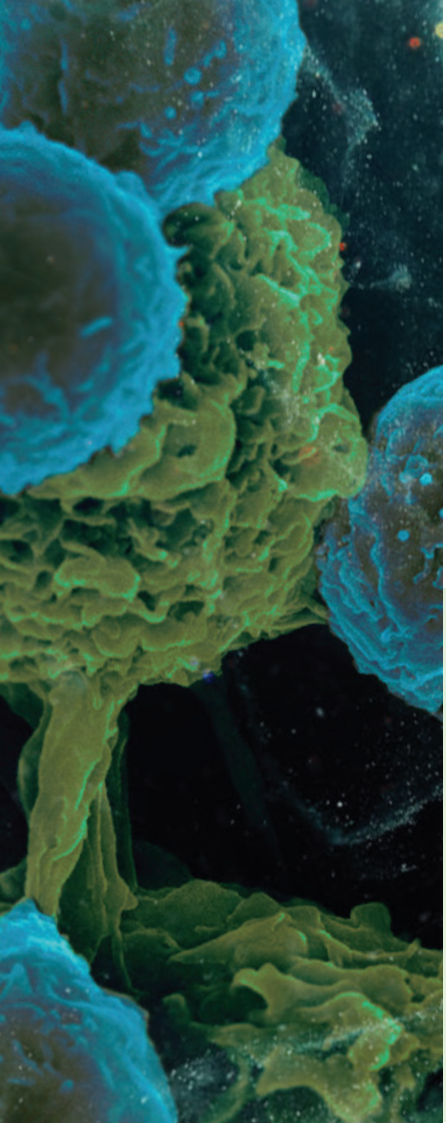

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Application Note

Discovery of Overlapping Immunogenic B-cell and T-cell Epitopes

ABSTRACT

This application note describes the use of PEPperCHIP® Peptide Microarrays combined with functional T-cell activation assays to discover coincident B-cell receptor and T-cell receptor epitopes (Figure 1). We applied this approach to unravel immunogenic epitopes in Epstein-Barr virus nuclear antigen-1 (EBNA-1) that activate B-cells to produce antibodies as well as T-cells to produce interferon gamma (IFN-γ). For this purpose, we analyzed sera and peripheral blood mononuclear cells (PBMCs) from two different donors. High resolution epitope mapping with the PEPperCHIP® Epstein-Barr Virus Peptide Microarray enabled the identification of promising B-cell receptor epitopes in EBNA-1 that were further evaluated in terms of T-cell activation in an IFN-γ ELISpot assay. This approach allowed the discovery of a peptide sequence that functions as B-cell as well as T-cell immunogenic epitope.

INTRODUCTION

Due to the huge B-cell and T-cell receptor repertoire, the lymphocyte-driven humoral and cellular immune response is highly specific (1). Although a foreign protein contains a large variety of different peptides that could evoke an immune response, only a few peptides are actually immunogenic and even less are immunodominant (2). Several peptide characteristics such as peptide length, amino acid sequence and human leukocyte antigen (HLA) affinity constitute a peptide as lymphocyte receptor epitope. Importantly, a B-cell epitope might also function as T-cell epitope. However, for most peptide sequences this is not the case (2).

Depending on the research topic, the simultaneous activation of B-cells and T-cells can be desired. In the development of a protective vaccines that induce contemporary as well as persisting immunity through humoral and cellular responses, overlapping epitopes for B-cell and T-cell receptor would be highly favorable.

For the selection of immunogenic peptide candidates, a variety of epitope prediction tools (such as the prediction tools from IEDB Analysis Resource) can help to forecast immunogenicity by using published data and/or applying knowledge on preferred amino acids lengths and sequences etc. (3). However, the veracity of predictions is unambiguously limited and cannot replace laboratory screenings of B-cell and T-cell receptor epitopes.

High-density peptide microarrays are a powerful tool to simultaneously screen tens of thousands of peptides against serum antibodies in a high-throughput context (4). Subsequently, identified B-cell receptor epitopes can serve as basis for the generation of synthetic overlapping peptide libraries for T-cell immunogenicity testing by ELISpot assays. ELISpots directly determine the frequency of antigen-specific T-cells by detecting released activation markers such as IFN-γ. The method is extremely sensitive and enables a quantitative single cell analysis that is impossible with supernatant-based assays such as ELISAs. Moreover, multiparametric ELISpot (or FluoroSpot) assays allow the detection of different markers in parallel and thereby the characterization of T-cell subtypes.

This application note describes an experimental workflow for exploring coincident B-cell receptor and T-cell receptor epitopes in EBNA-1 protein of EBV. B-cell receptor epitopes were identified with PEPperCHIP® Epstein-Barr Virus Peptide Microarrays. The corresponding peptides were incorporated in an overlapping mini peptide library and

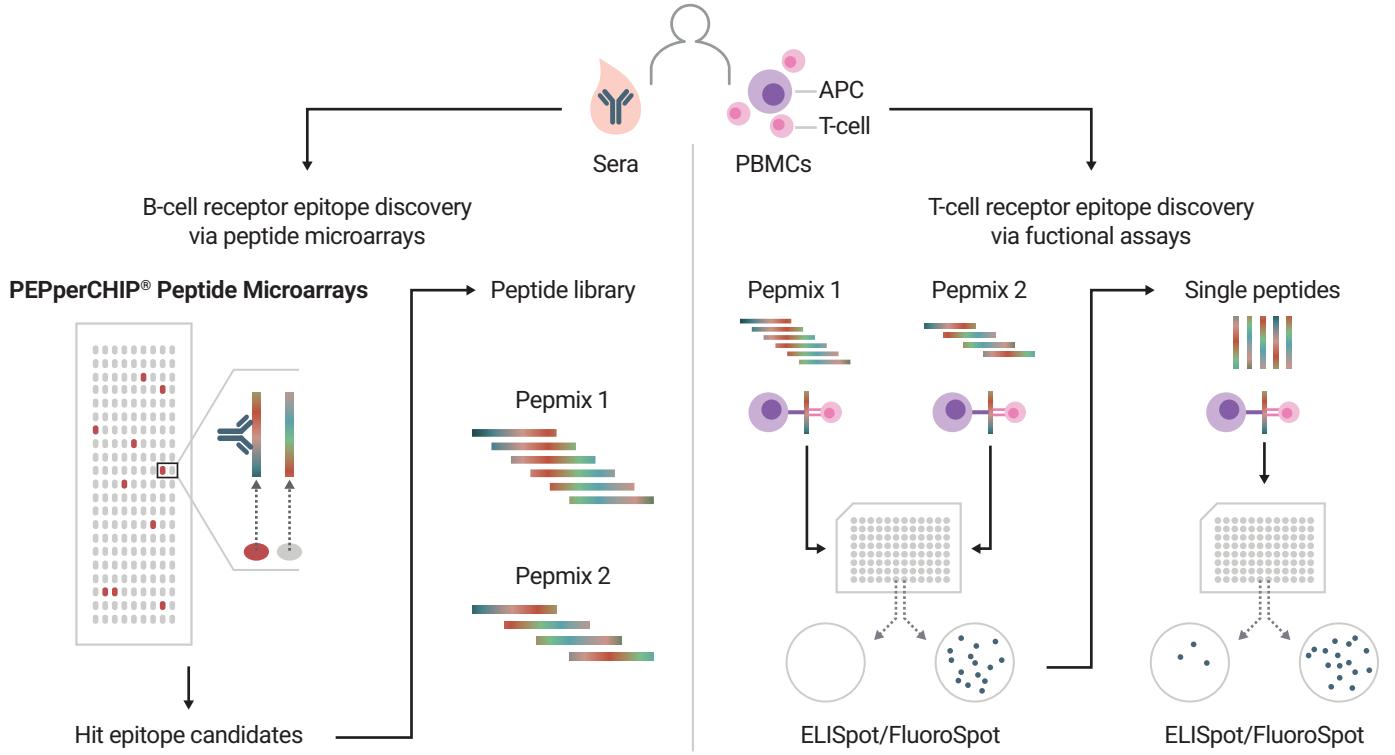

Figure 1. Workflow for the discovery of coincident B-cell and T-cell epitopes. Serum samples are screened with PEPperCHIP® Peptide Microarrays displaying peptide libraries of up to 35,000 different peptides printed in duplicate. Identified hit peptides are validated via literature review and T-cell activation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

tested for T-cell activation via IFN-γ ELISpot. Via this approach we were able to identify FHPVGEADY in EBNA-1 as a core sequence of B-cell as well as T-cell receptor epitope.

RESULTS AND DISCUSSION

In the study, we initially mapped the humoral immune responses of EBV-infected individuals. To determine the infection-elicited antibodies against EBNA-1, we screened sera of two donors with PEPperCHIP® Epstein-Barr Virus Peptide Microarrays (Figure 2). The PEPperCHIP® Epstein-Barr Virus Peptide Microarrays 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.

PEPperCHIP® Epstein-Barr Virus Peptide Microarrays were initially incubated with the secondary goat anti-human IgG (Fc) DyLight680 antibody to identify possible background interactions of the detection antibodies. Here, no background interactions were observed. Subsequently, the sera of donor 1 and donor 2 were diluted to 1:150 and incubated overnight at 4°C. After washing, detection was done using the secondary goat anti-human IgG (Fc) DyLight680 antibody. Fluorescence readout was performed using an INNOPSYS Imaging System, and 16-bit gray-scale images were analyzed with the PepSlide® Analyzer software.

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 (Figure 2).

To discover potential coincident T-cell receptor epitopes within the identified B-cell receptor epitopes, peptides RRPFFHPVGEADY, PFFHPVGEADYFE and FHPVGEADYFEYH were synthesized. In addition, peptides HPVGEADYFEY of EBNA-1 protein and DYCNVLNKEF from replication and transcription activator (BRLF1) were included in the study. Peptide HPVGEADYFEY is described to be highly immunogenic in EBV-exposed individuals expressing HLA-B*3501 (5-7), while peptide DYCNVLNKEF is known to activate T-cells in HLA-A*2402-expressing individuals (8).

Peptide-mediated T-cell activation was measured via interferon gamma (IFN-γ) ELISpot. IFN-γ is commonly used as T-cell activation marker in ELISpot and has a wide range of functions in the immune response, such as

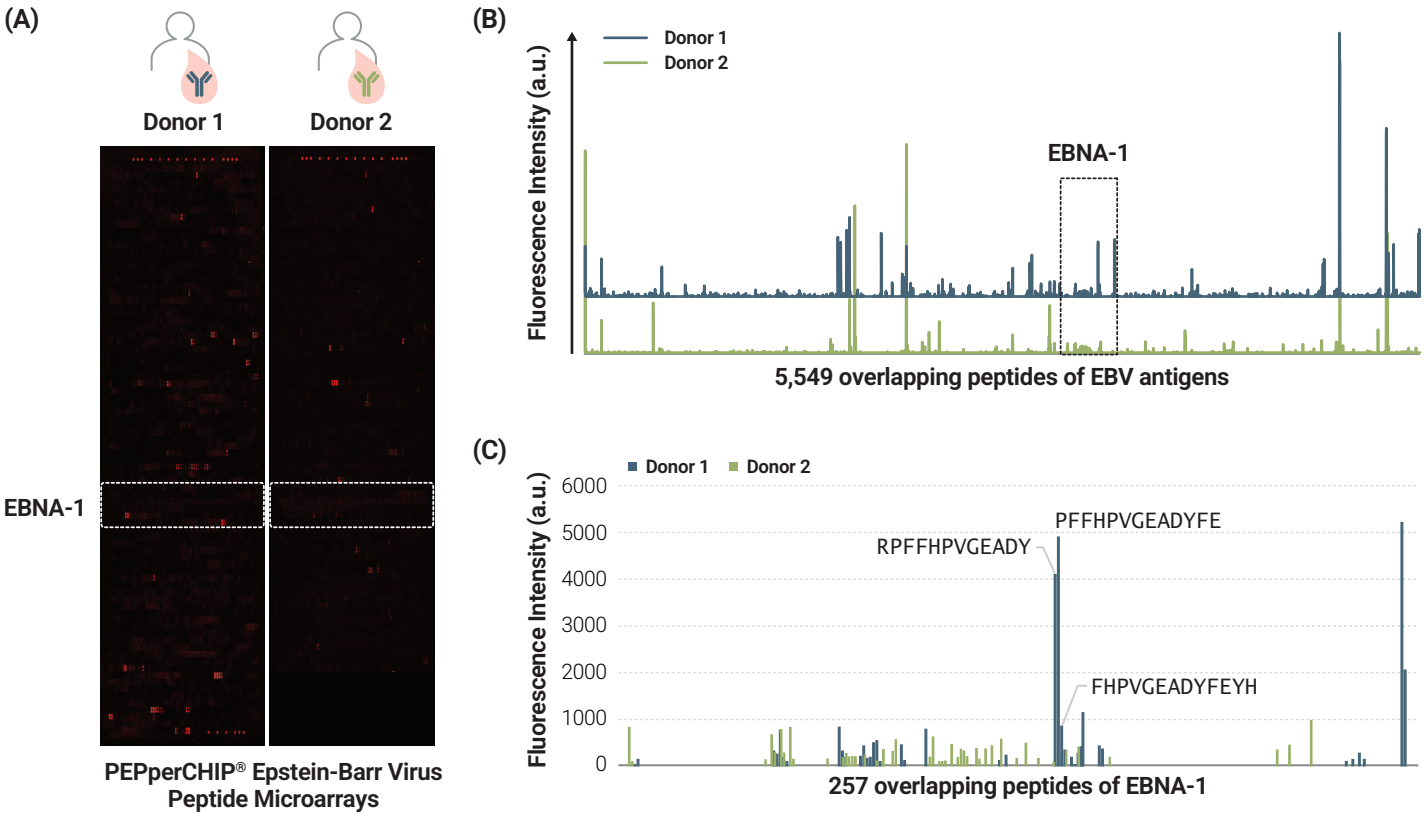

Figure 2. Discovery of B-cell receptor epitopes via peptide microarray. (A) Scans of the PEPperCHIP® Epstein-Barr Virus Peptide Microarrays and (B) fluorescence intensity plots. The microarrays were incubated with the sera of two donors at a dilution of 1:150 overnight at 4°C. Detection was done using the secondary goat anti-human IgG (Fc) DyLight680 antibody. Fluorescence readout was performed using an INNOPSYS Imaging System. Red spots=IgG responses. The frame corresponds 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 sorted from the N- to the C-terminus of the protein. While we observed moderate IgG responses of donor 1 with two clear epitopes, the serum of donor 2 exhibited only weak signals and no clear epitope.

activation of macrophages and induction of IgG production from activated B cells. IFN-γ is mainly produced and secreted by activated CD4 and CD8 T-cells which constitute about 45–70% of PBMCs in human peripheral blood (9,10).

Briefly, PBMCs were tested directly in a 96-well IFN-γ ELISpot plate for 24 hours. 500,000 PBMCs per well were stimulated in triplicates either with the mini peptide library made of the 5 different synthetic (Table 1) or with the single peptides in a final concentration of 10 µg/ml. As positive control, PBMCs were activated with a commercial EBNA-1 peptide mix containing 158 15 amino acid peptides with 11 amino acids peptide-peptide overlap (JPT, Berlin, Germany).

The assay was performed according to the manufacturer's protocol and analyzed on an ImmunoSpot® Analyzer (CTL ImmunoSpots Europe, Bonn, Germany). 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 (Figure 3). This result was in line with the peptide microarray data that highlighted a humoral response against FHPVGEADY-containing peptides in donor 1, but no corresponding response in donor 2. 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 PFFHPVGEADYFE FHPVGEADYFEYH HPVGEADYFEY	EBNA-1
DYCNVLNKEF	BRLF1

Peptides RRPFFHPVGEADY, PFFHPVGEADYFE and FHPVGEADYFEYH were identified as B-cell epitopes based on the previous epitope mapping with the serum samples of donor 1 and 2. Peptides HPVGEADYFEY of EBNA-1 protein and DYCNVLNKEF of replication and transcription activator (BRLF1) were chosen from literature. For T-cell stimulation, all five peptides were either applied simultaneously as mini peptide library or as individual peptides.

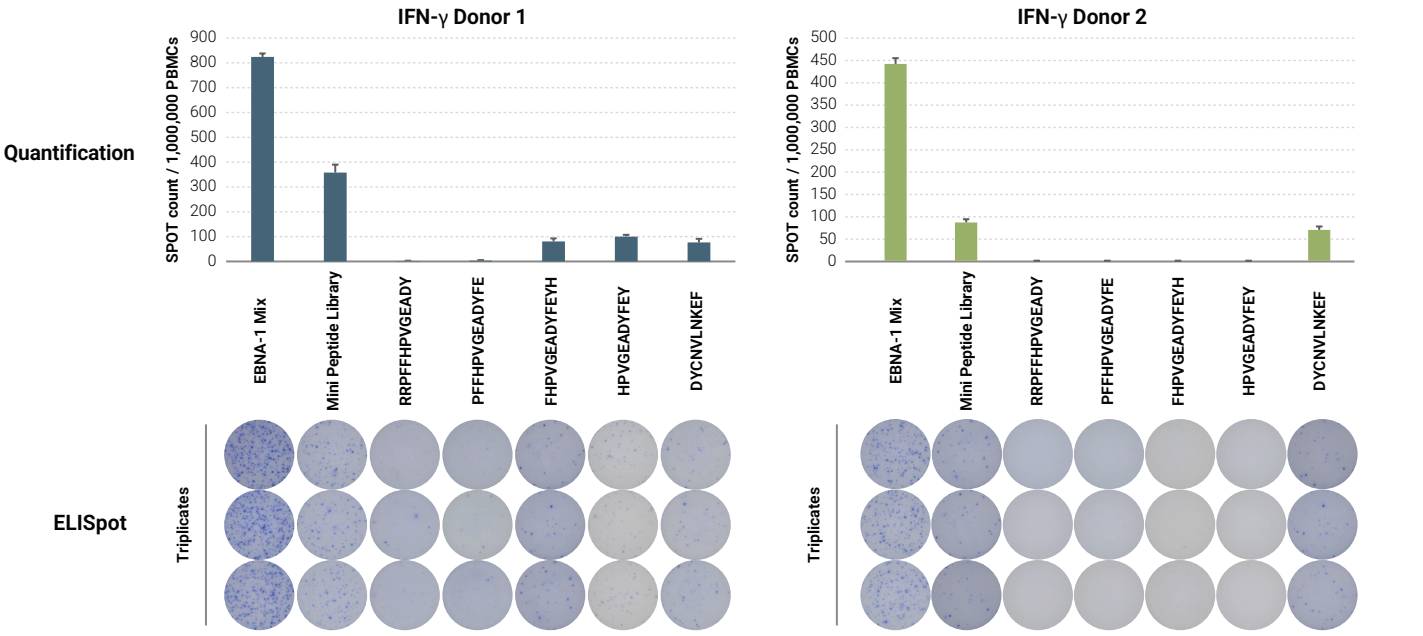

Figure 3. Discovery of T-cell receptor epitopes via functional assay. 500,000 PBMCs per well were stimulated in a 96-well IFN-γ ELISpot plate for 24 hours with a commercial EBNA-1 peptide mix (EBNA-1), the mini peptide library (Table 1) or the underlying single peptides in a final concentration of 10 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.

CONCLUSION

In conclusion, we were able to demonstrate that the combination of PEPperCHIP® Peptide Microarrays for antibody analysis and functional T-cell activation assays is ideally suited to unravel coincident B-cell receptor and T-cell receptor epitopes. The approach can be easily transferred to any other immunogenicity testing with custom antigens e.g. for vaccine development, cancer research or the analysis of anti-drug responses.

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