SUPPORTING INFORMATION

SUPPORTING METHODS

Healthy donor samples

The study was approved by the local institutional review board (*Ethikkommission der Charité – Universitätsmedizin Berlin*, protocol EA2/096/15 to LH). All participants provided written informed consent, and all research was conducted in accordance with the Declaration of Helsinki. Mononuclear cells for TCR transduction were isolated using Ficoll-Paque PLUS (GE Healthcare) and cryopreserved in RPMI-1640 (Thermo Fisher Scientific) with 50% fetal bovine serum (FBS, Thermo Fisher Scientific) and 10% dimethyl sulfoxide (DMSO, Carl Roth GmbH) before further processing.

Cloning of the EBV library

Amino acid sequences of the Epstein-Barr virus proteome (strain B95-8) were downloaded from UniProt (proteome ID UP000153037), separated into 50 amino acid long fragments with an overlap of 25 amino acids between adjacent fragments and reverse translated using EMBOSS (https://www.ebi.ac.uk/Tools/st/emboss_backtranseq/). An adapter sequence (TACCGGACTCAGAT), the XhoI restriction site and a start codon were added to the 5' end of each fragment. At the 3' end of each fragment, a cytosin for in-frame cloning with mCherry, the EcoRI restriction site and an adapter (TGCAGTCGACGGA) were added. Minigene constructs were synthesized as oligo pools (Twist Bioscience) and cloned into pcDNA6/V5-His A, which, if indicated, had previously been modified to encode mCherry linked via a sequence containing T2A (**GAATTC**TGCAGTCGACGG<u>AAGGCAGAGGATCTCTGCTGACATGCGGAGATGTGG AAGAGAACCCCGGACCT</u>GGGGATCCACCGGTC; EcoRI in bold; T2A underlined) at the 3' end of the insert. 10 ng of the Twist Bioscience oligo pool was amplified in 10 cycles using KAPA polymerase (Roche), purified using the PCR Purification Kit (Qiagen) and digested with

XhoI and EcoRI (both NEB) for 1 hour at 37 °C. pcDNA6/V5-His A was sequentially digested with XhoI and EcoRI (NEB) with an intermediate XhoI heat inactivation step at 65 °C for 20 minutes and dephosphorylated using rSAP (NEB). The digested vector and library fragments were purified using the PCR Purification Kit (Qiagen) and ligated overnight using T4 DNA Ligase (Invitrogen). The ligation product was purified by sodium acetate precipitation and transformed into 10-beta electrocompetent E. coli (NEB) according to the manufacturer's instructions. After 50 min of cultivation, 10 μl of the 1 ml competent cell suspension were diluted 1:5000 and 50 μl were plated to assess the number of transformed plasmids and therefore the representation of the library during cloning. At least 1000-fold coverage of each library fragment was maintained within each library throughout the cloning process. The remaining E. coli were transferred to 100 ml LB medium and cultured overnight. DNA was isolated using the NucleoBond Xtra Midi kit (Macherey-Nagel).

Cloning of minigenes

Minigenes not linked to mCherry were constructed as follows (5' → 3'): XhoI restriction site, start codon, minigene, stop codon, EcoRI restriction site. Minigenes linked to mCherry were constructed as follows (5' → 3'): XhoI restriction site, start codon, minigene, a cytosin for in-frame cloninig with mCherry, EcoRI restricition site. They were cloned into pcDNA6/V5-His A without mCherry or with mCherry, respectively, as described above.

EBV library sequencing and data processing

For deep sequencing, the EBV library was prepared by two PCR reactions adding library-specific indices, and P5 and P7 adapter sequences (Supporting Information Tab. 2). Libraries were sequenced 150 basepairs paired end using a 300 cycles v2 MiSeq Reagent Kit (Illumina). Reads were assigned to individual library preparations based on Illumina indices by bcl2fastq (https://emea.support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-

software.html). Forward and reverse reads were assembled using PEAR[1]. We selected all reads in which we identified a reading frame by 5' ATG and an in frame stop codon at the 3' end. Reads were translated into amino acid sequences and those that perfectly matched sequences of the constructed EBV library were counted for further analyses.

Recombinant HLA expression

HLA coding sequences (IPD accession numbers: HLA00005, HLA00237) were downloaded from IPD-IMGT/HLA (https://www.ebi.ac.uk/ipd/imgt/ hla/), synthesized (GeneArt, Life Technologies), and cloned into pHSE3' in-frame with mTagBFP2 as previously described [2]. HEK293T$_{HLA-KO}$ were transfected with FuGENE® HD (3:1 FuGENE® HD Transfection Reagent:DNA, Promega).

Transfection of minigene, HLA, and library constructs into antigen-presenting cells

We used FuGENE HD (Promega) for transfection according to the manufacturer's instructions. FuGENE HD delivers multiple plasmids per cell [2] resulting in high frequencies of cells presenting the potential target epitopes.

Recombinant TCR expression on human primary lymphocytes

Recombinant TCR expression in human lymphocytes was done as previously described [3, 4].

Co-cultures

HEK293T$_{HLA-KO}$ were transfected with minigene and HLA constructs 36 hours before co-culture experiments. 1.5×10^5 HEK293T$_{HLA-KO}$ were co-cultured with 1.5×10^5 human T cells or 1.2×10^5 TCR-recombinant $58\alpha^-\beta^-$ cells in 500 µl medium (RPMI-1640 supplemented with 10% FBS, 10,000 U/mL penicillin, and 10 mg/mL streptomycin) in a 24-well plate for 24 hours at 37 °C and 5% CO_2. Activation of TCR-transgenic $58\alpha^-\beta^-$ cells was determined by GFP

fluorescence detected by flow cytometry. Activation of primary human T cells was measured by detection of intracellular IL-2, IFN-γ, or cell surface CD137 expression by flow cytometry (flow cytometry reagents in Supporting Tab. 4), or by measurement of IFN-γ and granzyme B in cell culture supernatants by ELISA (IFN-γ ELISA Set BD OptEIA, BD Biosciences; human granzyme B DuoSet ELISA kit R&D Systems).

Flow cytometry

For intracellular staining, we used the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer's instructions. Analytical flow cytometry was performed on an Aurora (Cytek) instrument. Flow cytometry and data visualization were done following the Guidelines for the use of flow cytometry and cell sorting in immunological studies [5, 6].

Statistics

Statistics were calculated using R (R core team) version 4.0.3 [7]. Test details and P value thresholds are indicated in all figure descriptions.

SUPPORTING TABLES

Supporting Information Table 1

Label	Length (amino acids)	Nucleotide sequence
YVL_9	9	ATGTACGTGCTGGACCACCTGATCGTGGTGTAG
YVL_15	15	ATGGTGATGAGGTACGTGCTGGACCACCTGATCGTGGTGACCGACAGGTAG
YVL_30	30	ATGAACGACGCCTGCAGCATCGCCTGCCCCATCGTGATGAGGTACGTGCTGGACCACCTGATCGTGGTGACCGACAGGTTCTTCATCCAGGCCTGA
YVL_50	52	ATGAACTTCTGGGCCATCCTGAGGAACAACAGGGTGAGGAGGAGGGCCGAGAACGCCGGCAACGACGCCTGCAGCATCGCCTGCCCCATCGTGATGAGGTACGTGCTGGACCACCTGATCGTGGTGACCGACAGGTTCTTCATCCAGGCCCGAATTCAGTAG
YVL_9_T2A	32	ATGTACGTGCTGGACCACCTGATCGTGGTGCGAATTCTGCAGTCGACGGAAGGCAGAGGATCTCTGCTGACATGCGGAGATGTGGAAGAGAACCCCGGACCTGGGGATCCACCGGTC
YVL_15_T2A	38	ATGGTGATGAGGTACGTGCTGGACCACCTGATCGTGGTGACCGACAGGCGAATTCTGCAGTCGACGGAAGGCAGAGGATCTCTGCTGACATGCGGAGATGTGGAAGAGAACCCCGGACCTGGGGATCCACCGGTC
YVL_30_T2A	53	ATGAACGACGCCTGCAGCATCGCCTGCCCCATCGTGATGAGGTACGTGCTGGACCACCTGATCGTGGTGACCGACAGGTTCTTCATCCAGGCCCGAATTCTGCAGTCGACGGAAGGCAGAGGATCTCTGCTGACATGCGGAGATGTGGAAGAGAACCCCGGACCTGGGGATCCACCGGTC
YVL_50_T2A	72	ATGAACTTCTGGGCCATCCTGAGGAACAACAGGGTGAGGAGGAGGGCCGAGAACGCCGGCAACGACGCCTGCAGCATCGCCTGCCCCATCGTGATGAGGTACGTGCTGGACCACCTGATCGTGGTGACCGACAGGTTCTTCATCCAGGCCCGAATTCTGCAGTCGACGGAAGGCAGAGGATCTCTGCTGACATGCGGAGATGTGGAAGAGAACCCCGGACCTGGGGATCCACCGGTC

Supporting Information Table 1. YVL minigenes constructs.

The length indicates the number of amino acids between the start codon and the stop codon for constructs without T2A and between the start codon and the T2A cleavage site for constructs containing T2A. Nucleotide sequences extend from the start codon to the stop codon for constructs without T2A or from the start codon to the beginning of the mCherry sequence for constructs with T2A. The sequence encoding YVL is highlighted in red, the sequence between minigene and mCherry is highlighted in yellow and the T2A sequence is underlined. Non-highlighted nucleotides 5' and 3' of YVL encode BRLF1. YVL_50 had a length of 52 amino acids due to cloning procedures but was labeled as YVL_50 to maintain consistency within the manuscript text.

Supporting Information Table 2

PCR reaction	Primer 1	Primer 2
PCR reaction 1	ACACTCTTTCCCTACACGACG CTCTTCCGATCTNNNNCGGCG CGCCCTCGAG	GTGACTGGAGTTCAGACGTGTG CTCTTCCGATCTNNNNTCCGTCG ACTGCAGAATTC
PCR reaction 2, EBV library 1	AATGATACGGCGACCACCGAG ATCTACAC<u>TATAGCCT</u>ACACTC TTTCCCTACACGAC	CAAGCAGAAGACGGCATACGAG AT<u>ATTCAGAA</u>GTGACTGGAGTTC AGACGTG
PCR reaction 2, EBV library 2	AATGATACGGCGACCACCGAG ATCTACAC<u>ATAGAGGC</u>ACACT CTTTCCCTACACGAC	CAAGCAGAAGACGGCATACGAG AT<u>ATTCAGAA</u>GTGACTGGAGTTC AGACGTG
PCR reaction 2, EBV library 3	AATGATACGGCGACCACCGAG ATCTACAC<u>CCTATCCT</u>ACACTC TTTCCCTACACGAC	CAAGCAGAAGACGGCATACGAG AT<u>ATTCAGAA</u>GTGACTGGAGTTC AGACGTG

Supporting Information Table 2. Sequences of primers used for preparation of Illumina deep sequencing libraries.

Index sequences are underlined. N indicates random nucleotides.

Supporting Information Table 3

Label	HLA restriction	TRAV	CDR3α AA sequence	TRAJ	TRBV	CDR3β AA sequence	TRBJ
YVL	A*02:01	38-2/DV8*01	CAYRSAFKLTF	48*01	30*01	CAWSVPLGRREKLFF	1-4*01
CLG	A*02:01	21*01	CAILMDSNYQLIW	33*01	10-2*02	CASSEDGMNTEAFF	1-1*01
FLY	A*02:01	17*01	CATEGDSGYSTLTF	11*01	6-5*01	CASSYQGGNYGYTF	1-2*01
GLC	A*02:01	5*01	CAESTGKLIF	37*01	29-1*01	CSVGTGGTNEKLFF	1-4*01
HPV	B*35:01	5*01	CAESYTGGFKTIF	9*01	6-1*01	CASGTEAFF	1-1*01
EPL	B*35:01	1-2*01	CAVMSSGGSYIPTF	6*01	10-3*01	CAISTGDSNQPQHF	1-5*01

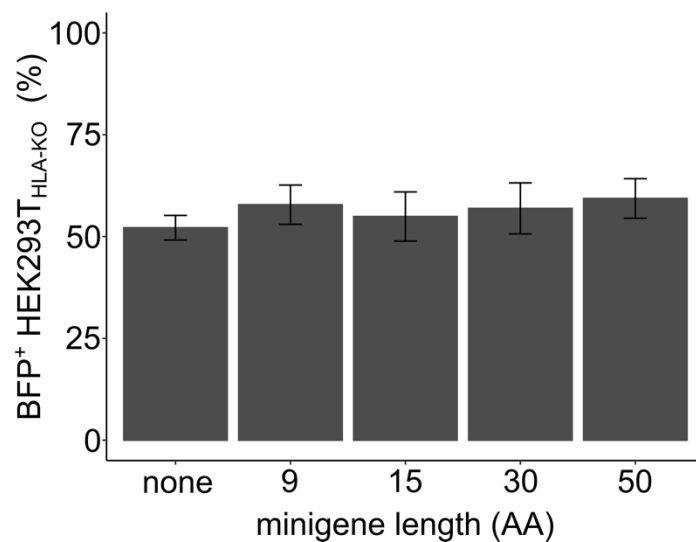**Supporting Information Table 3. TCRαβ sequences of six EBV epitope specific TCRs.**

AA, amino acid; TRAJ, TCRα J-gene and allele; TRAV, TCRα V-gene and allele; TRBJ, TCRβ J-gene and allele; TRBV, TCRβ V-gene and allele. Sequences of these TCRs were identified as part of a previous publication of our group [8].
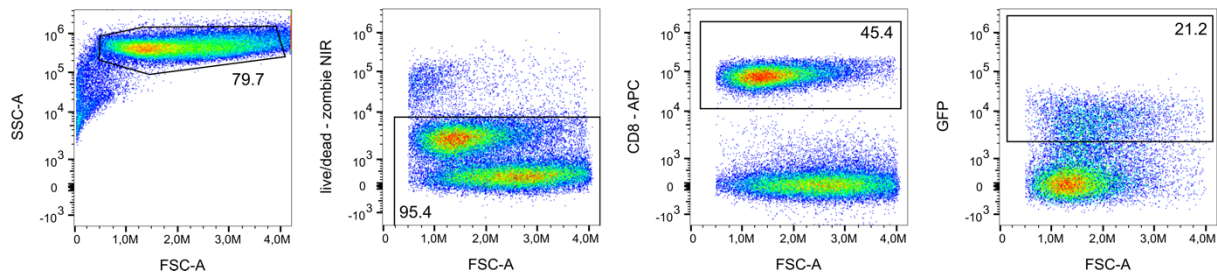
Supporting Information Table 4

Target	Clone	Fluorescent dye	Vendor
CD137	4-1BB	PE	Biolegend
IFN-γ	B27	PE	Biolegend
IL-2	REA689	PE	Miltenyi Biotec
Mouse TCR β	H57-597	APC	Biolegend
CD8	RPA-T8	FITC	Biolegend
Live/dead		Zombie NIR	Biolegend

Supporting Information Table 4. Flow cytometry reagents for co-culture experiments.
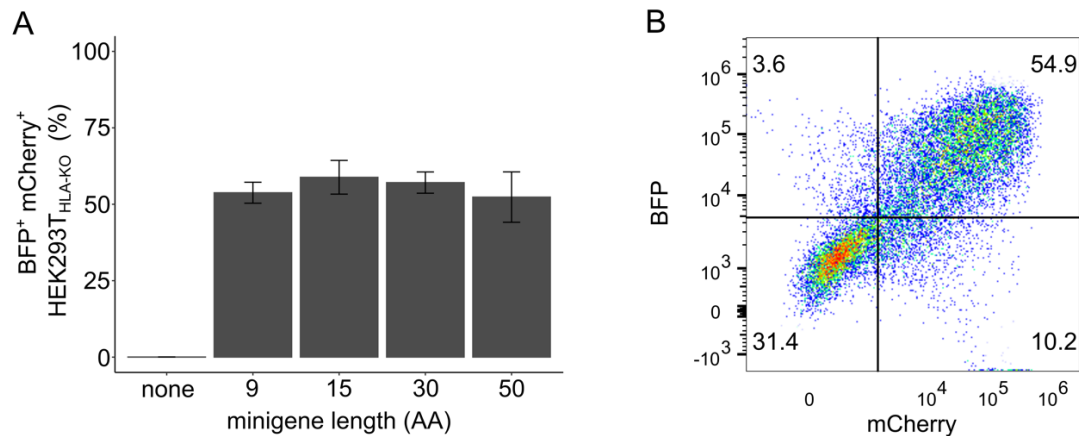
All reagents were used according to the manufacturer's instructions. APC indicates allophycocyanin; FITC, fluorescein isothiocyanate; and PE, phycoerythrin.

SUPPORTING FIGURES

Supporting Information Figure 1

Supporting Information Figure 1. Detection of recombinant HLA expression in HEK293T$_{HLA-KO}$.

HEK293T$_{HLA-KO}$ were co-transfected with a plasmid encoding HLA-A*02:01 and plasmids encoding minigenes of the EBV library. The plasmid encoding HLA-A*02:01 carried an mTagBFP2 (BFP) which was measured by flow cytometry to monitor transfection efficiency. Bar charts indicate mean ± standard error of n=2 independent experiments with three replicates per experiment. AA indicates amino acids.
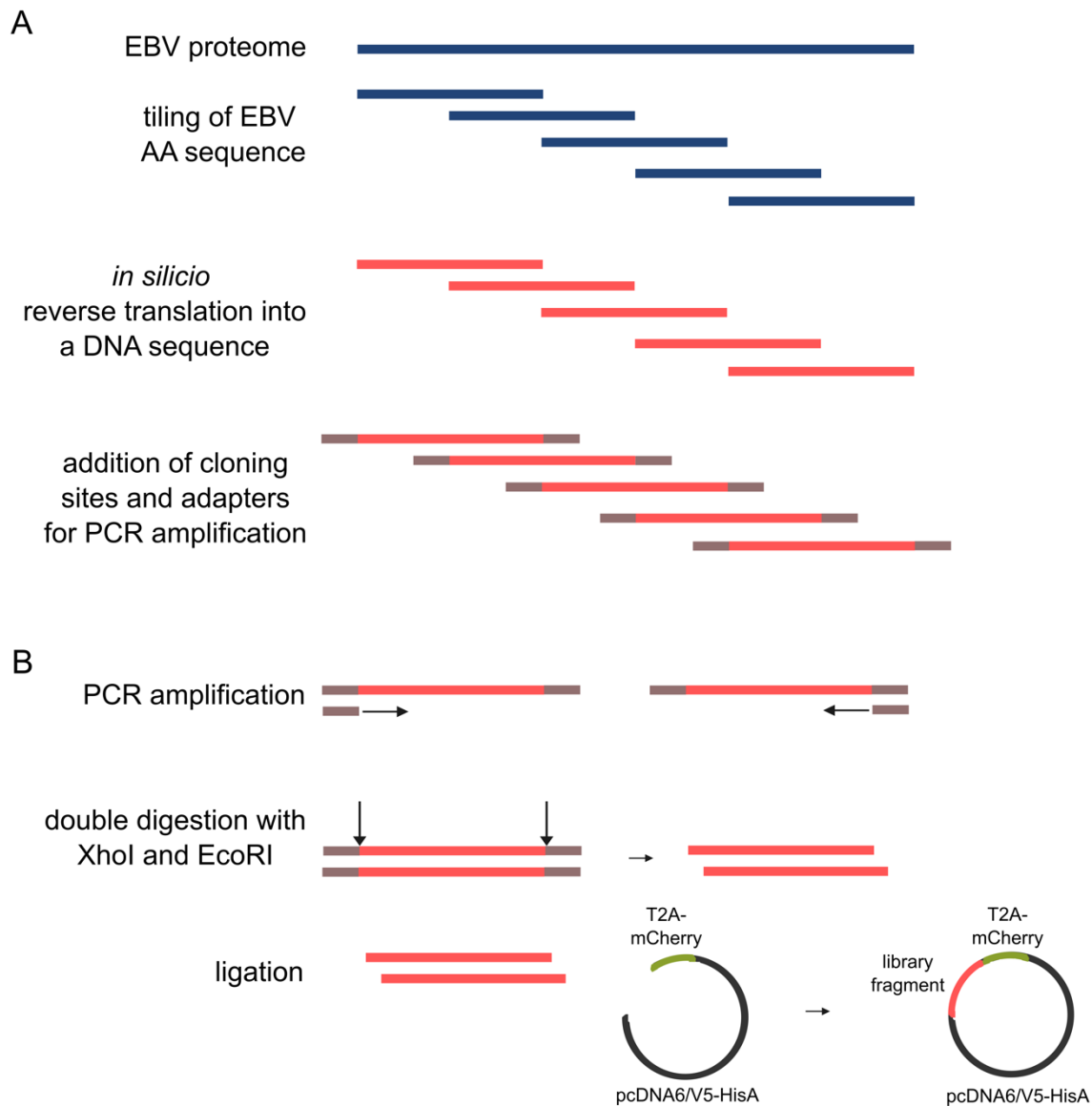
Supporting Information Figure 2

Supporting Information Figure 2. Detection of 58α⁻β⁻ reporter T-cell activation.

The figure illustrates the gating strategy of one co-culture experiment as a representative example. Pseudo-color plots from left to right show sequential gating on cellular events, exclusion of dead cells, gating on TCR-transgenic 58α⁻β⁻ reporter cells identified by recombinant expression human CD8. The GFP gate was defined based on non-stimulated controls. Numbers within gates indicate percentages. APC indicates allophycocyanin; GFP, green fluorescent protein.
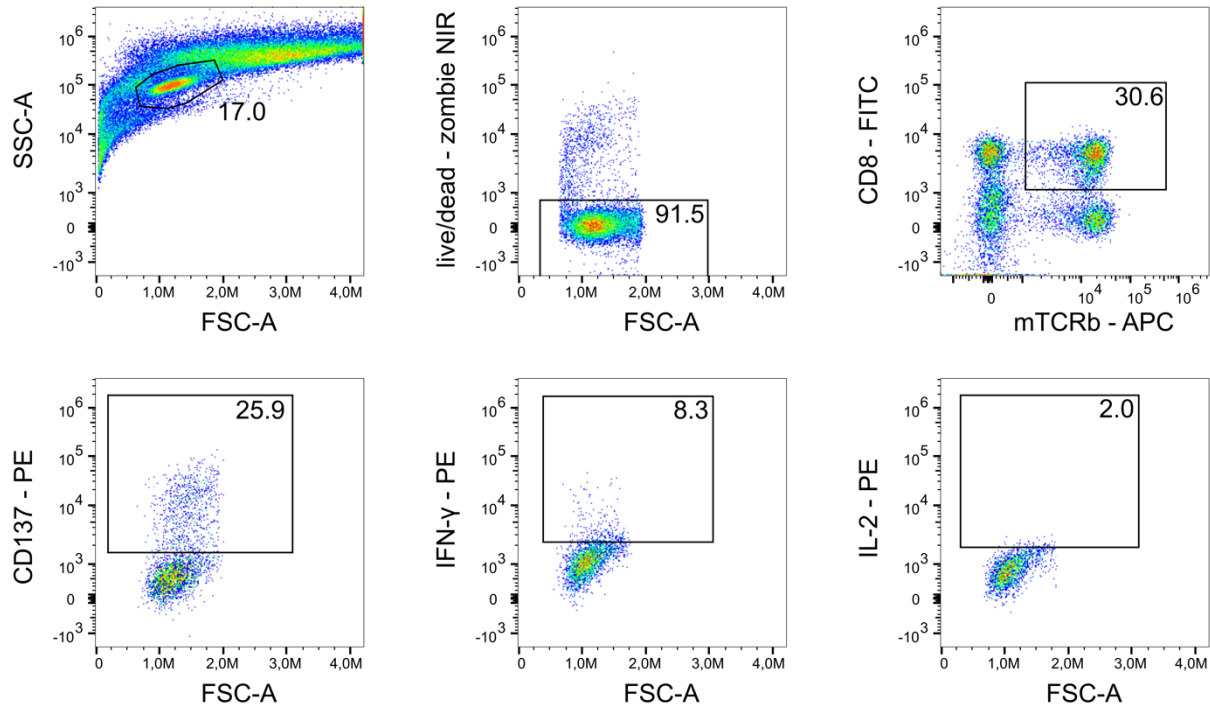
Supporting Information Figure 3

Supporting Information Figure 3. Detection of HLA and minigene expression in HEK293T$_{HLA-KO}$.
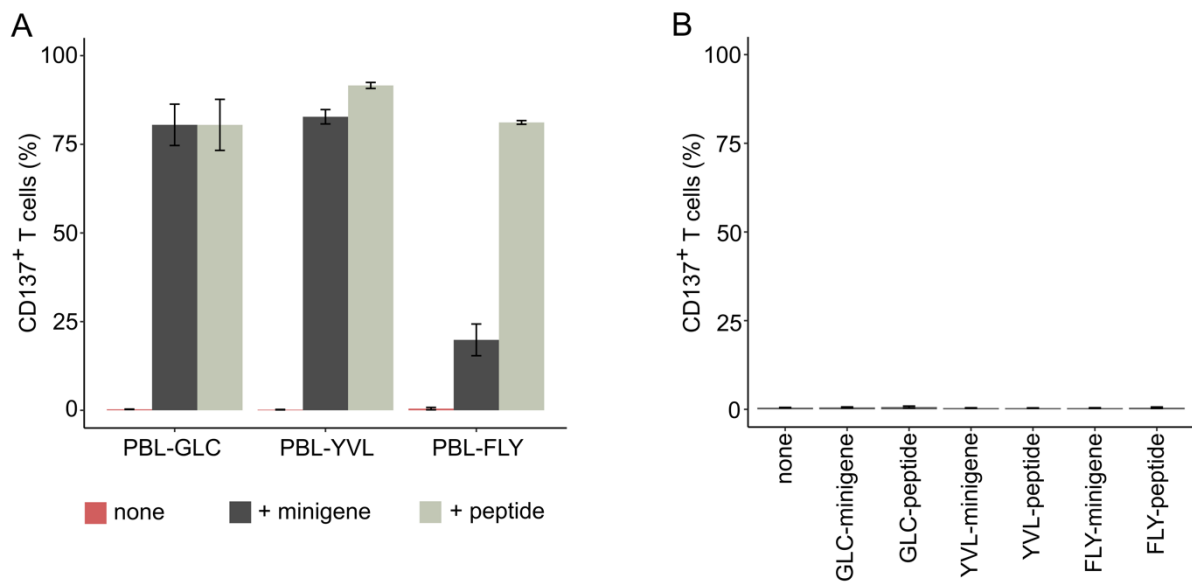
HEK293T$_{HLA-KO}$ were co-transfected with a plasmid encoding HLA-A*02:01 and plasmids encoding minigenes of the EBV library. The plasmid encoding HLA-A*02:01 carried an mTagBFP2 (BFP), and plasmids encoding the EBV library carried mCherry. Fluorescence was detected by flow cytometry. Bar charts indicate mean ± standard error of n=2 independent experiments with three replicates per experiment. AA indicates amino acids. (B) Flow cytometry data underlying the bar charts in figure part A. The plot is pre-gated on live single cells and shows data of one representative experiment as an example.

Supporting Information Figure 4

Supporting Information Figure 4. Detailed oligonucleotide library design and cloning strategy.

(A) Library design. The EBV proteome was separated into 50 amino acid fragments (blue) and reverse translated (red). Cloning sites, adapters for PCR amplification and a start codon (grey) were added. (B) The DNA pool (red) was PCR amplified, double digested and ligated into the pcDNA6/V5-His A expression plasmid, which already contained a T2A-mCherry sequence (green). AA indicates amino acids.

Supporting Information Figure 5

Supporting Information Figure 5. Detection of activation of TCR-transduced human T cells.

Pseudo-color plots of the upper panel show sequential gating on lymphocytes, exclusion of dead cells, identification of CD8$^+$ TCR-transduced cells. Pseudo-color plots of the lower panel show expression of CD137, IFN-γ and IL-2 on CD8$^+$ TCR-transduced cells; gates were set based on non-stimulated controls. The figure illustrates the gating strategy of one co-culture as a representative example. Numbers indicate percentages. APC indicates allophycocyanin; FITC, fluorescein isothiocyanate; and PE, phycoerythrin.

Supporting Information Figure 6

Supporting Information Figure 6. T-cell activation upon minigene or peptide stimulation.

(A) EBV-specific TCR-transduced human lymphocytes were incubated with HEK293T$_{HLA-KO}$ co-transfected with a plasmid encoding HLA-A*02:01 and the target epitope minigene, or only with a plasmid encoding HLA-A*02:01 and loaded with the target nonamer peptide (FLY: FLYALALLL; GLC: GLCTLVAML; YVL: YVLDHLIVV). To detect T-cell activation, CD137 expression on live TCR-transduced CD8$^+$ T cells was determined by flow cytometry. (B) Non-transduced human lymphocytes were incubated with HEK293T$_{HLA-KO}$ co-transfected with a plasmid encoding HLA-A*02:01 and the indicated minigene, or only with a plasmid encoding HLA-A*02:01 and loaded with the indicated peptide. To detect T-cell activation, CD137 expression on live CD8$^+$ T cells was determined by flow cytometry.

Supplementary References

1   **Zhang, J., Kobert, K., Flouri, T. and Stamatakis, A.,** PEAR: a fast and accurate
       Illumina Paired-End reAd mergeR. *Bioinformatics* 2014. **30**: 614-620.

2   **Siewert, K., Malotka, J., Kawakami, N., Wekerle, H., Hohlfeld, R. and Dornmair,
       K.,** Unbiased identification of target antigens of CD8+ T cells with combinatorial
       libraries coding for short peptides. *Nat Med* 2012. **18**: 824-828.

3   **Engels, B., Cam, H., Schuler, T., Indraccolo, S., Gladow, M., Baum, C.,
       Blankenstein, T. and Uckert, W.,** Retroviral vectors for high-level transgene
       expression in T lymphocytes. *Hum Gene Ther* 2003. **14**: 1155-1168.

4   **Welters, C., Welters, M. L., Stadler, S., Bullinger, L., Strobel, J., Hackstein, H.,
       Dhamodaran, A., Blankenstein, T. and Hansmann, L.,** HLA-C*04:09N is expressed
       at the cell surface and triggers peptide-specific T-cell activation. *Haematologica* 2023.

5   **Cossarizza, A., Chang, H. D., Radbruch, A., Abrignani, S., Addo, R., Akdis, M.,
       Andra, I., Andreata, F., Annunziato, F., Arranz, E., Bacher, P., Bari, S., Barnaba,
       V., Barros-Martins, J., Baumjohann, D., Beccaria, C. G., Bernardo, D., Boardman,
       D. A., Borger, J., Bottcher, C., Brockmann, L., Burns, M., Busch, D. H., Cameron,
       G., Cammarata, I., Cassotta, A., Chang, Y., Chirdo, F. G., Christakou, E., Cicin-
       Sain, L., Cook, L., Corbett, A. J., Cornelis, R., Cosmi, L., Davey, M. S., De Biasi,
       S., De Simone, G., Del Zotto, G., Delacher, M., Di Rosa, F., Santo, J. D., Diefenbach,
       A., Dong, J., Dorner, T., Dress, R. J., Dutertre, C. A., Eckle, S. B. G., Eede, P.,
       Evrard, M., Falk, C. S., Feuerer, M., Fillatreau, S., Fiz-Lopez, A., Follo, M., Foulds,
       G. A., Frobel, J., Gagliani, N., Galletti, G., Gangaev, A., Garbi, N., Garrote, J. A.,
       Geginat, J., Gherardin, N. A., Gibellini, L., Ginhoux, F., Godfrey, D. I., Gruarin,
       P., Haftmann, C., Hansmann, L., Harpur, C. M., Hayday, A. C., Heine, G.,**

Hernandez, D. C., Herrmann, M., Hoelsken, O., Huang, Q., Huber, S., Huber, J. E., Huehn, J., Hundemer, M., Hwang, W. Y. K., Iannacone, M., Ivison, S. M., Jack, H. M., Jani, P. K., Keller, B., Kessler, N., Ketelaars, S., Knop, L., Knopf, J., Koay, H. F., Kobow, K., Kriegsmann, K., Kristyanto, H., Krueger, A., Kuehne, J. F., Kunze-Schumacher, H., Kvistborg, P., Kwok, I., Latorre, D. et al., Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). *Eur J Immunol* 2021. **51**: 2708-3145.

6   **Cossarizza, A., Chang, H. D., Radbruch, A., Acs, A., Adam, D., Adam-Klages, S., Agace, W. W., Aghaeepour, N., Akdis, M., Allez, M., Almeida, L. N., Alvisi, G., Anderson, G., Andra, I., Annunziato, F., Anselmo, A., Bacher, P., Baldari, C. T., Bari, S., Barnaba, V., Barros-Martins, J., Battistini, L., Bauer, W., Baumgart, S., Baumgarth, N., Baumjohann, D., Baying, B., Bebawy, M., Becher, B., Beisker, W., Benes, V., Beyaert, R., Blanco, A., Boardman, D. A., Bogdan, C., Borger, J. G., Borsellino, G., Boulais, P. E., Bradford, J. A., Brenner, D., Brinkman, R. R., Brooks, A. E. S., Busch, D. H., Buscher, M., Bushnell, T. P., Calzetti, F., Cameron, G., Cammarata, I., Cao, X., Cardell, S. L., Casola, S., Cassatella, M. A., Cavani, A., Celada, A., Chatenoud, L., Chattopadhyay, P. K., Chow, S., Christakou, E., Cicin-Sain, L., Clerici, M., Colombo, F. S., Cook, L., Cooke, A., Cooper, A. M., Corbett, A. J., Cosma, A., Cosmi, L., Coulie, P. G., Cumano, A., Cvetkovic, L., Dang, V. D., Dang-Heine, C., Davey, M. S., Davies, D., De Biasi, S., Del Zotto, G., Dela Cruz, G. V., Delacher, M., Della Bella, S., Dellabona, P., Deniz, G., Dessing, M., Di Santo, J. P., Diefenbach, A., Dieli, F., Dolf, A., Dorner, T., Dress, R. J., Dudziak, D., Dustin, M., Dutertre, C. A., Ebner, F., Eckle, S. B. G., Edinger, M., Eede, P., Ehrhardt, G. R. A., Eich, M., Engel, P., Engelhardt, B., Erdei, A. et al.,**

Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur J Immunol* 2019. **49**: 1457-1973.

7   **R Core Team,** R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria 2020.

8   **Lammoglia Cobo, M. F., Welters, C., Rosenberger, L., Leisegang, M., Dietze, K., Pircher, C., Penter, L., Gary, R., Bullinger, L., Takvorian, A., Moosmann, A., Dornmair, K., Blankenstein, T., Kammertons, T., Gerbitz, A. and Hansmann, L.,** Rapid single-cell identification of Epstein-Barr virus-specific T-cell receptors for cellular therapy. *Cytotherapy* 2022. **24**: 818-826.