced T-cell activation in donor 1, whereas EBNA-1 peptides RRPFFHPVGEADY and PFFHPVGEADYFE did not. Therefore, the core T-cell epitope seemed to depend on appropriate C-terminal sequences.

**Table 1. Peptide sequences for validation of T-cell immunogenicity.**

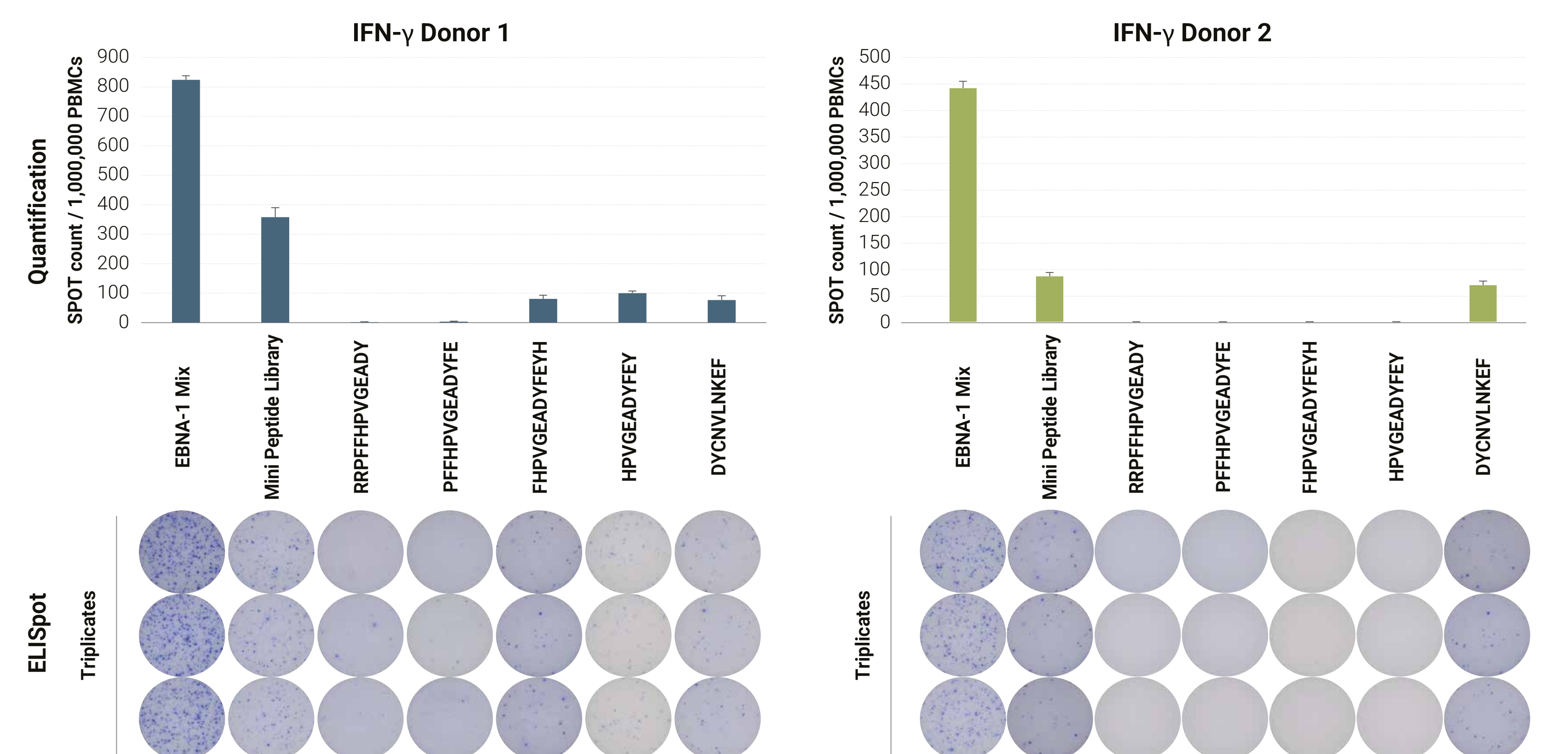
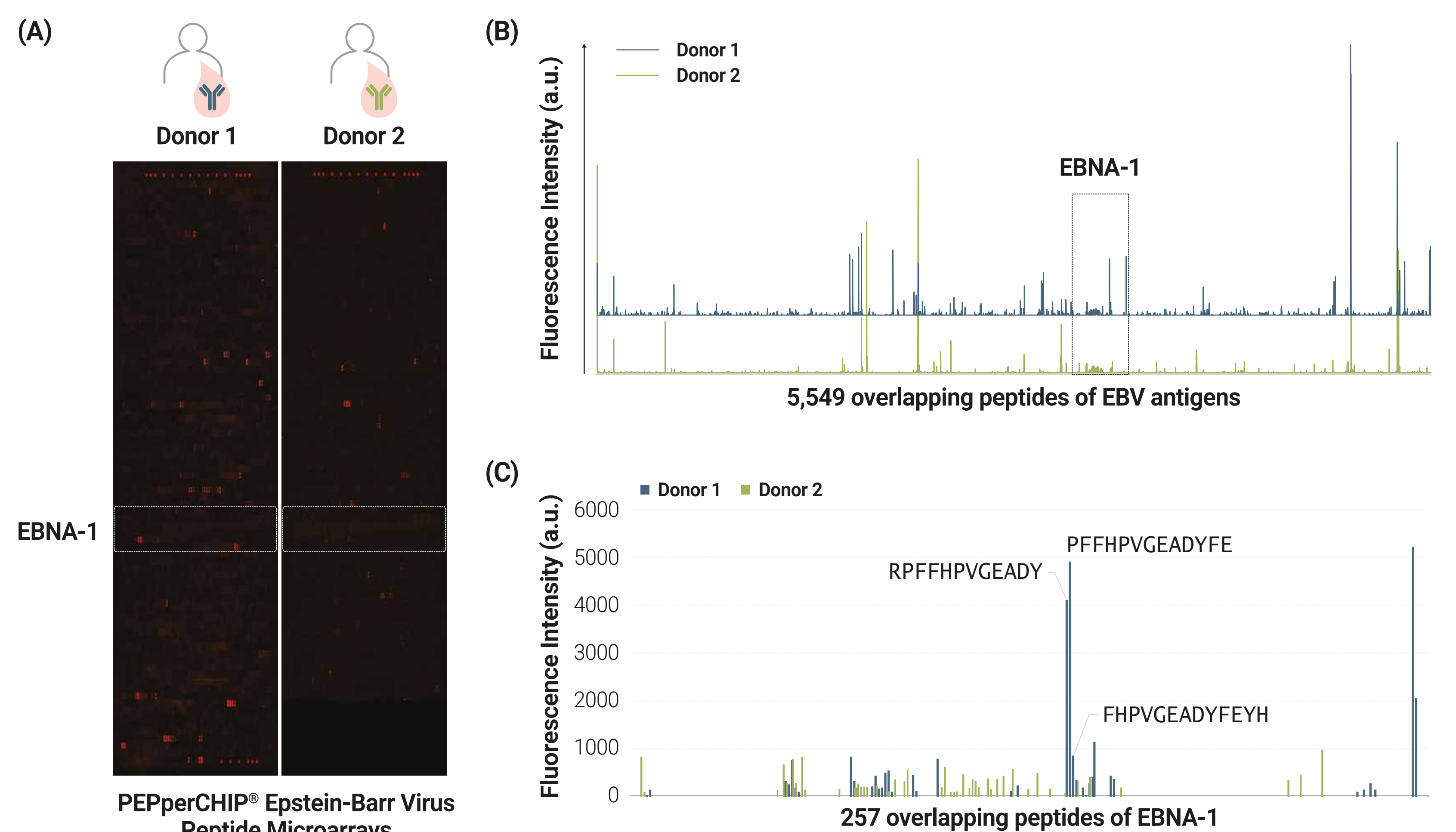
Peptide sequence	Protein
RRPFFHPVGEADY	EBNA-1
PFFHPVGEADYFE	
FHPVGEADYFEYH	
HPVGEADYFEY	
DYCNVLNKEF	BRLF1

## CONCLUSIONS

In conclusion, our proof of principle study shows that the combination of peptide microarrays and ELISpot assays is suitable to identify overlapping B cell and T cell epitopes. As the approach is applicable to any other antigen, it could generally help to identify new peptide vaccine candidates.



**Fig. 1. Workflow for the discovery of coincident B-cell and T-cell epitopes.** Serum samples are screened with PEPPERCHIP® Peptide Microarrays displaying peptide libraries of up to 35,000 different peptides printed in duplicate. Identified hit peptides are validated via literature review and T-cell activation prediction tools. Subsequently, promising peptide candidates are synthesized applied in ELISpot/FluoroSpot assays with PBMCs of the same donor.



**Fig. 4. Discovery of T-cell receptor epitopes via functional assay.** 500,000 PBMCs per well were stimulated in a 96-well IFN-γ ELISpot plate for 24 hours with a commercial EBNA-1 peptide mix (EBNA-1), the mini peptide library (Table 1) or the underlying single peptides in a final concentration of 10 μg/ml. ELISpot was analyzed on an ImmunoSpot® Analyzers. Top half: Quantification of ELISpots with the mean values and the standard deviation of triplicates of the counted spots per 1 million PBMCs. Bottom half: IFN-γ-dependent blue-colored immune complexes.