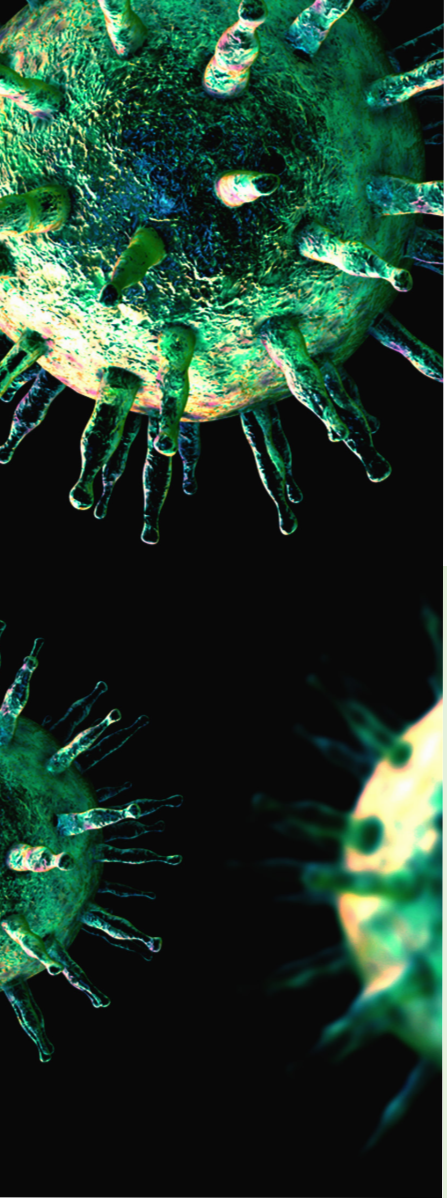

Application Note

Parean Biotechnologies
Deep Immune Phenotyping

PEPPERPRINT
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High-throughput Identification of Immunogenic B- and T-cell Epitopes Meets Deep Immune Cell Phenotyping

ABSTRACT

This application note describes an experimental workflow for the identification of immunogenic B- and/or T-cell epitopes in combination with an in-depth immune cell repertoire analysis. Applying high-density peptide microarrays combined with state-of-the-art ELISpot analyses discovered a highly immunogenic B- and T-cell overlapping epitope derived from the EBNA-1 protein of Epstein-Barr Virus (EBV). With a focus on T-cells, we next explored the T-cell receptor (TCR) immune repertoire specific to this epitope. With tailored-made dextramers, we sorted epitope-specific $CD8^+$ T-cells, sequenced the immune repertoire and analyzed the TCR alpha and beta chain structures with dedicated bioinformatic pipelines. Next-generation sequencing enabled the identification of TCRs specific to this EBNA-1-derived epitope. The gene composition and amino-acids structure analysis highlighted specific highly conserved motifs, signing a restricted clonotypic stimulation. The presented approach allows the discovery of TCR sequences that can be used as biomarkers and/or potential therapeutics for infectious diseases, vaccine development, and cell therapies.

INTRODUCTION

Identifying the antigenic determinants underlying an immune response and moreover, characterizing the cognate immune cell repertoires is of utmost importance for the development of vaccines and immunotherapies. In both areas, the synergy of humoral, B-cell mediated antibody responses and T-cell mediated cellular responses can be the base of success. Therefore, parallel investigation of both arms of the immune system is a conductive approach. In regards to humoral immunity, high-density peptide microarrays are a powerful tool to simultaneously screen tens of thousands of peptides against serum antibodies in a high-throughput context. Antibody responses to linear and conformational epitopes can rapidly be analyzed yielding high and low immunogenic epitopes with amino acid resolution (1).

For the evaluation of antigen-specific T-cell immunity and identifying T-cell epitopes, the enzyme-linked immune absorbent spot (ELISpot) assay is a gold standard (2). The assay determines the frequency of antigen-specific T-cells by detecting released activation markers such as cytokines and further effector molecules. In the ELISpot assay, T-cells are activated with antigens in PBMC culture, which allows binding to HLA molecules on antigen-presenting cells and presentation to T-cells. The method is very robust, accurate, and sensitive. It allows a quantitative single-cell analysis of low-frequency T-cells. Due to these features, ELISpots are widely utilized in immune diagnostics as well as diverse fundamental and clinical research areas that investigate specific immune responses (2).

For a deeper understanding of the antigen-mediated immune response and the development of therapeutic approaches such as immune cell therapy, analyzing the immune repertoire is of particularly high relevance (3). For T-cells, the specificity of the immune response is mediated by the T-cell receptor (TCR), which interacts with presented peptides through their complementary-determining-region-3 (CDR3) (4). The TCR is a heterodimer, composed of two paired chains whose loci are organized as gene segment families. By recombination, junctional diversity, and combinatory diversity, the TCR repertoire has the potential to be composed of more than 10^{19} different clonotypes, even estimated up to 10^{61}. Indeed, each individual has a unique TCR footprint, constantly impacted by new antigen challenges. In this way, the immune repertoire can serve as a molecular barcode for immunomonitoring in infection or cancer development. TCR sequencing

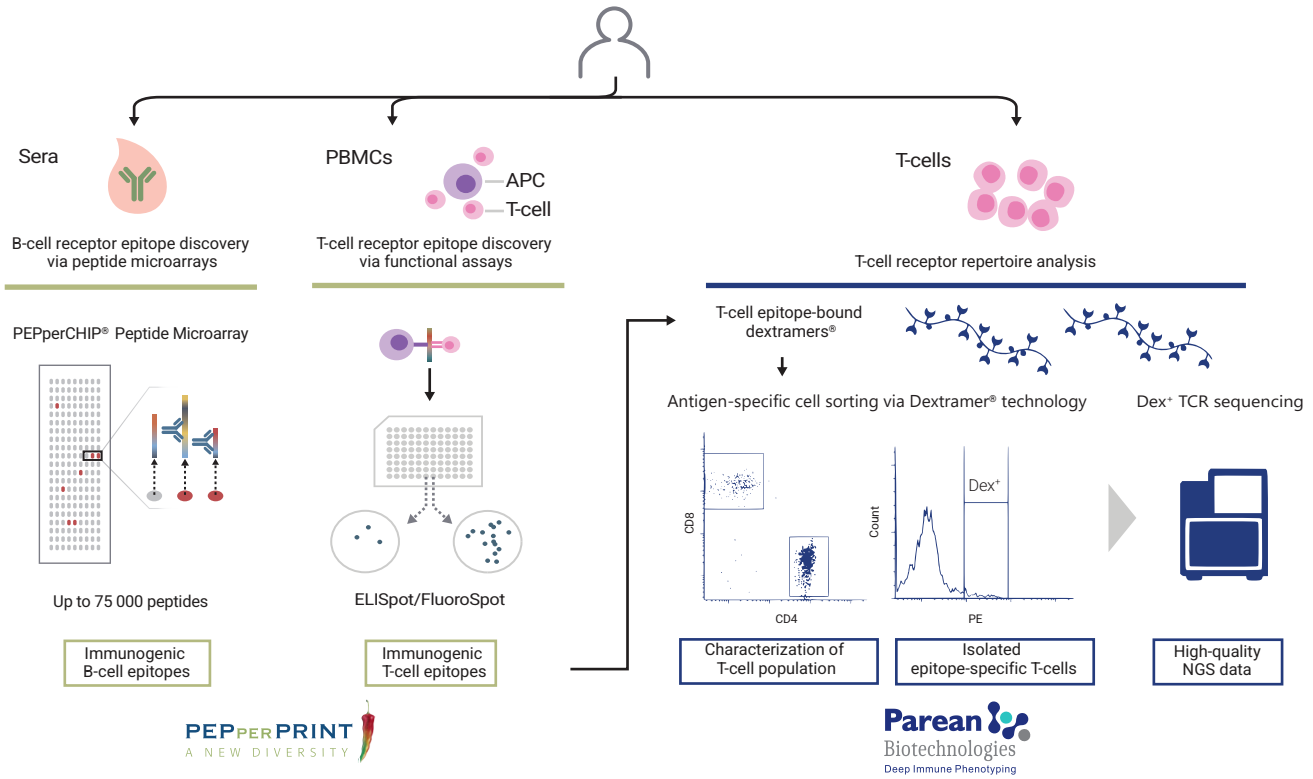

Figure 1. Combinational approach of PEPperPRINTs and Parean Biotechnologies competencies to unravel B-cell and T-cell epitopes and deep immune cell phenotyping. B-cell epitopes are identified via high-throughput screenings of sera for antibody-binding against tens of thousands of different peptides via PEPperCHIP® Peptide Microarrays. ELISpot assays in PBMC culture allow for testing peptides for T-cell antigenicity in 96 well plate format. For further analysis CD8⁺ epitope-specific T-cells are sorted with tailored-made dextramers®. Harvested epitope-specific T-cells are then used for immune repertoire analyses via next-generation sequencing of the TCR alpha and beta chain on dedicated bioinformatic pipelines.

at the single-cell level of epitope-specific T-cells can provide high valuable information for use in diagnostics or therapy (5, 1, 3).

In this study, we investigate immunity against Epstein-Barr virus (EBV) -encoded nuclear antigen-1 (EBNA-1). EBV is one of the most common human viruses (6). It establishes a persistent infection through latency in B-cells, where it occasionally reactivates. EBNA-1 is accused to play a role in EBV-associated complications and constitutes a marker for virus-associated cancer cells and thereby offering opportunities for targeted therapeutic intervention (6).

Here, we identified B-cell epitopes via PEPperCHIP® Epstein-Barr Virus Peptide Microarrays and further analyzed these for T-cell antigenicity via ELISpot assay.

Subsequently, identified epitope-specific T-cells were isolated via fluorescence-activated cell sorting. RNA from harvested cells was isolated, amplified, and sequenced, next-generation sequencing (NGS) data were analyzed to uncover epitope-specific T-cell receptor cognate repertoire. Applying this combinational approach, we discovered a highly immunogenic B- and T-cell overlapping epitope derived from the EBNA-1. TCR repertoire analysis highlighted a restricted clonotypic repertoire, with specific highly conserved motifs in the CDR3 sequences, signing an antigen-specificity.

RESULTS AND DISCUSSION

(1) Identification of an immunogenic B- and T-cell overlapping peptide in EBNA-1 of EBV

Initially, we mapped the humoral immune responses of an EBV-infected individual. To determine the infection-elicited antibodies against EBNA-1, we screened the serum of an EBV-infected, healthy individual with PEPperCHIP® Epstein-Barr Virus Peptide Microarrays (Figure 2). The peptide microarray contains 5,549 linear peptides of the most immunogenic EBV antigens printed in duplicates and were framed by poliovirus VP1 protein 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 revealed that one of the main B-cell epitopes was based on peptides with the consensus motif FHPVGEADY (individual peptides RRPFFHPVGEADY, PFFHPVGEADYFE, FHPVGEADYFEYH) (Figure 2).

To identify potential overlapping T-cell receptor epitopes within the identified B-cell receptor epitopes, peptides RRPFFHPVGEADY, PFFHPVGEADYFE, and FHPVGEADYFEYH were synthesized. Additionally, 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

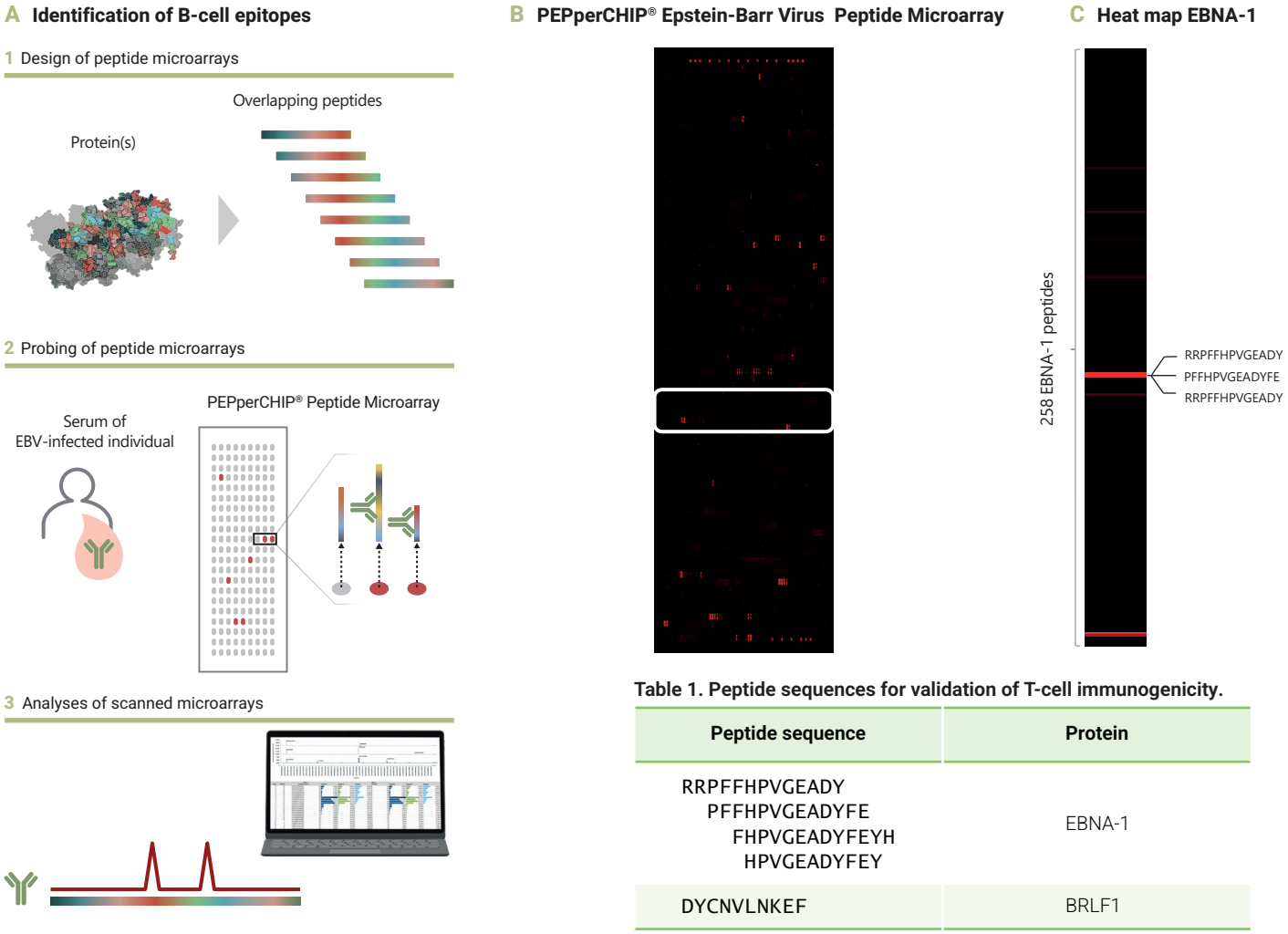

Table 1. Peptide sequences for validation of T-cell immunogenicity.

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

Figure 2. Discovery of B-cell receptor epitopes via peptide microarray. (A) Workflow of B-cell epitope mapping. Protein sequences of interest are subdivided into overlapping sequences spanning the whole protein. The microarray is incubated with sera and formed peptide-antibody complexes are visualized via fluorescently labeled secondary antibodies. Microarrays are scanned and fluorescent intensity is quantified (B) Scans of the PEPperCHIP® Epstein-Barr Virus Peptide Microarrays. The microarrays were incubated with the sera of an EBV-infected individual 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 highlights the position of the EBNA-1 peptides. (C) Heatmap of antibody response profile against 258 EBNA-1-derived peptides. Scanned images were analyzed with the PepSlide® Analyzer software. The heatmap shows the fluorescence intensities of 257 overlapping EBNA-1 peptides sorted from the N- to the C-terminus of the protein. Color code: black = FI below 200; red = FI above 3000.

HLA-B*3501 (7, 8), while peptide DYCNVLNKEF is known to activate T-cells in HLA-A*2402- expressing individuals (9).

Peptide-mediated T-cell activation was measured via interferon gamma (IFN-γ) ELISpot (Figure 3). Briefly, PBMCs were stimulated with the mini peptide library (Table 1) or with the single peptides. As positive control, PBMCs were activated with an EBNA-1 peptide mix containing 158 15-mer amino acid peptides with 11 amino acids peptide-peptide overlap. 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. The mini peptide library also activated T-cells. Based on the tests with the single peptides, this activation was due to immunogenicity of peptides FHPVGEADYFEYH, HPVGEADYFEY and DYCNVLNKEF. Peptides FHPVGEADYFEYH and HPVGEADYFEY induced T-cell activation, whereas RRPFFHPVGEADY and PFFHPVGEADYFE did not. Therefore, the core T-cell epitope seemed to depend on appropriate C-terminal sequences.

(2) Isolation of peptide-specific CD8⁺ T-cells

To subsequently investigate the TCR immune repertoire for the identified immunogenic EBNA-1 peptide, we first isolated antigen-specific CD8⁺ T-cells from an EBV-infected individual expressing HLA-B*3501, known to present HPVGEADYFEY (7, 8). As a control, we used CD8⁺ T-cells from an HLA-A*2402-expressing donor to investigate the DYCNVLNKEF specific repertoire (10). To purify peptide-specific T-cells, single cell suspension of human CD3⁺ T-cells were isolated from peripheral blood mononuclear cells (PBMCs) using negative immunomagnetic separation techniques. T-cells were labelled with recombinant antibodies and we sorted dextramer HPVGEADYFEY and DYCNVLNKEF specific cells, based on the following

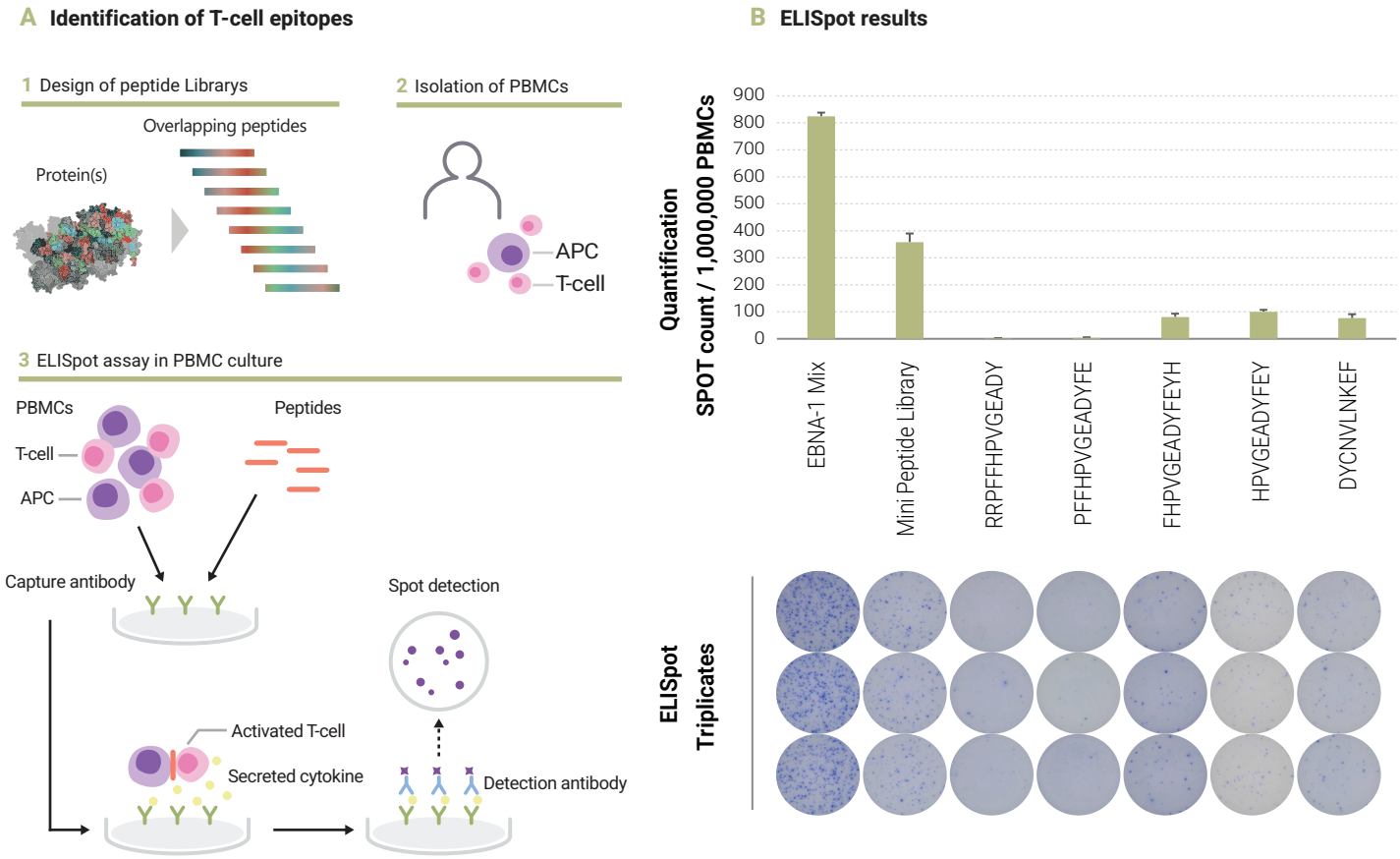

Figure 3. Discovery of T-cell epitopes. (A) Workflow of T-cell epitope identification. 1. Identification of immunogenic epitopes is facilitated via testing overlapping peptide libraries that span the whole protein sequence of interest. 2. PBMCs are isolated from the blood with lymphocyte separation media and density gradient centrifugation. 3. ELISpot assay determines the frequency of antigen-specific T-cells by detecting released activation markers such as cytokines via the sandwich enzyme-linked immunosorbent assay technique. In the assay, T-cells are activated with the peptides in PBMC culture. Immuncomplex formation mediates a color reaction, manifested as spots. Spots are quantified via an ELISpot reader. (B) 500,000 PBMCs per well were stimulated in a 96-well IFN-γ ELISpot plate for 24 hours with an 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. 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.

phenotypes: $CD3^+$/ $CD8^+$/ $CD4^-$/ Tet^+. For the HPVGEADYFEY, 1,431 cells were sorted. For the DYCNVLNKEF, 2,850 cells were sorted (Figure 4). The phenotype of the virus-specific $CD8^+$ T-cells showed that the response towards EBV-peptides consisted mainly of activated $CD8^+$ T-cells ($CD3^+$ / $CD8^+$ / $CD4^-$/ $CD45RA^-$).

(3) TCR repertoire analysis

The TCR is a transmembrane heterodimer composed of two glycoprotein chains: nearly 95% of T-cells express the α and β chain dimer and 5% carry the γ and δ chains (3). The hypervariable or "Complementary Determining Regions" (CDRs) which are involved in contact with the pHLA complex and in the antigen-recognition are localized in the N-terminal domains of the TCR chains. Each chain consists of three CDRs. The sequence of the CDR1 and CDR2 depend on the V gene, while the CDR3 is the product of the recombination of the V and J segments for the α chain and V, D and J segments for the β chain, respectively. The CDR3 region on α and β chains are respectively the result of the VJα or VDJβ junction and recombination region. Thereby, the CDR3 exhibits the greatest degree of variability of a TCR. By recombination, junctional diversity and combinatory diversity, the TCR repertoire has the potential to be composed of more than 10^{19} clonotypes, even estimated up to 10^{61} clonotypes (11,12).

Deep sequencing of TCRα and TCRβ repertoires was performed as described previosly (13) on sorted dextramer® $CD8^+$ positive T-cells. Briefly, RNA was extracted, reverse transcription was perfomed and further extended with a template-switching oligonuclotide. cDNAs were then amplified following two semi-nested PCRs and then purified to produce libraries, sequencing adapter and sample index sequences were added. Libraries were sequenced using 2x250bp read length on MiSeq instrument (Illumina®). FASTQ raw data were aligned using MiXCR providing error correction and occurrence of each unique TCR as well as alignment and sequence quality scores (14). Identical TCR sequences will be grouped as clonotypes (TRV-CDR3nt-TRJ) and the relative frequencies of each clonotyoe will be assessed.

We first analyzed the V and J genes usage in TRA and TRB repertoires of HPVGEADYFEY and DYCNVLNKEF-specific $CD8^+$ T-cells (Figure 5). As expected, HPVGEADYFEY and

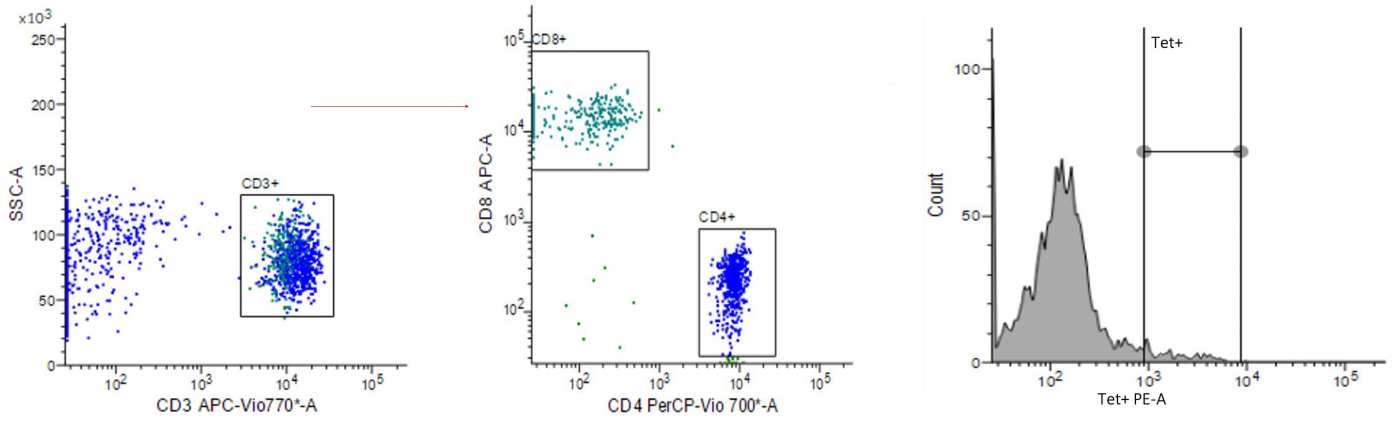

Figure 4. Gating strategy for antigen-specific CD8⁺ T-cell sorting. Cells were gated in an FSC-H and FSC-A dot plot to eliminate doublets. Lymphocytes were selected by a SSClow gate which also excluded debris. T-lymphocytes were gated on the expression of CD3 (left plot). CD8⁺ T-cells were identified by expression of CD4⁻ and CD8⁺ (middle plot). The Tet⁺ cells are the CD8⁺ antigen-specific cells that are sorted for NGS analysis (right plot).

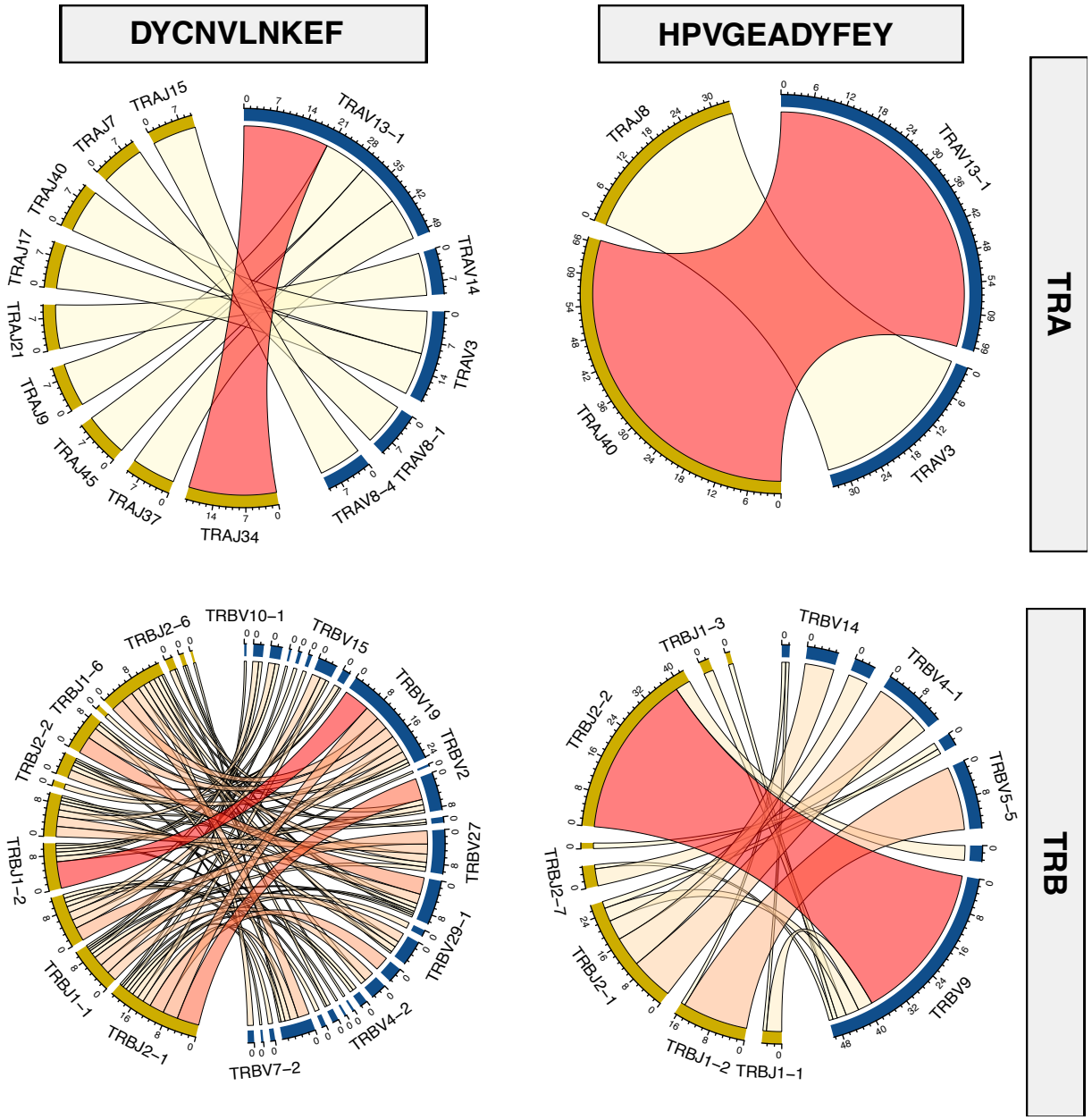

Figure 5. EBV specific V–J gene usage. Chord diagram showing frequency (%) of the given V and J usage for HPVGEADYFEY (right) and DYCNVLNKEF (left). TRA (top) and TRB (bottom) repertoires are represented by the two peptides. Only V (blue) and J (gold) genes that are detected are represented. The colors and sizes of the segments represent frequencies.

DYCNVLNKEF-specific CD8+ TCR repertoires demonstrated inter-individual differences. The frequency of TRAV, TRAJ, TRBV and TRBJ genes found in tetramer-sorted cells varied greatly. The HPVGEADYFEY-specific TCR repertoire showed a predominant expression of TRAV13/ TRAJ40 for α chain and TRBV9/ TRBJ2-2 for the β chain of the TCR while the DYCNVLNKEF-specific TCR repertoire showed a predominant expression of TRAV13-1/ TRAJ34 for the alpha chain and TRBV19/ TRBJ1-2 for the beta chain with a broad expression of other TRBV and TRBJ combination.

Next, spectratyping analysis was carried out to further examine the CDR3 regions of tetramer-positive T-cells (Figure 6). A typical Gaussian distributions of CDR3β lengths was observed for DYCNVLNKEF tetramer-positive T-cells. Whereas DYCNVLNKEF-specific CDR3α lengths are a bit more restricted. On the other hand, in a very clear way, the HPVGEADYFEY-specific TCR α and β spectratypes showed a highly restricted non-parametric distribution. Spectratypes of CDR3α and CDR3β show the presence of expanded clonotypes of 15 and 12 amino acids in length, respectiveley with preferential use of the V genes TRAV13-1 (66%) and TRBV9 (45%), describing a highly oligoclonal α and β repertorie.

CDR3s regions were examined for conserved amino acid residues. The TRB chain, CDR3β of 12 amino acids, is represented as a logo in Figure 7 (right). In the hypervariable region, a motif composed of a hydrophobic amino acid on position 5, a basic amino acid on position 6 and polar amino acids on positions 7 and 8 was found in 76% of the CDR3β. For the TRA chain, CDR3α 15 amino acids, is represented as a logo in Figure 7 (left). There is a broader diversity but a hydrophobic position at position 6, a polar region from amino acid 7 to 11 and a basic position at amino acid 12 were found for 80% of the CDR3α.

These results are important to develop new strategies for infectious disease biomarkers and therapeutics. Based on these analyses, T-cell-based tests can be developed to quantify virus-specific T-cell immune responses. TCR repertoire can be employed to monitor the response to vaccines down to the clonal level. This approach paves the way for the use of the TCR repertoire as a novel powerful biomarker to track exposure, predict the vaccine response and monitor the immune response of individuals to antigens.

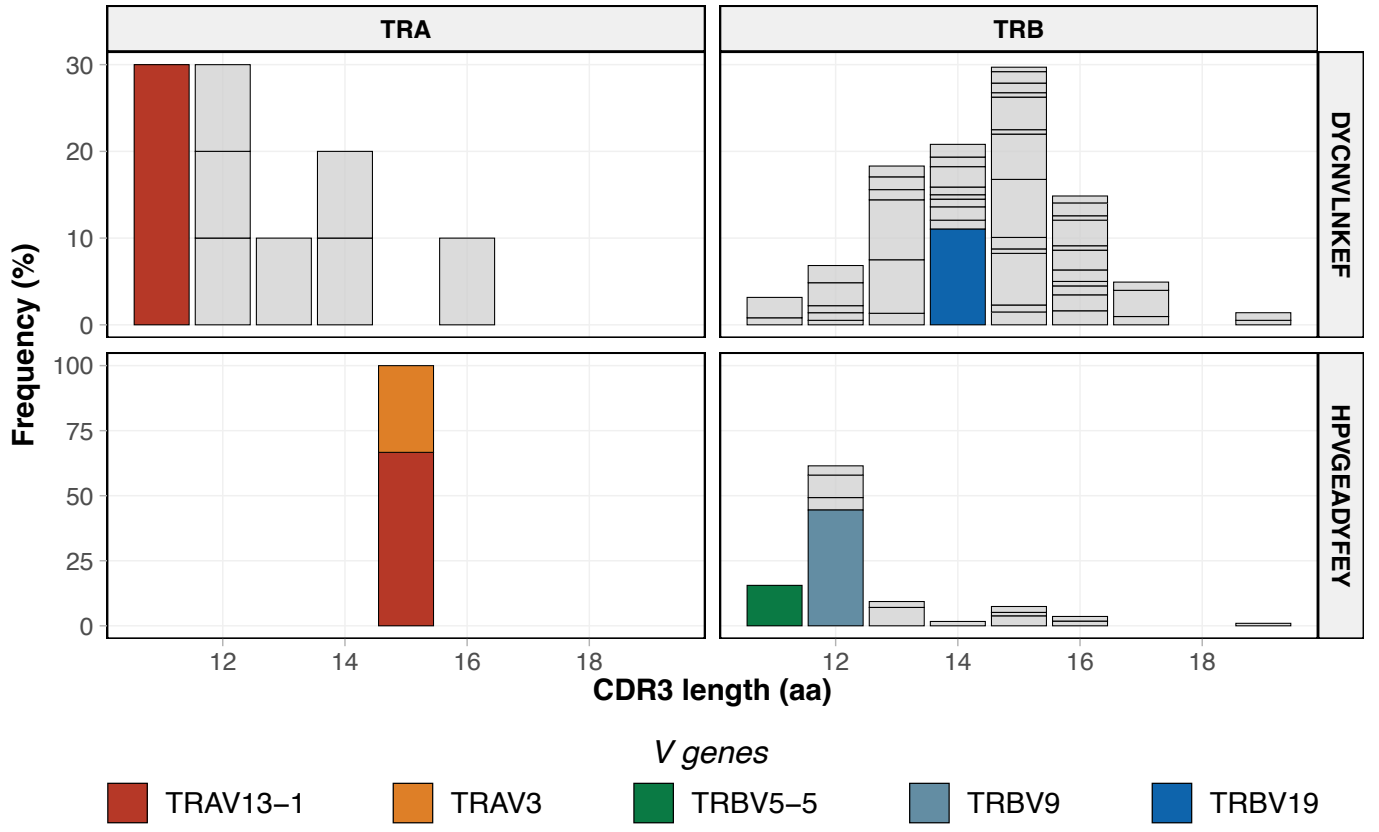

Figure 6. Spectratyping analysis of TCR CDR3α and CDR3β. On the left, the DYCNVLNKEF CDR3β (up) presents a classical Gaussian distribution. The CDR3α lengths are depicted below are a bit more restricted. For the HPVGEADYFEY specific CDR3, the distribution of the CDR3α (down) and CDR3β (up) are highly restricted. For both α and β, the spectratype highlight the presence of expanded clonotypes of respectively 15 and 12 amino acid length.

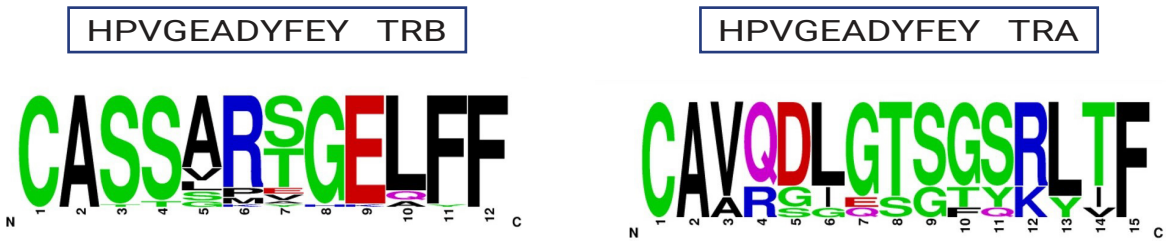

Figure 7. Structure of the TRB and TRA CDR3 of the most expanded TCR. Frequency plot for the sequences of CDR3 β (left) and α (right) chain motifs found in tetramer-positive T-cell repertoires.

CONCLUSIONS

In conclusion, we were able to demonstrate that the combination of PEPperPRINT T-cell activation assays and Parean® T-cell repertoire tools are complementary to decipher the T-cell receptor repertoire in immune response. This approach can be transferred to any other applications for vaccine development, cancer research or the analysis of anti-drug responses, e.g. immunogenicity prediction.

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