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Technical Report

Oligonucleotide library screening for identification of virus-specific T-cell receptors

T cells have critical roles in a variety of malignant, infectious, inflammatory, and autoimmune conditions. While T-cell infiltration of tumors can correlate with therapeutic responses and prognosis, antigen specificities of disease-infiltrating T cells remain largely unknown [1, 2]. Characteristic immune phenotypes, especially of clonally expanded T cells, suggest reactivity with antigens associated with persistent viral infections such as Epstein–Barr virus (EBV) or cytomegalovirus. Indeed, expanded T-cell clones in tumor environments and autoimmune diseases have been determined virus reactive [3–6]; however, there is no straightforward approach to systematically study the virus reactivity of a given set of T-cell clones.

A variety of elegant methodologies promise the identification of unknown T-cell receptor (TCR) ligands [7–9]; however, these methodologies are laborious and not applicable for screening larger numbers of TCRs. Techniques for up-front identification of virus reactivity would be beneficial and allow focusing on TCRs of interest for sophisticated downstream analyses.

We present an oligonucleotide library-based approach that allows systematic screening of selected TCRs for virus reactivity, without specification of individual target epitopes, using EBV as an example (schematic in Fig. 1A).

An efficient screening approach required optimized oligonucleotide library design in combination with highly sensitive detection of T-cell activation. We aimed to construct an oligonucleotide library covering the entire EBV proteome with partially overlapping equally sized oligonucleotide fragments. We chose EBV as a proof-of-principle because of its proteome size and high prevalence. Previously published elegant approaches for the definition of EBV-reactive TCRs could not cover the entire viral proteome [10]. For antigen presentation, the oligonucleotide library and HLA allele in question were expressed in HEK293T that lacked HLA-A, HLA-B, and HLA-C expression (named $HEK293T_{HLA\text{-}KO}$) [3]. To maximize library diversity and sensitivity of T-cell activation detection, we (1) determined the optimal length of oligonucleotide library fragments, (2) confirmed reproducible library cloning into expression vectors, and (3) compared sensitivities of five T-cell activation read-outs. Finally, we applied the optimized methodology for screening six EBV-specific TCRs as an example.

The library design considered the following: (1) the larger the size of each oligonucleotide the lower the total number of oligonucleotides required to cover the EBV proteome resulting in a higher representation of each oligonucleotide in the final library, and (2) assay compatibility with straight-forward DNA preparation and deep sequencing at a reasonable cost.

To determine the effects of the length of library oligonucleotides on T-cell activation, we designed minigenes encoding the EBV BRLF1-derived epitope YVLDHLIVV (YVL) presented on HLA-A*02:01 as part of 9, 15, 30, or 50 amino acid long peptides (Supporting information Table S1). Minigenes were cloned into pcDNA6/V5-His A and transfected into $HEK293T_{HLA\text{-}KO}$ together with pHSE3' encoding HLA-A*02:01 linked to BFP. Recombinant HLA expression was consistent between different transfections (Supporting information Fig. S1). To determine T-cell activation, minigene-transfected $HEK293T_{HLA\text{-}KO}$ were co-cultured with a YVL-specific TCR [11] expressed in the $58\alpha^{-}\beta^{-}$ reporter cell line [12], named 58-YVL. 58-YVL were grown from a single-cell clone [12] to ensure consistency on the T-cell component of the assay. T-cell activation was determined by NFAT-driven GFP expression within 58-YVL (gating in Supporting information Fig. S2). We detected the strongest T-cell activation in the context of minigenes encoding peptides of 9 or 50 amino acids in length (Fig. 1B), whereas 15 or 30 amino acid minigenes resulted in comparatively weak T-cell activation (Fig. 1B).

To exclude that differences in T-cell activation were due to varying transfection efficiency, we linked the different-length YVL-encoding minigenes to mCherry separated by T2A (Supporting information Table S1), cloned them into pcDNA6/V5-His A, and repeated the co-culture experiments. HLA and minigene expression were consistent between transfections (Supporting information Fig. S3), and, in line with the previous experiment, target minigenes encoding 9 or 50 amino acids resulted in strongest T-cell activation. Increased T-cell activation by 15 or 30 amino acid minigenes linked to mCherry compared with identical fragments without mCherry (Fig. 1B and C) was most likely due to inevitable elongation of the fragments up to the T2A cleavage site with potential effects on antigen processing.

We concluded that minigenes with a length of 50 amino acids enabled the strongest T-cell activation and reasonable library design to cover larger proteomes.

To construct an oligonucleotide library covering the entire EBV proteome (called "EBV library"), 1649 oligonucleotides (50 amino acids in length, 25 amino acids overlap between adjacent fragments) were synthesized and cloned into pcDNA6/V5-His A in frame with T2A-mCherry (schematic in Fig. 1A, Supporting information Fig. S4, Supporting information Appendix).

Assuming an equal distribution, frequencies of individual oligonucleotides within the EBV library were expected

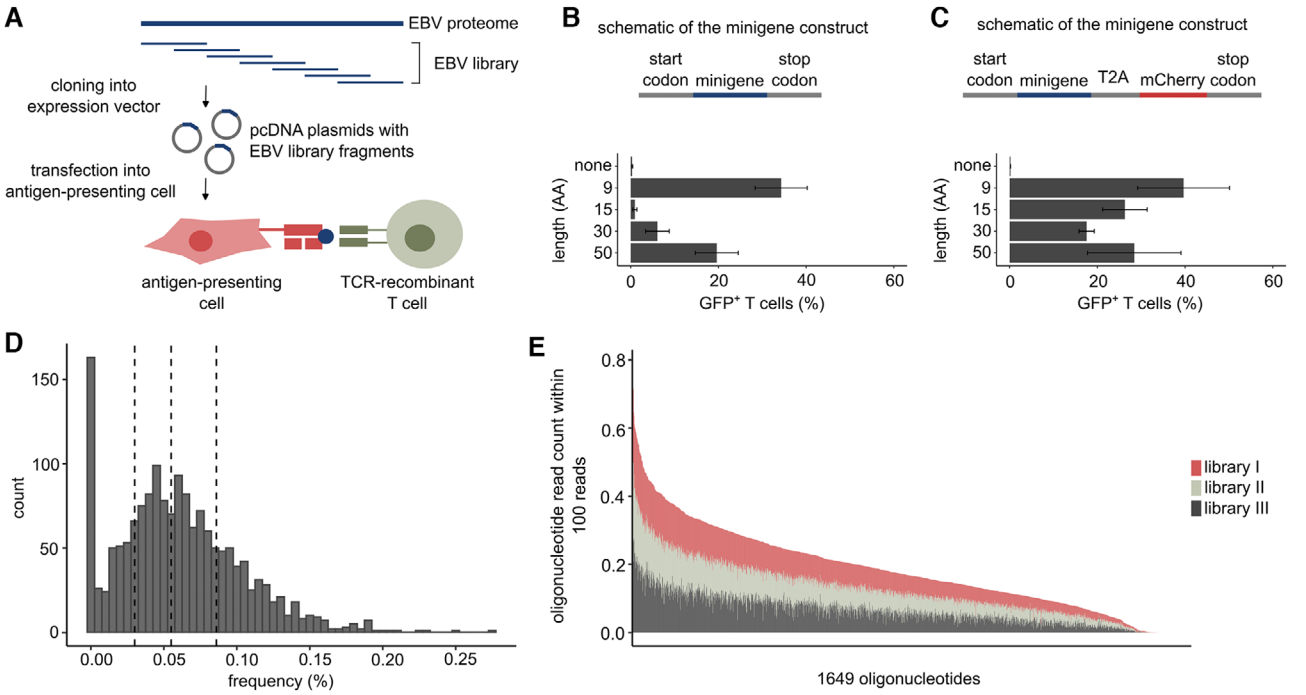

Figure 1. EBV library construction, cloning, and quality assessment. (A) Experimental strategy. Overlapping oligonucleotide fragments were cloned into pcDNA6/V5–His A and co-transfected with plasmids encoding the respective HLA allele (not shown) into HEK293T$_{HLA-KO}$. Activation of TCR-recombinant T cells indicated antigen recognition. (B and C) Determination of the optimal length of oligonucleotide minigenes. 58-YVL were co-cultured with HEK293T$_{HLA-KO}$ co-transfected with a plasmid encoding a YVL-minigene (without T2A-mCherry in (B) and linked to T2A-mCherry in (C)) of different length and a plasmid encoding HLA-A*02:01. In conditions labeled with "none", HEK293T$_{HLA-KO}$ were only transfected with the HLA-A*02:01 plasmid. Bar charts indicate mean ± SD of $n = 2$ independent experiments with three replicates per experiment. AA indicates amino acids. (D) Frequencies of individual oligonucleotide fragments of one EBV library as a representative example. Dashed lines indicate 25th percentile, median, and 75th percentile. (E) Counts of individual oligonucleotide fragments within 100 reads of three independently cloned EBV libraries. Library fragments were ordered by cumulative count (highest to lowest).

at 0.060%. To investigate the consistency of the library cloning process, we determined the representation of individual oligonucleotides within three independently cloned EBV libraries by deep sequencing (primers in Supporting information Table S2). On average 93.8% of total library oligonucleotides were detectable, and frequencies were determined with 0.055 ± 0.002% (median ± SD; Fig. 1D). Individual fragments showed similar frequencies within all three libraries and those not expressed in one library were likely not to be detected in the other libraries (Fig. 1E). Under- and overrepresentation of selected fragments was rather due to the nature of the individual fragments (e.g. potentially inefficient synthesis or limited PCR amplification due to repetitive sequences, among others) than random inconsistencies during the cloning process. Detailed frequencies of fragments of all three libraries can be found in the Supporting information Appendix.

In conclusion, oligonucleotide library production delivered consistent results across independent experiments. Screening individual TCRs against diverse oligonucleotide libraries required highest possible sensitivity for detection of T-cell activation. The sensitivity of TCR-transduced human T cells can be assumed to be substantially higher than 58$\alpha^-\beta^-$ cells due to species compatibility and efficient co-stimulation. Therefore, TCRs of interest were transduced into human T cells as previously described [3, 11, 13] and tested for activation in co-culture with our EBV library.

To identify the most sensitive T-cell activation read-out, we compared the detection of the following parameters: IFN-γ and granzyme B secretion by ELISA, as well as intracellular IL-2, intracellular IFN-γ, or cell surface CD137 expression by flow cytometry. All parameters were determined after co-culture of YVL-specific TCR-transduced human T cells (named PBL-YVL, Supporting information Table S3) with HEK293T$_{HLA-KO}$ co-transfected with (1) the 50 amino acid target minigene and the respective HLA allele as a positive control, or (2) EBV library and the respective HLA allele to determine whether sensitivity of the assay was sufficient if the target epitope was expressed as part of a diverse oligonucleotide library (Supporting information Table S4).

Activation of PBL-YVL by HEK293T$_{HLA-KO}$ co-transfected with the target epitope minigene and the respective HLA allele was reliably detected by all read-outs except intracellular IL-2 staining (Fig. 2A, gating strategy in Supporting information Fig. S5). Activation of PBL-YVL with EBV library-transfected cells was detectable by granzyme B in cell culture supernatants, intracellular IFN-γ staining, and, in our hands most robustly, cell surface CD137 staining (Fig. 2A). Based on these data, we chose CD137 staining for detection of T-cell activation in the following experiments.

For a proof of principle, we expressed six EBV-specific TCRs (restricted for HLA-A*02:01 or HLA-B*35:01, Supporting Information Table S3) [11] in human lymphocytes. As a positive control, TCR-transduced human lymphocytes were cultured with HEK293T$_{HLA-KO}$ and co-

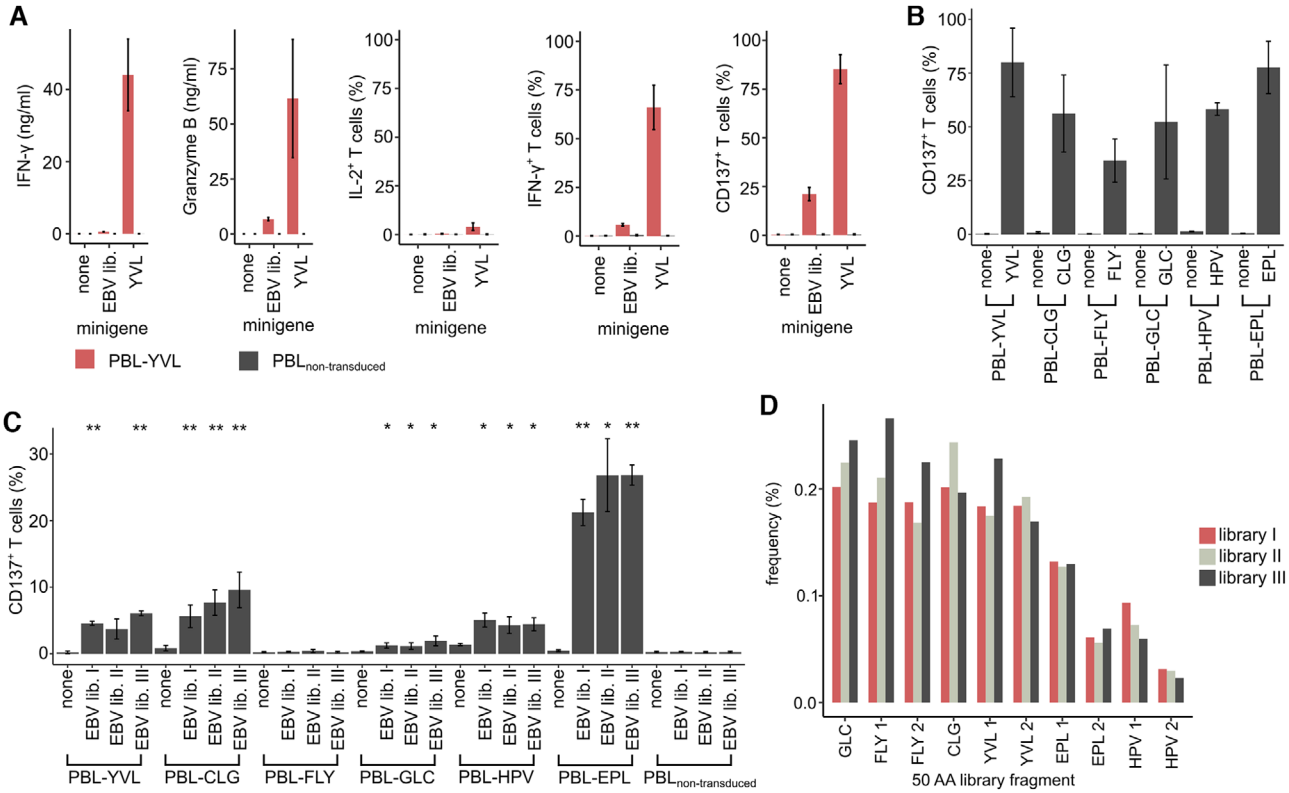

Figure 2. Sensitive detection of T-cell activation and EBV library screening. (A) PBL-YVL were incubated with HEK293T$_{HLA\text{-}KO}$ co-transfected with either the EBV library or the YVL-minigene, and a plasmid encoding HLA-A*02:01. T-cell activation was determined by IFN-γ or granzyme B ELISA of cell culture supernatants or by intracellular IL-2, intracellular IFN-γ, or cell surface CD137 expression detected by flow cytometry. Bar charts indicate mean ± SD of one experiment with $n = 3$ replicates. (B) EBV-specific TCR-transduced human lymphocytes were incubated with HEK293T$_{HLA\text{-}KO}$ co-transfected with the target epitope minigene and the appropriate HLA allele. To detect T-cell activation, CD137 expression on live TCR-transduced CD8$^+$ T cells was determined by flow cytometry. (C) EBV epitope-specific TCR-transduced human lymphocytes were incubated with HEK293T$_{HLA\text{-}KO}$ expressing the EBV library and the appropriate HLA allele. In conditions labeled with "none", HEK293T$_{HLA\text{-}KO}$ were only transfected with the plasmid encoding the respective HLA allele. CD137 expression on live TCR-transduced CD8$^+$ T cells was determined by flow cytometry. Bar charts indicate mean ± SD. Data are representative of $n = 3$ (PBL-YVL, PBL-CLG, PBL-FLY, PBL-HPV, PBL-EPL) and $n = 4$ (PBL-GLC, PBL$_{non\text{-}transduced}$) independent experiments. Statistical significance was determined by paired Student's *t*-test. $^*p < 0.05$; $^{**}p < 0.01$. (D) Frequencies of selected library oligonucleotides determined by deep sequencing of three independently cloned EBV libraries. Due to overlap between neighboring oligonucleotides, target epitopes could be part of one or two oligonucleotides indicated with "1" and "2".

transfected with minigenes containing the respective target epitope and HLA allele. As expected, all TCR-transduced human lymphocytes were activated by antigen-presenting cells transfected with the respective target minigene and HLA allele (Fig. 2B, activation after peptide stimulation for comparison in Supporting information Fig. S6).

Subsequently, we cultured human lymphocytes individually transduced with six different EBV-specific TCRs with HEK293T$_{HLA\text{-}KO}$ co-transfected with EBV library and the respective HLA allele. For screening TCRs with unknown specificity and HLA restriction, HEK293T$_{HLA\text{-}KO}$ could be transfected with all potential target HLA alleles simultaneously. Five out of six TCR-transduced cell lines showed activation above background (Fig. 2C). Background activation could be assumed to depend on TCR sequence, transduction batch, and T-cell donor, and had, therefore, to be determined for each TCR individually. We could not detect activation of PBL-FLY although target epitopes of all tested TCRs (including FLY) were present within the library (Fig. 2D). Of note, activation of PBL-FLY by the target minigene was already lower compared with the other five tested TCRs (Fig. 2B), and a possible explanation could be preferentially immunoproteasome-dependent processing [14], competition with other epitopes encoded by the oligonucleotide library, or target epitope-flanking nucleotide sequences [15].

In conclusion, we provide systematic data on oligonucleotide library design in combination with a T-cell assay for sensitive detection of activation by an oligonucleotide library. The methodology can be applied to screen TCRs of choice for EBV reactivity. We are aware that nonreactivity of a given TCR cannot exclude specificity for epitopes included in the library; however, our approach delivers consistent results and enables systematic screening of larger numbers of TCRs at a reasonable workload. If working with substantially larger libraries would be desired, splitting oligonucleotides into multiple libraries could be considered. The methodology can potentially be expanded to other viruses, for example, cytomegalovirus, provides valuable information upon the design of any oligonucleotide library of choice in combination with assays for T-cell activation and will contribute to our understanding of T-cell immunology in various physiological and disease conditions.

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Abbreviations: **EBV**: Epstein–Barr virus · **GFP**: green fluorescent protein · **mTagBFP2, BFP**: methyltransferase-directed transfer of activated groups blue fluorescent protein 2 · **NFAT**: nuclear factor of activated T cell · **TCR**: T-cell receptor

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