Risks and potential benefits of adoptively transferred virus-specific T cells

Huisman, W.


BENEFITS

CHAPTER 5

Identification of functional HLA-A*01:01-restricted Epstein-Barr Latent Membrane Protein 2-specific T-cell receptors

Wesley Huisman
Ilse Gille
Lieve E. van der Maarel
Lois Hageman
Laura T. Morton
Rob C.M. de Jong
Mirjam H.M. Heemskerk
Derk Amsen
J.H. Frederik Falkenburg
Inge Jedema

Journal of Infectious Diseases 2022;
Vol. 226, Issue 5, Pages 833–842

ABSTRACT

Background

Adoptive transfer of genetically engineered T cells expressing antigen-specific T-cell receptors (TCRs), is an appealing therapeutic approach for Epstein-Barr virus (EBV)-associated malignancies of latency type II/III that express EBV-antigens (LMP1/2). Patients who are HLA-A*01:01^{pos} could benefit from such products, since no T cells recognizing any EBV-derived peptide in this common HLA allele have been found thus far.

Methods

HLA-A*01:01-restricted EBV-(LMP2)-specific T-cells were isolated using peptide-MHC-tetramers. Functionality was assessed by production of IFNγ and cytotoxicity when stimulated with EBV-LMP2-expressing cell-lines. Functionality of primary T cells transduced with HLA-A*01:01-restricted EBV-LMP2-specific TCRs was optimized by knocking out the endogenous TCR of primary T cells (ΔTCR) using CRISPR-Cas9 technology.

Results

EBV-LMP2-specific T cells were successfully isolated and their TCRs were characterized. TCR gene-transfer in primary T cells resulted in specific peptide-MHC-tetramer binding and reactivity against EBV-LMP2-expressing cell-lines. The mean-fluorescence intensity of peptide-MHC-tetramer binding was increased 1.5-2 fold when the endogenous TCR of CD8^{pos} T cells was knocked out. CD8$^{pos/\Delta TCR}$ T cells modified to express EBV-LMP2-specific TCRs showed IFNγ secretion and cytotoxicity towards EBV-LMP2-expressing malignant cell-lines.

Discussion

We isolated the first functional HLA-A*01:01-restricted EBV-LMP2-specific T-cell populations and TCRs, which can potentially be used in future TCR gene-therapy to treat EBV-associated latency type II/III malignancies.

INTRODUCTION

Epstein-Barr virus (EBV) is associated with the development of a broad range of malignancies, including Burkitt's lymphoma, Hodgkin lymphoma (HL), B-, T- and NK-cell lymphomas, post-transplant lymphoproliferative disorder (PTLD), nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC)(1, 2). Although the outcome for most patients with EBV^{pos} lymphomas and NPC is favorable, patients with refractory or relapsed lymphomas have a poor prognosis. Likewise, malignancies of epithelial origin like advanced GC are known to have a very poor prognosis(3).

In healthy individuals, EBV enters a latent phase after primary infection. Upon infecting a resting naïve B cell, EBV first enters the immunogenic latency phase III where it expresses all viral proteins (e.g. EBV Nuclear Antigen 1-3 (EBNA1-3) and latent membrane proteins (LMP) 1 and 2)(4). This results in the activation of the naïve B cell, followed by entrance to the second latency phase (II) with a restricted gene expression of only EBNA1, LMP1 and LMP2. This induces the activated B cell to differentiate into a memory B cell, resulting in the establishment of the latency phase I, where only EBNA1 and BARF1 RNAs are expressed(5). Malignancies associated with this virus often exhibit one of these latency phases. EBV-driven PTLD is associated with latency phase III, resulting in expression of all immunogenic antigens by EBV infected B cells (1, 6). Treatment of PTLD with EBV-specific T-cells has been proven successful after allogeneic hematopoietic stem cell transplantation, with low rates of graft versus host disease (7). Although Burkitt's lymphomas only express weakly immunogenic EBV antigens (latency type I), EBV^{pos} lymphomas and malignancies of epithelial origin, including HL, diffuse large B-cell lymphoma (DLBCL), GC and NPC, additionally express latency type II proteins LMP1 and LMP2 (8, 9). Treatment of EBV^{pos} latency type II lymphomas using adoptively transferred EBV-LMP1/2-specific T cells was recently demonstrated (10-12).

It has been reported that patients expressing HLA-A*01:01 and/or HLA-B*37:01 have an increased risk of developing EBV^{pos} HL and infectious mononucleosis, while patients expressing HLA-A*02:01 have a decreased risk of developing EBV^{pos} HL (13, 14). Strikingly, no EBV-specific T cells recognizing any of the EBV antigens in the context of HLA-A*01:01 have been characterized to date, whereas HLA-A*02:01-restricted EBV-specific T-cell responses have been frequently found (13, 15). It was therefore suggested that HLA-A*01:01-restricted EBV-specific T cells are absent or only present at very low frequencies in the normal T-cell repertoire, although the reasons for this were not clear.

In this study, we aimed to isolate HLA-A*01:01-restricted EBV-specific T cells from healthy $HLA\text{-}A*01:01^{pos}$ EBV^{pos} individuals to allow development of T-cell therapy strategies for patients that harbor an EBV^{pos} malignancy and have an HLA-A*01:01 genotype. Although

such T cells were found to be present at extremely low frequencies in peripheral blood of healthy individuals, we succeeded in the characterization and isolation of several HLA-A*01:01-restricted EBV-LMP2-specific T-cell receptors (TCRs) that can be used for TCR gene therapy.

MATERIALS AND METHODS

Collection of donor peripheral blood mononuclear cells

After informed consent according to the Declaration of Helsinki, healthy donors homozygous for HLA-A*01:01 were selected from the Sanquin database and Leiden University Medical Center (LUMC) biobank of the department of Hematology. Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Isopaque separation and used either directly or thawed after cryopreservation in the vapor phase of liquid nitrogen. Donor characteristics (HLA typing and EBV serostatus) are provided in **Supplementary Table 1**.

Generation of HLA-A*01:01-restricted EBV peptide-MHC complexes

Selected peptides (**Table 1**) were synthesized in-house using standard Fmoc chemisty. Recombinant HLA-A*01:01 heavy chain and human β2m light chain were in-house produced in Escherichia coli. MHC-class-I refolding was performed as previously described with minor modifications(16). MHC-class-I complexes were purified by gel-filtration using FPLC. Peptide-MHC (pMHC) tetramers EBV-BZLF1^{FTP}, EBV-EBNA3A^{YTD} and EBV-LMP2^{LTE} were generated by labeling of biotinylated pMHC-monomers with streptavidin-coupled phycoerythrin (PE; Invitrogen, Carlsbad, USA) and pMHC tetramers EBV-LMP2^{ESE} and EBV-EBNA3A^{FLQ} were generated with streptavidin-coupled allophycocyanin (APC; Invitrogen). Complexes were stored at-80°C. Formation of stable pMHC-monomers was assessed using UVexchange technology(17) according to a previously described protocol(18).

Isolation and expansion of HLA-A*01:01-restricted EBV-specific T cells

PBMCs (30*10^6) from healthy donors were first incubated with in-house produced peptide-MHC-tetramer (pMHC) complexes for 30 min at 4°C before adding Peridinin Chlorophyll Protein Complex (PerCp)-labeled CD8 (BD, Franklin, USA) and fluorescein isothiocyanate-labeled (FITC) CD4 and CD14 (BD, Franklin, USA) antibodies at 4°C for 30 min. The pMHC-tetramers used and generated are shown in **Table 1**. Tetramer positive, CD8^{pos}, CD4^{neg} and CD14^{neg} T cells were sorted into U-bottom microtiter plates for the generation of T-cell populations. Virus-specific T cells were first specifically expanded in T-cell medium: Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Verviers, Belgium) containing 5% heat-inactivated fetal-bovine serum (FBS; Lonza), 5% heat-inactivated human serum (ABOS; Sanquin Reagents, Amsterdam, The Netherlands), 100 U/mL

penicillin (Lonza), 100 µg/mL streptavidin (Lonza) , 2.7mM L-glutamine (Lonza), 100 IU/mL Interleukine-2 (IL-2; Chiron, Emeryville, USA). T-cell medium was supplemented with 10^{-7}M specific peptide and with 5-fold 35 Gy irradiated autologous PBMCs. T cells that use a specific TCR-Variable β (TCR-Vβ) family were sorted subsequently from the virus-specific T-cell populations using the monoclonal antibodies from the TCR-Vβ kit (Beckman Coulter, Fullerton, USA) and were then non-specifically expanded using the aforementioned feeder mixture, replacing specific peptide with 0.8 µg/mL phytohemagglutinin (PHA; Oxoid Limited, Basingstoke, UK). To analyze the purity of the FACsorted T-cell populations, virus-specific T cells were first incubated with tetramers for 10 min at 37°C prior to staining with PerCp-labeled CD8 (BD) and FITC-labeled CD4(BD) antibodies. T-cell populations were qualified as peptide-specific if ≥ 97% of the populations were tetramer positive. Isolated T-cell populations that used one specific TCR-Vβ family were considered single TCR-Vβ positive if ≥95% of the populations were positive for that TCR-Vβ family. Sorting was performed on a FACS ARIA (BD) and analyzed using Diva software (BD). All analyses were performed on a FACS Calibur (BD), and analyzed using Flowjo Software (TreeStar, Ashland, USA).

Table 1. pMHC-tetramers used for the isolation of EBV-specific T cells restricted to HLA-A*01:01 from peripheral blood of healthy donors.

Viral antigen	Epitope	netMHC affinity (nM)*	Isolated (n/n)
BZLF-1	FTPDPYQVPF	36.51	0/6
EBNA3A	YTDHQTTPT	66.17	0/6
LMP2	ESEERPPTPY	97.43	5/6
EBNA3A	FLQRTDLSY	123.91	0/6
LMP2	LTEWGSGNRTY	271.52	0/6

(n/n) : Number of successful isolations out of a number of donors
*netMHC server4.0

TCR-sequencing of EBV-LMP2^{ESE}-specific T-cell populations

In short, mRNA was extracted from EBV-LMP2^{ESE} specific T-cell populations using magnetic beads (Dynabead mRNA DIRECT kit; Invitrogen). During cDNA synthesis, a non-templated 3'polycytosine terminus was added, which created a template for extension of the cDNA with the Template Switching Oligo (TSO). PCR was then performed with cDNA using Phusion Flash (Thermo Fisher Scientific) with anchor-specific primers. Both forward and reverse PCR primers contained overhanging sequences suitable for barcoding. Barcoded amplicons were purified, quantified and pooled into one library for paired-end sequencing of 125bp on an Illumina HiSeq4000. Deep sequencing was performed by GenomeScan (Leiden, The Netherlands)

TCR gene transfer into primary T cells and Jurkat E6 cells

TCR variable alpha(α) and TCR variable beta(β) sequences used by EBV-LMP2^{ESE}-specific T-cell populations were determined using ARTISAN PCR adapted for TCR PCR as previously described (19, 20). Primary CD4^{pos} and CD8^{pos} T cells were isolated with magnetic activated cell sorting (MACS) using CD4 and CD8 T-cell isolation kits with LS columns from Miltenyi Biotec (Bergisch Gladbach, Germany). Additional CD25-beads (Miltenyi) were added during CD4^{pos} T-cell isolation to deplete regulatory T cells. CD4^{pos} and CD8^{pos} T cells were non-specifically activated for 48 hours using an autologous feeder mixture and PHA as described before. After 48 hours these cells were transduced with retroviral supernatant that contained the TCRα and TCRβ sequence in rectronectin-coated 24 wells-plates (100,000 cells per well). These retroviral vectors were constructed on an MP71 backbone with murineTCR constant (mTCR-C) α/β, codon-optimized and cysteine-modified TCRα and TCRβ chain joined by a P2A sequence as previously described(21) and ordered from Baseclear (Leiden, The Netherlands). ϕ-NX-amphotropic packaging cells were transfected with MP71 vectors and pCL-ampho retrovirus packaging using FuGENE HD (Roche, Basel, Switzerland) according to the manufacturer's instructions and retroviral supernatant was harvested after 48 hours. In specific experiments, the endogenous TCR of primary T cells was knocked out according to a previously described protocol(22) (TRAC/TRBV knock-out; ΔTCR) prior to transduction. The endogenous TCR of Jurkat E6 (Clone E6-1 ATCC® TIB-152) cells, cultured in stimulator medium, was knocked-out using the same approach and these cells were used in all Jurkat E6 experiments.

MACS enrichments using APC-labeled mTCR-Cβ antibodies (BD) and anti-APC-microbeads (Miltenyi) were performed in order to purify TCR-transduced populations. Transduction efficiencies and purities after MACS enrichments were assessed by staining transduced cells with APC-labeled mTCR-Cβ-specific antibodies (BD) for 30 min at 4°C. Prior to mTCR-Cβ staining, cells were stained with PE-labeled HLA-A*01:01/pMHC-LMP2^{ESE} pMHC-tetramers to determine the specificity of the introduced TCRs. As a control, cells were stained with PE-labeled HLA-A*01:01/pMHC-CMV-pp50^{VTE} or PE-labeled HLA-A*02:01/pMHC-CMV-pp65^{NLV} pMHC-tetramers.

Stimulator cells for functional analyses

EBV-transformed lymphoblastic cell-lines (EBV-LCLs) were generated according to a standard protocol [15]. EBV-associated Burkitt's lymphoma cell-line Namalwa (ATCC CRL-1432) was kindly provided by Prof. Dr. Emmanuel Wiertz (Utrecht Medical Center, The Netherlands) and EBV-associated Burkitt's lymphoma cell-line mutu-III (c148 and c176) was kindly provided by Dr. Maaike Ressing (Leiden University Medical Center, The Netherlands) and Deborah Croom-Carter (University of Birmingham, United Kingdom). All Burkitt's lymphoma cell-lines and Raji (ATCC CCL-86), EBV-LCLs and HLA-deficient K562 cell-lines (ATCC CCL-243) were all cultured in stimulator medium: IMDM (Lonza;

Verviers, Belgium) supplemented with 10% heat-inactivated FBS (Lonza), 100U/mL penicillin (Lonza), 100µg/mL streptavidin (Lonza) and 2.7mM L-glutamine (Lonza).

Retroviral transduction of stimulator cells

Constructs encoding the HLA-A*01:01 and EBV-LMP2 sequence were coupled to an IRES sequence with mouseCD19 (mCD19) or a truncated form of the nerve growth factor receptor (tNGFR), respectively. Both constructs were cloned into LZRS plasmids. Constructs were verified using reverse transcriptase polymerase chain reactions (RT-PCR) and Sanger sequencing. As an additional control, tNFGR only was cloned into an LZRS plasmid (mock). Retroviral transduction was performed as previously described [4]. Mutu-3 cell-lines were only transduced with constructs encoding the HLA-A*01:01 sequence. K562 wildtype cell-lines, Namalwa cell-lines and Raji cell-lines were transferred to wells containing stable retroviral particles, generated using a puromycin selected stable ϕ-NX-amphotropic packaging cell-line, and incubated for 24 hrs at 37°C [5]. Transduced cell-lines were subsequently enriched by Fluorescent Activated Cell Sorting (FACS) for expression of mCD19 and tNGFR using PE-labeled mCD19 (BD) and APC-labeled tNGFR antibodies (CD271; Southern Biotech Associations, Alabama, USA).

Cytokine production assays to determine functionality

Interferonγ (IFNγ) production by virus-specific T cells or TCR-transduced $CD4^{pos}$/$CD8^{pos}$ T cells was measured and quantified using standard enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (Sanquin Reagents). Responder T cells were co-cultured with stimulator cells at a ratio of 1:5 (R:S) in T-cell medium supplemented with 25IU IL-2/mL for 16 hours at 37°C. EBV-LCLs were kept in culture for 5 days without new medium prior to experiments to upregulate the LMP2 expression in specific experiments (23).

Flow-based activation assay of Jurkat E6 cells

To measure activation of TCR-transduced Jurkat E6ΔTCR cell-lines upregulation of activation marker CD69 was analyzed using flow-based cytometry. Responder TCR-transduced Jurkat E6ΔTCR cells were stimulated with HLA-A*01:01-transduced K562 cell-lines with or without exogenous peptide loading (10-6M) at a ratio of 1:10 (responder:stimulator, R:S) in stimulator medium for 16 hours at 37°C. After O/N incubation, cells were washed twice before adding CD69-PE (Invitrogen), mTCR-Cβ-APC (BD) and CD8-PerCP (BD) monoclonal antibodies for 30 min at 4°C. All analyses were performed on a FACS Calibur (BD), and analyzed using Flowjo Software (TreeStar, Ashland, USA).

Cytotoxicity assay

Cytotoxicity was determined by 51-chromium (51Cr)-release assays. 51Cr-labeled

EBV-LCLs and 51Cr-labeled malignant cell-lines were exposed (3:1, 10:1 and 30:1 Effector:Target ratios) to virus-specific T-cell populations for 20hrs at 37°C in the same medium used for cytokine production assays. 51Cr release was measured on a γ-counter. Spontaneous 51Cr release of the target cells was determined in medium alone, and maximum 51Cr release was determined by adding Triton (1%; Sigma, Saint Louis, USA). Percentages of specific lysis were determined by the following calculation: ((experimental 51Cr release – averaged spontaneous 51Cr release) / (averaged maximal 51Cr release – averaged spontaneous 51Cr release)) x 100. Values for specific 51Cr lysis represent the mean plus and minus standard deviation of triplicate wells. Spontaneous and maximum release represents the mean of sextuplicate wells.

Quantitative polymerase chain reaction

Total RNA was isolated using the ReliaPrep kit (Promega; Madison,Wisconsin, USA) and quantity and quality was directly measured on the NanoDrop (ThermoFisher). cDNA was synthesized using M-MLV Reverse Transcriptase and Oligo(dT) primers. Quantitative RT-PCR(qPCR) was performed using FastStart Taq DNA Polymerase kit (Roche) and with EvaGreen® qPCR master mix. Amplification was measured and analyzed in real-time using LightCycler 480 (Roche). Data were normalized using two reference genes: VSP29 and GUSB. Amplifications started with denaturation: 10 min at 95°C, followed by 45 cycles of 10 seconds for denaturing at 95°C, 30 seconds of annealing at 65°C and 20 seconds extension at 72°C. See supplementary table 2 for primer sequences used.

RESULTS

Isolation of HLA-A*01:01-restricted EBV-specific CD8^{pos} T cells by pMHC-tetramer enrichment

To investigate whether HLA-A*01:01-restricted EBV-specific T-cells are present in peripheral blood of healthy EBV-seropositive donors, HLA-A*01:01-binding peptides derived from different immunogenic EBV antigens (EBNA3A, BZLF1, and LMP2) were identified based on an MHC class I peptide binding prediction algorithm (24, 25) (**Table 1**). HLA-A*01:01/pMHC-tetramer complexes were subsequently synthesized (BZLF1^{FTP}, EBNA3A^{YTD}, LMP2^{ESE}, EBNA3A^{FLQ} and LMP2^{LTE}) and HLA-A*01:01/pMHC-tetramer positive CD8^{pos} T cells were sorted by flow cytometry from PBMCs of 6 HLA-A*01:01^{pos} healthy donors. HLA-A*01:01-restricted EBV-specific T cells were detected at extremely low frequencies (not exceeding background staining in most cases) in total PBMCs from all 6 donors (representative example for 1 donor in **Figure 1A;** upper panel). After flow cytometric cell sorting, only EBV-LMP2^{ESE}-specific T cells (**Table 1**) could be expanded from 5 of 6 donors (representative example for 1 donor in **Figure 1A;** lower panel). After a second round of sorting, pure EBV-LMP2^{ESE}-specific pMHC^{pos}/CD8^{pos} T-cell populations

were obtained (**Figure 1B**). Sequence analysis of the T-cell receptor beta variable chain (TRBV) showed that these EBV-LMP2^{ESE}-specific T-cell populations used different TCRs. T cells from donor A, B and C used the same TCRβ Variable and Joining chain, but with small differences in the CDR3β region (**Table 2**). All populations were clonal except those from donor B and donor C. Two sub-populations could be purified from donor B using FACSorting based on expression of TRBV6-3 (population B^1) and TRBV13 (population B^2). Sub-populations from donor C could not be purified due to expression of the same TRBV6-3 (**Table 2**). Sequence analysis revealed that TRBV6-3 expressing TCRs from donor A and B used the same TCRα chain with an identical CDR3α sequence, while the TCRs from the two populations in donor C used the same α Variable and Joining chain, but with small differences in the CDR3α region. Surprisingly, 4 out of 5 donors harbored a T-cell population expressing TRBV6-3, indicating that diversity within the EBV-LMP2^{ESE}-specific T-cell repertoire is limited.

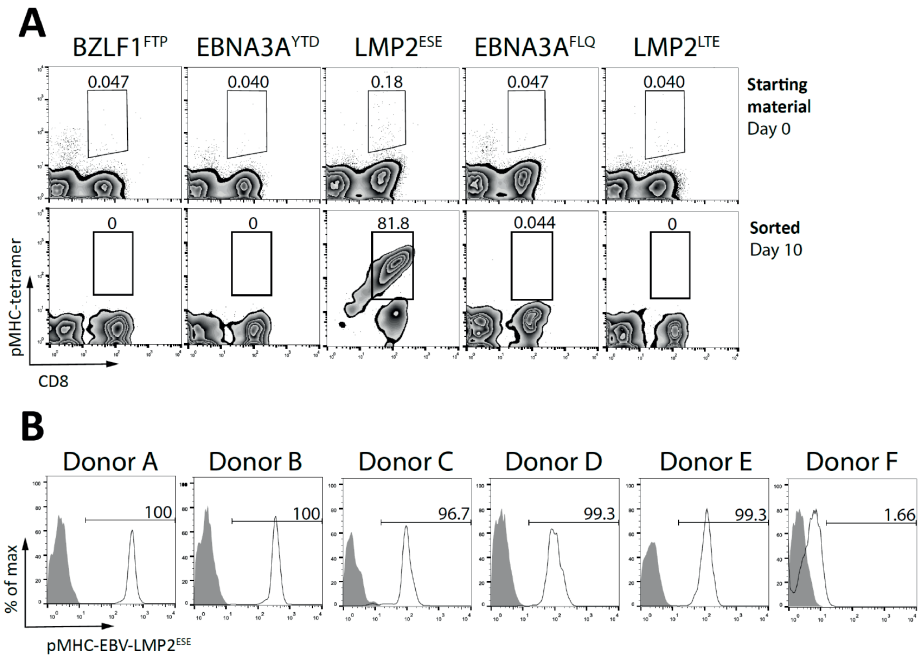


Figure 1. pMHC-tetramer staining of HLA-A*01:01-restricted EBV-specific T cells.
PBMCs from six healthy EBV^{pos} HLA-A*01:01^{pos} donors were incubated with different pMHC-tetramers that were predicted to be strong binders specific for EBV and restricted to HLA-A*01:01. **A**) Representative examples for donor B are shown. Total PBMCs were stained with pMHC-tetramers and CD8. Tetramer positive cells were sorted and expanded for 2 weeks. Only EBV-LMP2^{ESE}-specific T cells could be expanded **B**) Histograms of pMHC-tetramer EBV-LMP2^{ESE} (black-line) or unstained (grey) of all sorted EBV-LMP2^{ESE}-specific T-cell populations after a second enrichment and two weeks of expansion.

Table 2. Characteristics of EBV-LMP2^{ESE}-specific T cells isolated from peripheral blood of healthy HLA-A*01:01^{pos} donors

Donor ID	TRBV	CDR3-beta	TRBJ	TRAV	CDR3-alpha	TRAJ
A	TRBV6-3	CASSWEGQYNEQFF	TRBJ2-1	TRAV12	CVVTGYSSASKIIF	TRAJ3
B^1	TRBV6-3	CASSSEGQFNEQFF	TRBJ2-1	TRAV12	CVVTGYSSASKIIF	TRAJ3
B^2	TRBV13	CASSFWAVTGELFF	TRBJ2-2	TRAV4	CLVGDM_RGSTLGRLYF	TRAJ18
C^1	TRBV6-3	CASSPEGVFNEQFF (73,5%)	TRBJ2-1	TRAV30	CGTGSGGGADGLTF	TRAJ45
C^2	TRBV6-3	CASSYGIYEQFF (24,4%)	TRBJ2-1	TRAV30	CGTEDGRGGADGLTF	TRAJ45
D	TRBV6-3	CASSYGWAEAFF	TRBJ1-1	TRAV2	CAGNNARLMF	TRAJ31
E	TRBV12-3/4	CASSSSWTSGSGETQYF	TRBJ2-5	TRAV26	CIVSGGKLIF	TRAJ23

Amino acids replaced by an underscore could not be determined due to an additional nucleotide insertion retrieving an even number of nucleotides. Donor B harbored two distinct populations that could be separated by fluorescent activated cell sorting based on expression of TRBV6-3 (population B^1) and TRBV13 (population B^2). Donor C also harbored two distinct populations, but these could not be separated due to expression of the same TRBV6-3 gene. The frequencies within this C^{1+2} population are shown between brackets.
TRBV; T-cell Receptor Beta Variable, TRBJ; T-cell Receptor Beta Joining, TRAV; T-cell Receptor Alpha Variable, TRAJ; T-cell Receptor Alpha Joining, CDR3; Complementary Determining Region 3

Specific exogenous and endogenous EBV-LMP2^{ESE} recognition

Next, we aimed to determine the functionality of these EBV-LMP2^{ESE}-specific T-cell populations. The T-cell populations were stimulated with K562 cells transduced with HLA-A*01:01 and pulsed with various concentrations of EBV-LMP2^{ESE} peptide. Five out of 6 EBV-LMP2^{ESE}-specific T-cell populations recognized up to 10^{-10}M of exogenously pulsed peptide (**Figure 2A**). Similarly, when stimulated with K562 cells transduced with both HLA-A*01:01 and the full coding sequence of EBV-LMP2, all T-cell populations except TRBV13-expressing T cells from donor B recognized endogenously processed and presented EBV-LMP2^{ESE} peptide (**Figure 2B**). To analyze recognition of naturally processed and presented EBV-LMP2^{ESE} peptide, EBV-LCLs were cultured for 5 days without refreshing media to increase LMP2 expression(23) (**Supplementary Figure 1**). Production of IFNγ upon coculture of HLA-A*01:01^{pos} EBV-LCLs demonstrated that EBV-LMP2-specific T cells were capable of recognizing EBV-LMP2^{ESE} peptide processed and presented under physiological conditions (**Figure 2C**).

TCR gene transfer in primary CD4^{pos} and CD8^{pos} T cells

To study the introduction of EBV-LMP2^{ESE}-specific reactivity by TCR gene transfer, different EBV-LMP2^{ESE}-specific TCRs were cloned. Since 4 out of 5 functional T-cell populations harbored EBV-LMP2^{ESE}-specific T cells expressing TRBV6-3 and TRBJ2-1 (i.e. Donor A, B and C), we compared the EBV-LMP2^{ESE}-specific reactivity of these TCRs. TCRs were codon optimized and modified with a murine constant domain to increase preferential pairing of the introduced TCRα and TCRβ chain and cloned into a modified MP71-flex vector (**Figure 3A**). First, we introduced EBV-LMP2^{ESE}-specific TCRs into CD8^{pos} primary T cells. CD8^{pos} T cells were isolated from peripheral blood of two unrelated healthy donors using MACS separation (>95% pure; data not shown). High transduction

efficiencies (range 35-42%) were obtained for all TCRs, resulting in 89-98.4% transduced T cells after purification. Specific binding of HLA-A*01:01/pMHC-EBV-LMP2^{ESE} tetramers was demonstrated for all EBV-LMP2^{ESE}-specific TCR-transduced CD8^{pos} T cells (**Figure 3B**). However, CD8^{pos} T cells transduced with TCR-B1 exhibited heterogenous staining with the HLA-A*01:01/pMHC-EBV-LMP2^{ESE} tetramer. TCR-transduced CD8^{pos} T cells did not stain with a control pMHC-tetramer containing an irrelevant CMV peptide.

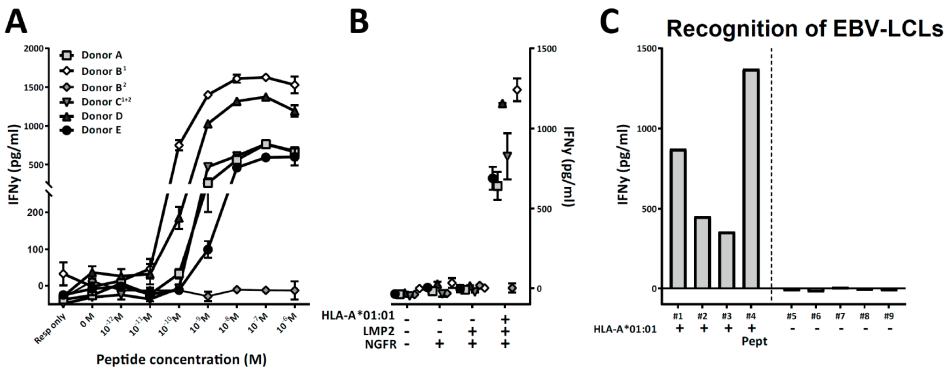


Figure 2. Recognition of exogenously and endogenously presented EBV-LMP2^{ESE} peptide by EBV-LMP2-specific T cells. **A**) Six different EBV-LMP2^{ESE}-specific T-cell populations were stimulated with HLA-A*01:01-transduced K562 cells loaded with titrated concentrations of the respective peptide for 16hrs. IFNγ production was measured by standard ELISA. **B**) EBV-LMP2^{ESE}-specific T cells were stimulated with K562 cells that were retrovirally transduced with the full coding sequence of LMP2 and/or HLA-A*01:01. K562 cells transduced with only the marker gene NGFR were used as additional control. **C**) Representative example of an EBV-LMP2^{ESE}-specific T-cell population (Donor A) that was stimulated with HLA-A*01:01^{pos} and HLA-A*01:01^{neg} EBV-LCLs that expressed LMP2 under physiological conditions.
Shown are means with standard deviations of one experiment carried out in triplicate (A/B).

To determine their dependence on CD8 for binding to pMHC-EBV-LMP2^{ESE} tetramers, all four EBV-LMP2^{ESE}-specific TCRs were introduced in CD4^{pos} T cells. Similar transduction efficiencies and enrichments were obtained as for CD8^{pos} T cells. Lower intensities of pMHC-EBV-LMP2^{ESE}-specific tetramer staining were observed for CD4^{pos} T cells transduced with EBV-LMP2^{ESE}-specific TCRs compared to similarly transduced CD8^{pos} T cells. TCR-B1-transduced CD4^{pos} T cells virtually lacked pMHC-EBV-LMP2^{ESE}-specific tetramer staining (**Figure 3C**). This shows that 3 out of 4 TCRs do not fully depend on the co-receptor CD8 to bind pMHC-EBV-LMP2^{ESE}-specific tetramer, but the intensity of tetramer staining is significantly reduced in the absence of CD8.

Finally, we investigated the functionality of primary CD8^{pos} and CD4^{pos} T cells transduced with the EBV-LMP2^{ESE}-specific TCRs. Both CD8^{pos} and CD4^{pos} T cells produced IFNγ (and

GMCSF and IL-4; data not shown) upon stimulation with HLA-A*01:01-transduced K562 cells exogenously loaded with different concentrations of EBV-LMP2^{ESE} peptide (**Figure 3D**). EBV-LMP2^{ESE}-specific T-cell populations recognized up to 10^{-8}M and 10^{-9}M of exogenously pulsed peptide. In summary, these data show that all EBV-LMP2^{ESE}-specific TCRs, despite differences in the level of pMHC-tetramer staining, recognize exogenously loaded EBV-LMP2^{ESE} peptide. These findings were confirmed when EBV-LMP2^{ESE}-specific TCRs were introduced in Jurkat E6 cells with and without CD8 (**Supplementary Figure 2**).

Reduction of endogenous TCR expression increases EBV-LMP2^{ESE}-specific tetramer binding in CD8^{pos} T cells

Knocking-out the endogenous TCR of primary T cells can increase the functionality of the introduced TCR(22, 26). Therefore, we knocked out the endogenous TCR (ΔTCR) of CD4^{pos} and CD8^{pos} T cells using CRISPR-Cas9 technology targeting the *TRAC/TRBC* loci prior to transduction with EBV-LMP2^{ESE}-specific TCRs (**Supplementary Figure 3**). Similar transduction efficiencies were observed as before, ranging from 20-40% and all populations were successfully enriched for the introduced TCR (data not shown). The mean fluorescence intensity (MFI) of EBV-LMP2^{ESE} tetramer binding was substantially increased in 3 out of 4 TCR-transduced CD8$^{pos/\Delta TCR}$ T cells. In contrast, in CD4$^{pos/\Delta TCR}$ T cells the MFI of EBV-LMP2^{ESE} tetramer binding was not increased (**Figure 4A; Supplementary Figure 4**), while the MFI of the expression of the introduced (mTCR-Cβ) was similar for CD4^{pos} and CD8^{pos} T cells (data not shown).

Functionality was assessed by stimulating CD8$^{pos/\Delta TCR}$ T cells with different EBV-associated cell-lines and measurement of IFNγ production. It has been shown that some EBV-associated malignant cell-lines, excluding EBV-LCLs and the Burkitt's lymphoma cell-line mutu-III, lose their EBV genome *in vitro*(27, 28). Therefore, we used EBV-LCLs and two sub-clones of the mutu-III cell-line that should express EBV-LMP2 at physiological levels and transduced the EBV-associated malignant cell-lines Namalwa and Raji with HLA-A*01:01 and the full-coding EBV-LMP2 sequence. All CD8$^{pos/\Delta TCR}$ T cells expressing EBV-LMP2^{ESE}-specific TCRs recognized the malignant cell-lines transduced with EBV-LMP2 and HLA-A*01:01 (**Figure 4B**), but not those without transduction of LMP2. In accordance, LMP2 expression was relatively higher in LMP2-transduced Namalwa cells compared to LMP2-transduced Raji cells (**Supplementary Figure 5**). All CD8$^{pos/\Delta TCR}$ T cells expressing EBV-LMP2^{ESE}-specific TCRs recognized HLA-A*01:01^{pos} EBV-LCLs that expressed LMP2 at physiological levels, while no recognition of HLA-A*01:01^{neg} EBV-LCLs was observed (**Figure 4C; Supplementary Figure 5**). The EBV-related Burkitt's mutu-III sub-clone c148 showed the highest physiological LMP2 expression, whereas the mutu-III sub-clone c176 had a >100 fold lower LMP2-expression (Supplemental Figure S5). In accordance with this, all CD8$^{pos/\Delta TCR}$ T cells expressing EBV-LMP2^{ESE}-specific TCRs recognized the HLA-A*01:01-transduced sub-clone c148, but not sub-clone c176 (**Figure 4D**).

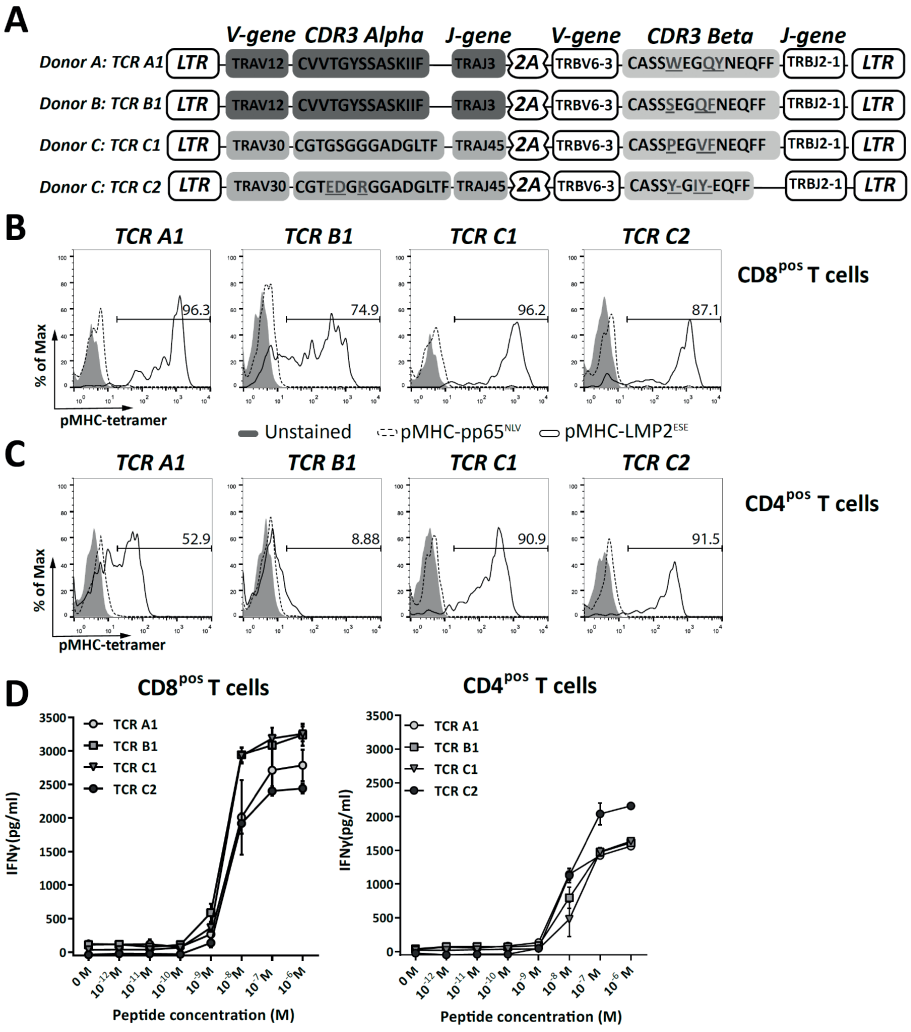


Figure 3. TCR gene transfer introduced EBV-LMP2^{ESE} specificity and reactivity into primary CD8^{pos} and CD4^{pos} T cells.

Primary CD8^{pos} and CD4^{pos} T cells were isolated from healthy EBV^{neg} HLA-A*01:01^{pos} donor G and donor H, using MACS isolations. T cells were transduced with retroviral supernatant containing the constructs of the TRBV6-3/TRBJ2-1 expressing EBV-LMP2^{ESE}-specific TCRs. Transduced T cells were purified and enriched based on expression of the murine-TCR-Cβ domain using MACS isolation. Shown are results and data from donor H. **A**) Design of the MP71-flex retroviral expression vector. Underscores reflect differences between CDR3 regions. **B and C**) Shown are histograms of a specific HLA-A*01:01/pMHC-LMP2^{ESE} tetramer (black-line) or irrelevant HLA-A*01:01/pMHC-pp65^{NLV} tetramer (dotted-line) staining of CD8^{pos} (**B**) or CD4^{pos}(**C**) T cells transduced and enriched based on expression of EBV-LMP2^{ESE}-specific TCRs. Numbers in the middle-right represent percentage of EBV-LMP2^{ESE} -specific T cells binding HLA-A*01:01/pMHC-LMP2^{ESE} tetramer. **D and E**) EBV-LMP2^{ESE}-specific TCR-transduced CD8^{pos} (**D**) and CD4^{pos} (**E**) T-cell populations were stimulated with HLA-A*01:01 transduced K562 cells loaded with titrated concentrations of the respective peptide for 16hrs. IFNγ was measured by standard ELISA.

Data is representative of three separate experiments, performed in 2 different donors.

2A; self-cleaving peptide side, LTR; Long terminal Repeat

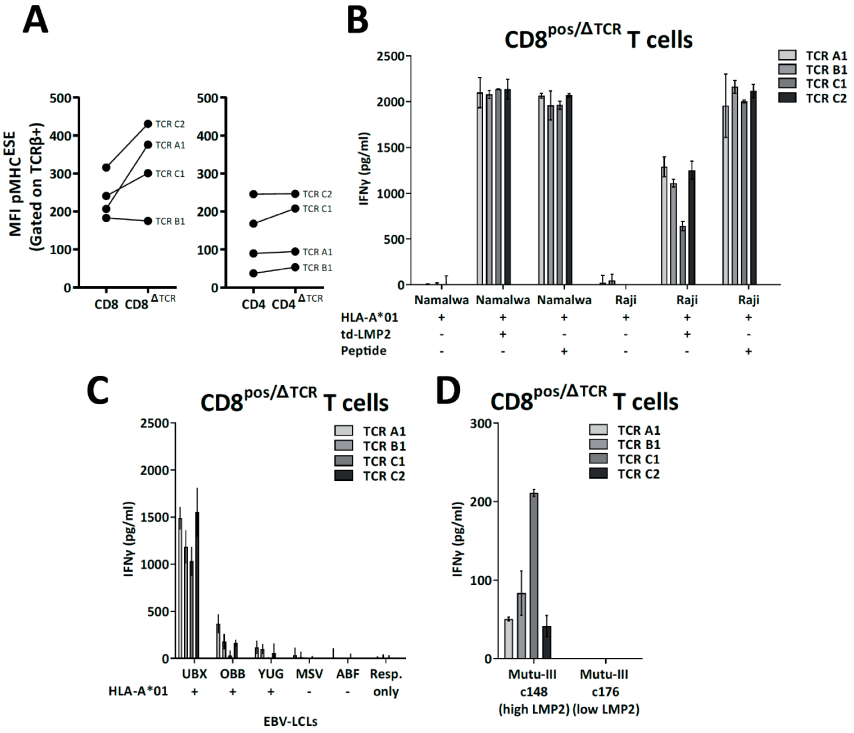


Figure 4: CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs effectively recognize endogenously processed and presented LMP2^{ESE} peptide.
A and **B**) Mean-Fluorescence Intensity (MFI) of pMHC-EBV-LMP2^{ESE} tetramer binding was assessed for CD8^{pos} (**A**) and CD4^{pos} (**B**) T cells with and without (ΔTCR) endogenous expressing TCRs. **C**) CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs were stimulated for 16 hours with HLA-A*01:01-transduced EBV-associated malignant cell-lines Namalwa and Raji in a responder: stimulator ratio of 1:5. These cell-lines were additionally transduced with LMP2 and the cell-lines without LMP2 were exogenously pulsed with 10^{-6}M of EBV-LMP2^{ESE} peptide. **D**) CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs were stimulated for 16 hours with HLA-A*01:01^{pos} and HLA-A*01:01^{neg} EBV-LCLs that express LMP2 under physiological conditions in a responder: stimulator ratio of 1:5. E) CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs were stimulated for 16 hours with two HLA-A*01:01-transduced Burkitt's lymphoma mutu-III cell-lines that were expected to express LMP2 under physiological conditions. However, mutu-III sub-clone c148 expressed LMP2 >100fold higher compared to sub-clone c176.
*Data shown are from separate experiments carried out in triplicate (**C, D and E**) with T cells from donor H.*

Primary CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs lyse LMP2 expressing target cells

Finally, we investigated the ability of these EBV-LMP2^{ESE}-specific TCR-transduced T cells to lyse EBV-LMP2^{pos} target cells. CD8$^{pos/\Delta TCR}$ T cells transduced with CMV-pp65^{NLV}-specific TCRs were used as a negative control. Efficient lysis by CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs was observed for cell-lines pulsed with EBV-LMP2^{ESE} peptide (**Supplementary Figure 6**). Specific lysis was also observed when tested against

an HLA-A*01:01/LMP2-transduced cell-line and HLA-A*01:01^{pos} EBV-LCLs (**Figure 5A**). Modest lysis by CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCR-C1 was observed of the HLA-A*01:01-transduced mutu-III sub-clone c148 with the highest LMP2-expression (**Figure 5B**). As expected, no or only limited lysis of LMP2-expressing target cells was observed with the CMV-pp65^{NLV}-specific TCR transduced CD8^{pos} T cells (**Figure 5**) and no lysis was observed of HLA-A*01:01^{neg} EBV-LCLs. In summary, these findings demonstrate that HLA-A*01:01-restricted EBV-specific T cells do exist, and shows that their TCR was capable of producing IFNγ and lysis of LMP2-expressing cells upon transduction into primary CD8^{pos} T cells.

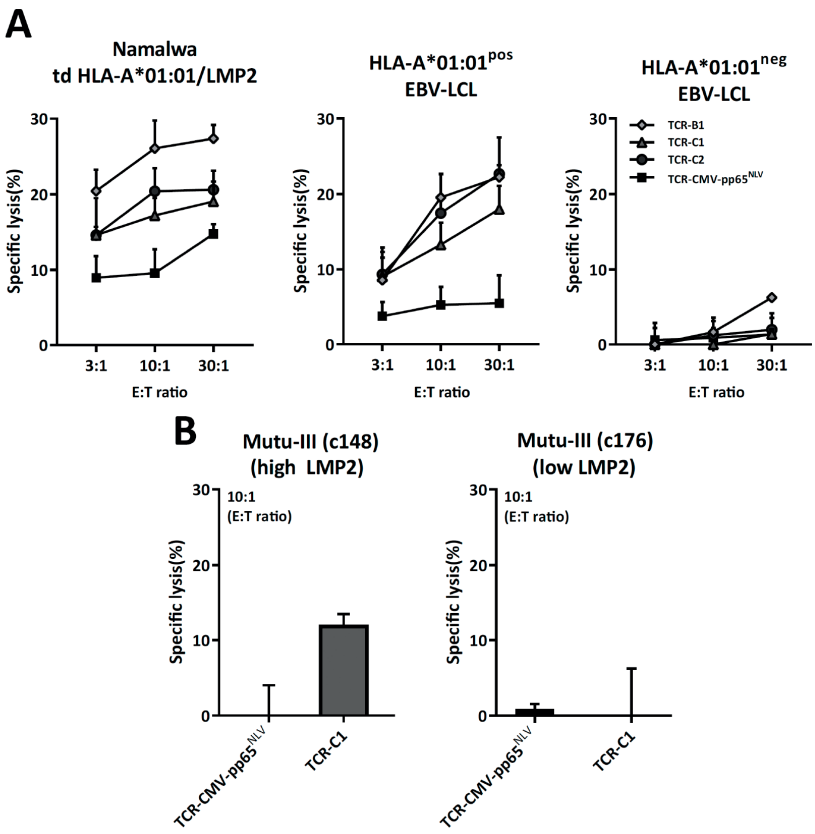


Figure 5. CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs lyse target cells presenting endogenous LMP2 peptide. **A**) Representative examples are shown of CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs that were tested for their lytic capacity against malignant cells transduced with HLA-A*01:01 and LMP2 or against HLA-A*01:01$^{pos/neg}$ EBV-LCLs. CD8$^{pos/\Delta TCR}$ T cells transduced with a TCR targeting HLA-A*02:01/CMV-pp65^{NLV} were used as control. **B**) Primary CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCR-C1 were tested for their lytic capacity against two HLA-A*01:01-transduced sub-clones of the malignant Burkitt's lymphoma cell-line mutu-III. Mutu-III sub-clone c148 expressed LMP2 >100fold higher compared to sub-clone c176. CD8$^{pos/\Delta TCR}$ T cells transduced with a TCR targeting HLA-A*02:01/CMV-pp65^{NLV} were used as negative control.
Data are shown from one experiment carried out in triplicate

DISCUSSION

In this study we successfully isolated the first HLA-A*01:01-restricted EBV-specific T cells that recognized the LMP2 epitope ESE. These T cells could be isolated from 5 out of 6 healthy donors and TCR-sequencing analyses revealed limited TCR diversity. All EBV-LMP2^{ESE}-specific T-cell populations, except the TRBV13 expressing sub-population from donor B, were functional against stimulator cells that express LMP2 under physiological conditions. Furthermore, retroviral introduction of EBV-LMP2^{ESE}-specific TCRs into CD8^{pos} and CD4^{pos} T cells permitted specific recognition of EBV-LMP2 expressing cell-lines and knock-out of the endogenous TCR (ΔTCR) using CRISPR-Cas9 technology increased pMHC-EBV-LMP2^{ESE}-specific tetramer binding in CD8^{pos} T cells. These CD8$^{pos/\Delta TCR}$ T cells lyse EBV-LMP2-expressing target cells, illustrating that these novel TCRs may be exploited to enhance the immunotherapy of HLA-A*01:01^{pos} EBV-associated malignancies.

So far, no HLA-A*01:01-restricted EBV-specific T-cell populations have been described, which lead to the assumption that they do not exist or that they are present at only very low frequencies(15). Peptide-binding predictions revealed 5 strong binding HLA-A*01:01-restricted peptides (**Table 1**). The frequencies in total PBMCs of the analyzed HLA-A*01:01-restricted EBV-specific T-cell populations did not exceed background staining and T cells recognizing the LMP2^{ESE} peptide could only be successfully isolated and expanded after a round of positive selection, again underscoring the very low frequencies of HLA-A*01:01-restricted EBV-specific T cells and confirming the reported difficulty to isolate HLA-A*01:01-restricted EBV-specific T cells(14). The TCR repertoire of the EBV-LMP2^{ESE}-specific T cells appeared to be very skewed, with preferential expression of TRBV12-3/4 (n=1/5 donors) , TRBV13 (n=1/5) or TRBV6-3 (n=4/5). TRBV13-expressing EBV-LMP2^{ESE}-specific T cells were found to be not functional, although this population did exhibit proper pMHC-EBV-LMP2^{ESE}-specific tetramer binding. Such dysfunctional T cells might may result in an overestimation of the functional HLA-A*01:01-restricted EBV-specific T cells when pMHC-tetramers are used. However, others report that pMHC-tetramers can also fail to detect functional T cells, resulting in an underestimation(29).

The TCR-repertoire of the TRBV6-3-expressing T-cell populations was found to be extremely skewed, with almost identical CDR3β sequences. Although these TCRs were very similar, differences in pMHC-EBV-LMP2^{ESE}-tetramer binding were observed when these TCRs were introduced in primary CD8^{pos} and CD4^{pos} T cells. A lower intensity of pMHC-EBV-LMP2^{ESE}-tetramer binding was observed for all TCRs transduced in CD4^{pos} T cells, suggesting that these TCRs are not completely CD8 independent. Differences in pMHC-tetramer binding of the different TCRs transduced in CD8^{pos} T cells could be the result of competition for CD3 with the endogenous TCR(30, 31). However, when we knocked-out the endogenous TCR, a subtle increase in intensity of tetramer staining for

the introduced TCR was seen, but this did not result in increased functional reactivity. Introduction of EBV-LMP2^{ESE}-specific TCRs in CD4^{pos} T cells also resulted in recognition of peptide-pulsed stimulator cells, but no recognition of LMP2-expressing cells was observed. In conclusion, both CD8^{pos} and CD4^{pos} T cells can be modified by TCR gene transfer to contribute to recognition of LMP2-expressing target cells, which could be beneficial for TCR-gene transfer immunotherapies.

It is evident that virus-specific TCRs recognize antigens processed and presented in HLA by virus-infected cells. Both the TCR and the expression of the viral antigen play an important role in recognition of virus-infected cells. We demonstrated that all primary CD8^{pos} T cells transduced with EBV-LMP2^{ESE}-specific TCRs showed good recognition of peptide-pulsed and LMP2 expressing cell-lines, suggesting sufficient avidity of the TCR. However, limited recognition was observed of EBV-LCLs expressing LMP2 at physiological levels and there was no clear correlation between EBV-LMP2-specific reactivity and LMP2-expression. In contrast to the full-coding LMP2-transduced cell-lines, EBV-LCLs are known to express LMP2 as two splice variants (i.e. LMP2a and LMP2b)(32). Our qPCR was not able to distinguish LMP2a from LMPb. Since the LMP2^{ESE} epitope is located in the LMP2b splice-variant, it is possible that the EBV-LCLs OBB and YUG were less well recognized because of the unpredictable ratio between these two variants(33). Additionally, differential expression per cell could result in less overall recognition, only showing recognition of cells that sufficiently express LMP2. We showed that LMP2 expression can be increased by exhausting EBV-LCLs(23), resulting in recognition and lysis. EBV-LCL-specific lysis by our EBV-LMP2^{ESE}-specific T cells did not exceed 40%, which resembles results shown by others for EBV-LMP1/2-specific T cells restricted to HLA-A*02:01(34) and HLA-A*11:01(27). In line with this, others reported that EBV-EBNA1-specific T cells were not able to lyse EBV-LCLs within short-term cytotoxicity assays, but they could prevent the longer term outgrowth of these EBV-LCLs (35).

Overall, our findings demonstrate that although present in very low frequencies, HLA-A*01:01-restricted EBV-LMP2-specific T cells do exist and are capable of killing LMP2-expressing malignant cells and LMP2 expressing EBV-LCLs. Therefore, gene transfer of these LMP2-specific TCRs may be exploited to enhance the immunotherapy of HLA-A*01:01^{pos} EBV-associated malignancies of latency type II/III.

Acknowledgement
This work was supported by Sanquin Research and Landsteiner Laboratory for Blood Cell research [PPO 15-37/Lnumber 2101]. The sponsor of this study is a nonprofit organization that supports science in general. They had no role in gathering, analyzing, or interpreting the data

REFERENCES

1. Shannon-Lowe C, Rickinson AB, Bell AI. Epstein-Barr virus-associated lymphomas. Philos Trans R Soc Lond B Biol Sci. 2017;372(1732).
2. Neparidze N, Lacy J. Malignancies associated with epstein-barr virus: pathobiology, clinical features, and evolving treatments. Clin Adv Hematol Oncol. 2014;12(6):358-71.
3. Brenner H, Rothenbacher D, Arndt V. Epidemiology of stomach cancer. Methods in molecular biology (Clifton, NJ). 2009;472:467-77.
4. Amon W, Farrell PJ. Reactivation of Epstein-Barr virus from latency. Rev Med Virol. 2005;15(3):149-56.
5. Dugan JP, Coleman CB, Haverkos B. Opportunities to Target the Life Cycle of Epstein-Barr Virus (EBV) in EBV-Associated Lymphoproliferative Disorders. Frontiers in oncology. 2019;9:127.
6. Montone KT, Hodinka RL, Salhany KE, Lavi E, Rostami A, Tomaszewski JE. Identification of Epstein-Barr virus lytic activity in post-transplantation lymphoproliferative disease. Mod Pathol. 1996;9(6):621-30.
7. Gottschalk S, Heslop HE, Rooney CM. Adoptive immunotherapy for EBV-associated malignancies. Leuk Lymphoma. 2005;46(1):1-10.
8. Greijer AE, Ramayanti O, Verkuijlen SA, Novalic Z, Juwana H, Middeldorp JM. Quantitative multi-target RNA profiling in Epstein-Barr virus infected tumor cells. J Virol Methods. 2017;241:24-33.
9. Brooks L, Yao QY, Rickinson AB, Young LS. Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. J Virol. 1992;66(5):2689-97.
10. McLaughlin LP, Rouce R, Gottschalk S, Torrano V, Carrum G, Wu MF, et al. EBV/LMP-specific T cells maintain remissions of T- and B-cell EBV lymphomas after allogeneic bone marrow transplantation. Blood. 2018;132(22):2351-61.
11. Bollard CM, Gottschalk S, Torrano V, Diouf O, Ku S, Hazrat Y, et al. Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein-Barr virus latent membrane proteins. J Clin Oncol. 2014;32(8):798-808.
12. Roskrow MA, Suzuki N, Gan Y, Sixbey JW, Ng CY, Kimbrough S, et al. Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's disease. Blood. 1998;91(8):2925-34.
13. Niens M, Jarrett RF, Hepkema B, Nolte IM, Diepstra A, Platteel M, et al. HLA-A*02 is associated with a reduced risk and HLA-A*01 with an increased risk of developing EBV+ Hodgkin lymphoma. Blood. 2007;110(9):3310-5.
14. Jones K, Wockner L, Brennan RM, Keane C, Chattopadhyay PK, Roederer M, et al. The impact of HLA class I and EBV latency-II antigen-specific CD8(+) T cells on the pathogenesis of EBV(+) Hodgkin lymphoma. Clin Exp Immunol. 2016;183(2):206-20.
15. Brennan RM, Burrows SR. A mechanism for the HLA-A*01-associated risk for EBV+ Hodgkin lymphoma and infectious mononucleosis. Blood. 2008;112(6):2589-90.
16. Garboczi DN, Hung DT, Wiley DC. HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in Escherichia coli and complexed with single antigenic peptides. Proc Natl Acad Sci U S A. 1992;89(8):3429-33.
17. Rodenko B, Toebes M, Hadrup SR, van Esch WJ, Molenaar AM, Schumacher TN, et al. Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. Nat Protoc. 2006;1(3):1120-32.
18. Eijsink C, Kester MG, Franke ME, Franken KL, Heemskerk MH, Claas FH, et al. Rapid assessment of the antigenic integrity of tetrameric HLA complexes by human monoclonal HLA antibodies. J Immunol Methods. 2006;315(1-2):153-61.
19. van Bergen CA, van Luxemburg-Heijs SA, de Wreede LC, Eefting M, von dem Borne PA, van Balen P, et al. Selective graft-versus-leukemia depends on magnitude and diversity of the alloreactive T cell response. J Clin Invest. 2017;127(2):517-29.
20. Koning MT, Nteleah V, Veelken H, Navarrete MA. Template-switching anchored polymerase chain reaction reliably amplifies functional lambda light chain transcripts of malignant lymphoma. Leuk Lymphoma. 2014;55(5):1212-4.
21. van der Lee DI, Reijmers RM, Honders MW, Hagedoorn RS, de Jong RC, Kester MG, et al. Mutated nucleophosmin 1 as immunotherapy target in acute myeloid leukemia. The Journal of clinical investigation. 2019;129(2):774-85.
22. Morton LT, Reijmers RM, Wouters AK, Kweekel C, Remst DFG, Pothast CR, et al. Simultaneous Deletion of Endogenous TCRαβ for TCR Gene Therapy Creates an Improved and Safe Cellular Therapeutic. Molecular Therapy. 2019.
23. Wasil LR, Tomaszewski MJ, Hoji A, Rowe DT. The effect of Epstein-Barr virus Latent Membrane Protein 2 expression on the kinetics of early B cell infection. PLoS One. 2013;8(1):e54010.
24. Andreatta M, Nielsen M. Gapped sequence alignment using artificial neural networks: application to the MHC class I system. Bioinformatics. 2016;32(4):511-7.
25. Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, Buus S, et al. Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. Protein Sci. 2003;12(5):1007-17.
26. Schober K, Muller TR, Gokmen F, Grassmann S, Effenberger M, Poltorak M, et al. Orthotopic replacement of T-cell receptor alpha- and beta-chains with preservation of near-physiological

T-cell function. Nat Biomed Eng. 2019.
27. Zheng Y, Parsonage G, Zhuang X, Machado LR, James CH, Salman A, et al. Human Leukocyte Antigen (HLA) A*1101-Restricted Epstein-Barr Virus-Specific T-cell Receptor Gene Transfer to Target Nasopharyngeal Carcinoma. Cancer Immunol Res. 2015;3(10):1138-47.
28. Zuo L, Yu H, Liu L, Tang Y, Wu H, Yang J, et al. The copy number of Epstein-Barr virus latent genome correlates with the oncogenicity by the activation level of LMP1 and NF-kappaB. Oncotarget. 2015;6(38):41033-44.
29. Rius C, Attaf M, Tungatt K, Bianchi V, Legut M, Bovay A, et al. Peptide-MHC Class I Tetramers Can Fail To Detect Relevant Functional T Cell Clonotypes and Underestimate Antigen-Reactive T Cell Populations. J Immunol. 2018;200(7):2263-79.
30. Thomas S, Stauss HJ, Morris EC. Molecular immunology lessons from therapeutic T-cell receptor gene transfer. Immunology. 2010;129(2):170-7.
31. Heemskerk MH, Hagedoorn RS, van der Hoorn MA, van der Veken LT, Hoogeboom M, Kester MG, et al. Efficiency of T-cell receptor expression in dual-specific T cells is controlled by the intrinsic qualities of the TCR chains within the TCR-CD3 complex. Blood. 2007;109(1):235-43.
32. Ikeda M, Ikeda A, Longan LC, Longnecker R. The Epstein-Barr virus latent membrane protein 2A PY motif recruits WW domain-containing ubiquitin-protein ligases. Virology. 2000;268(1):178-91.
33. Lynch DT, Zimmerman JS, Rowe DT. Epstein-Barr virus latent membrane protein 2B (LMP2B) co-localizes with LMP2A in perinuclear regions in transiently transfected cells. J Gen Virol. 2002;83(Pt 5):1025-35.
34. Cho HI, Kim UH, Shin AR, Won JN, Lee HJ, Sohn HJ, et al. A novel Epstein-Barr virus-latent membrane protein-1-specific T-cell receptor for TCR gene therapy. Br J Cancer. 2018;118(4):534-45.
35. Lee SP, Brooks JM, Al-Jarrah H, Thomas WA, Haigh TA, Taylor GS, et al. CD8 T cell recognition of endogenously expressed epstein-barr virus nuclear antigen 1. J Exp Med. 2004;199(10):1409-20.

SUPPLEMENTARY MATERIAL

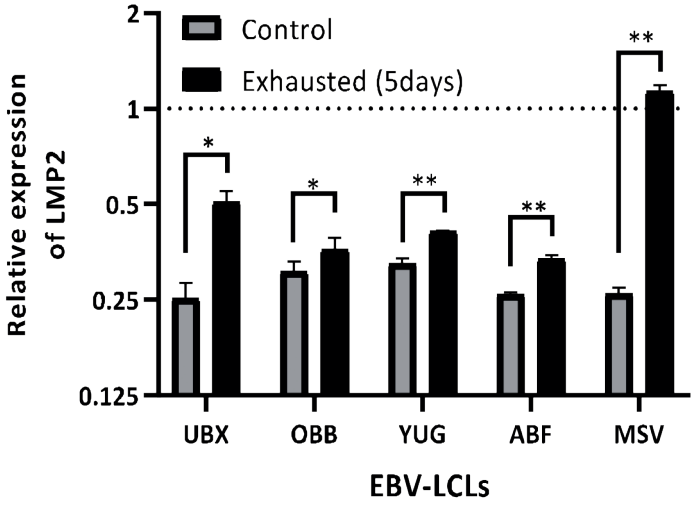
Supplementary Table 1. HLA-typing and EBV-serostatus of healthy donors.

Donor	EBV	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ		HLA-DP	
A	Pos	01:01	-	08:01	-	07:01	-	03:01	-	02:01	-	04:01	-
B	Pos	01:01	-	08:01	-	07:01	-	03	-	02	-	N.D	
C	Pos	01:01	-	08:01	-	07:01	-	03	-	02	-	N.D	
D	Pos	01:01	-	08:01	-	07:01	-	03:01	-	02:01	-	01:01	09:01
E	Pos	01:01	-	08:01	-	07:01	-	03	-	02	-	N.D	
F	Pos	01:01	-	08:01	-	07:01	-	03:01	-	02:01	-	04:01	05:01
G[1]	Neg	01:01	24	35:02	37:01	04:01	06:02	10:01	11:04	03:01	05:01	04:01	-
H[1]	Neg	01:01	02	15:01	40:01	03:03	03:04	N.D	N.D	03	06	04	-

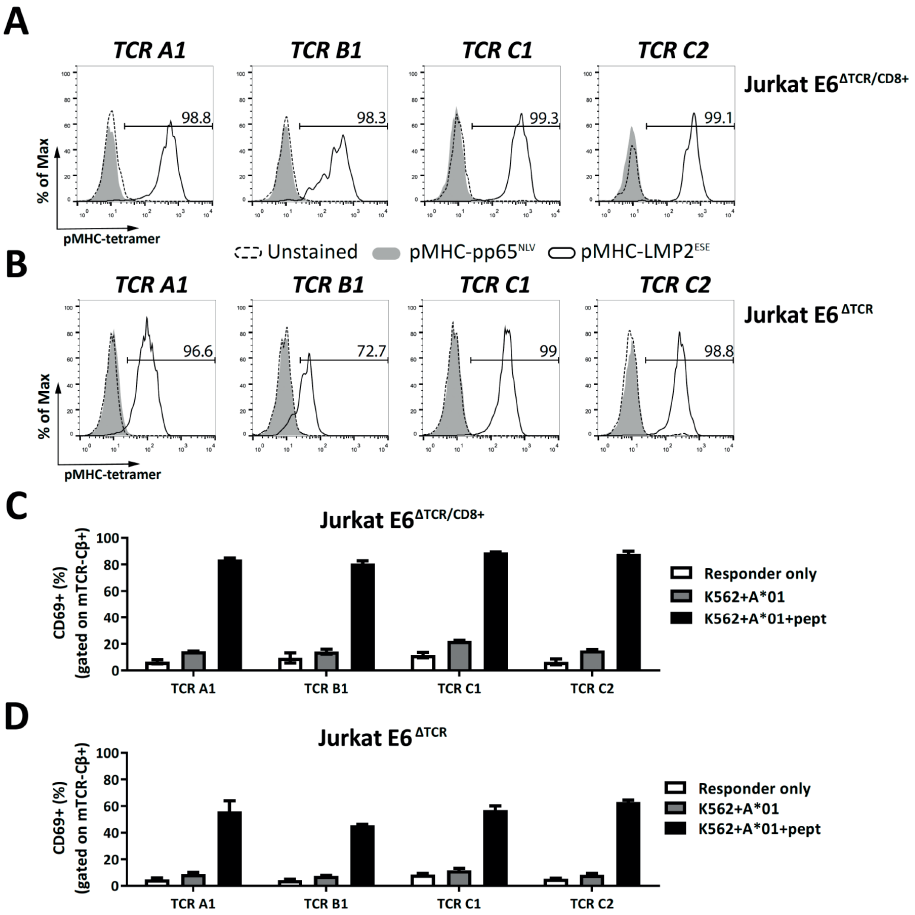
EBV-specific T cells restricted to HLA-A*01:01 were isolated from donors A-F. High resolution HLA-class-I typing was performed for all donors. Some HLA-class-II alleles were not determined, indicated by N.D.
[1]indicates donors used for isolation of $CD4^{pos}$ and $CD8^{pos}$ T cells for TCR-gene transfer of EBV-$LMP2^{ESE}$-specific TCRs

Supplementary Table 2. Primers used for qPCR

Primer name	Primer sequence
forward_LMP2	GAC-ACC-GGT-GAC-AGT-GCT-TA
reverse_LMP2	GGC-CAG-CAA-TGC-AAA-CAG-AA
forward_VSP29	TGA-GAG-GAG-ACT-TCG-ATG-AGA-ATC
reverse_VSP29	TCT-GCA-ACA-GGG-CTA-AGC-TG
forward_GUSB	ACT-GAA-CAG-TCA-CCG-ACG-AG
reverse_GUSB	GGA-ACG-CTG-CAC-TTT-TTG-GT



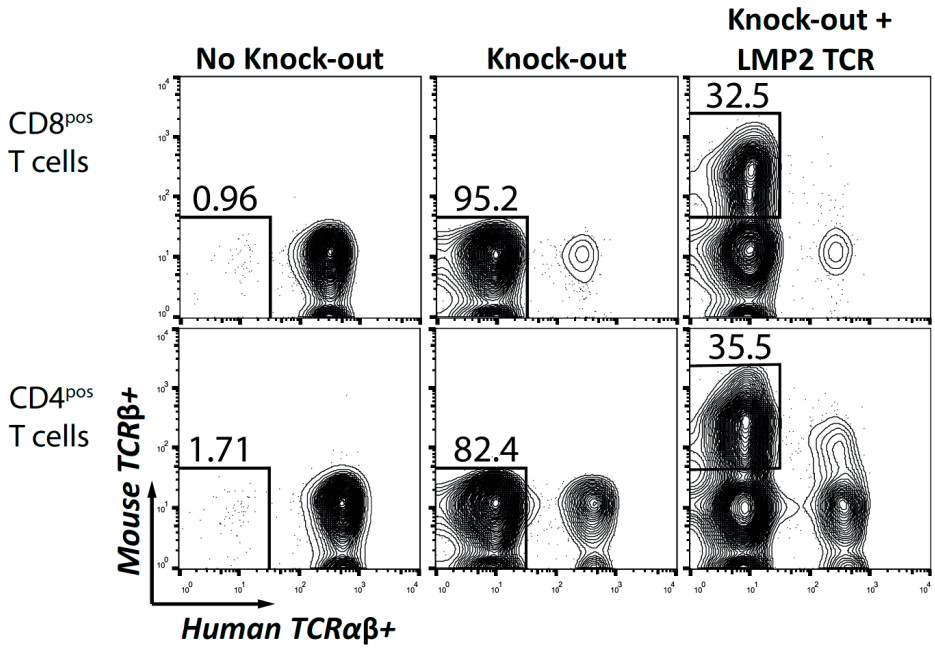
Supplementary Figure 1. Induced LMP2 expression in EBV-LCLs.
Five different EBV-LCLs were cultured under normal conditions and refreshed every 2-3 days or not refreshed for 5 days (exhausted) to induce LMP2 expression. LMP2 mRNA expression was determined by qRT-PCR. Expression shown was calculated as relative to the household genes VSP29 and GUSB, which was set to 1.
Statistical differences were assessed with the paired *t* test. **P* <.05; ***P* <.01
Shown are means with standard deviations of one experiment carried out in triplicate



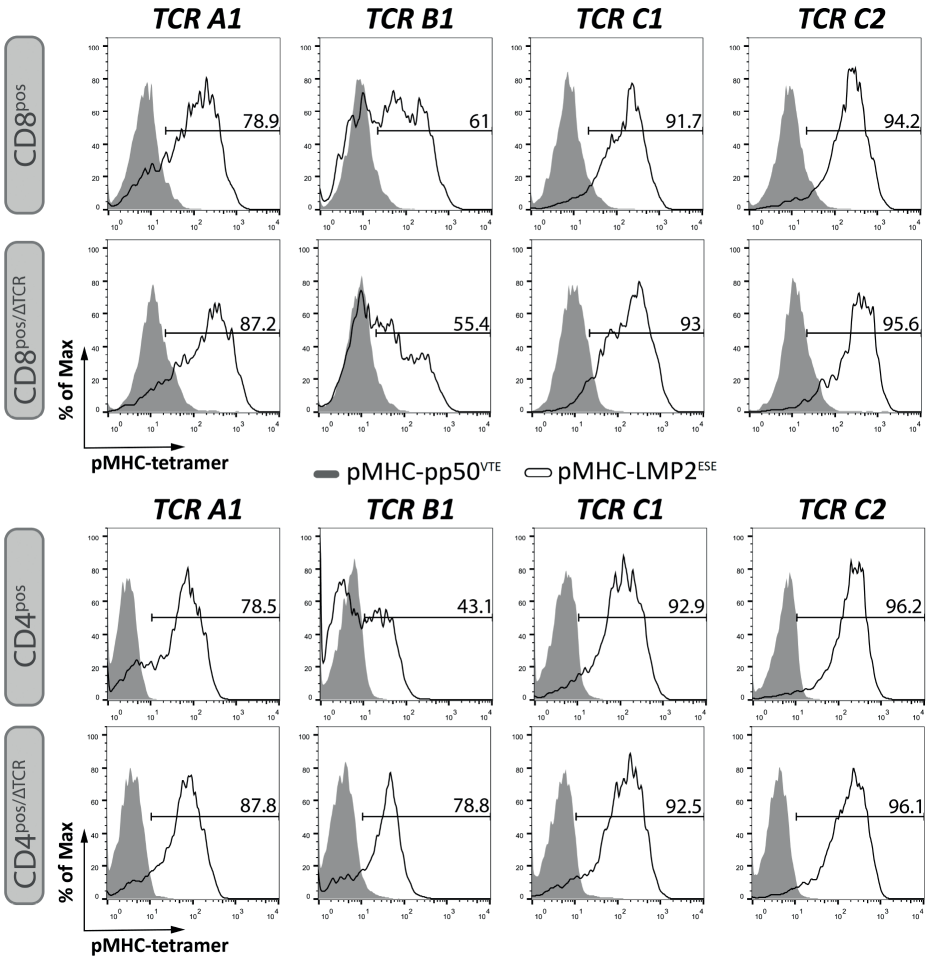
Supplementary Figure 2. TCR gene transfer introduced EBV-LMP2^{ESE} specificity and reactivity into Jurkat E6 cells.

The endogenous TCR of Jurkat E6 cells was knocked out using Crispr-Cas9 technology (ΔTCR). Jurkat E6$^{\Delta TCR}$ cells were transduced with CD8 to simulate CD8^{pos} T cells (Jurkat E6$^{\Delta TCR/CD8+}$). Transduced cells were purified based on expression of murine-TCR-Cβ using MACS and expanded. **A and B**) Shown are histograms of a specific HLA-A*01:01/pMHC-EBV-LMP2^{ESE} tetramer (black-line) or irrelevant HLA-A*02:01/pMHC-pp65^{NLV} tetramer (grey) staining of (**A**) Jurkat E6$^{\Delta TCR/CD8+}$ and (**B**) Jurkat E6$^{\Delta TCR}$. Numbers in the middle-right represent percentage of EBV-LMP2^{ESE} -specific T cells binding tetramer. **C**) Jurkat E6 $^{\Delta TCR/CD8+}$ and (**D**) Jurkat E6 $^{\Delta TCR}$ cells were tested for recognition of K562 cells transduced with HLA-A*01:01 and pulsed with and without EBV-LMP2^{ESE} peptide. Recognition was measured by upregulation of activation marker CD69 after stimulation for 16 hours.

Shown are means with standard deviations of one experiment carried out in triplicate (C and D)

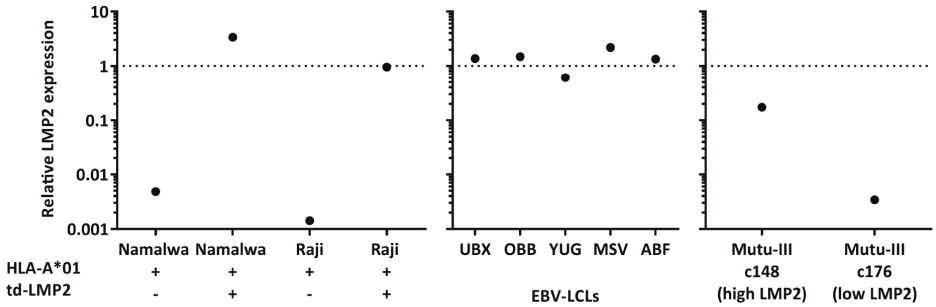


Supplementary Figure 3. Simultaneous CRISPR/Cas9 targeting of the endogenous TCRα and TCRβ. $CD8^{pos}$ and $CD4^{pos}$ T cells were isolated from healthy EBV^{neg} HLA-A*01:01^{pos} donor H, using MACS isolations. The endogenous TCR was knocked out using the CRISPR-Cas9 technology prior to introduction of EBV-$LMP2^{ESE}$-specific TCRs. The endogenous TCR of $CD8^{pos}$ (top row) and $CD4^{pos}$ (lower row) T cells were either not knocked out by CRISPR-Cas9(left panel) or TRAC/TRBC knocked out (ΔTCR; middle panel). The T cells without endogenous TCR were subsequently transduced with EBV-$LMP2^{ESE}$-specific TCRs (right panel; Representative example). Presence of endogenous (human) TCR was measured by expression of human TCRαβ (x-axis) and presence of introduced EBV-$LMP2^{ESE}$-specific TCR was measured by expression of murine-TCR-Cβ (y-axis)

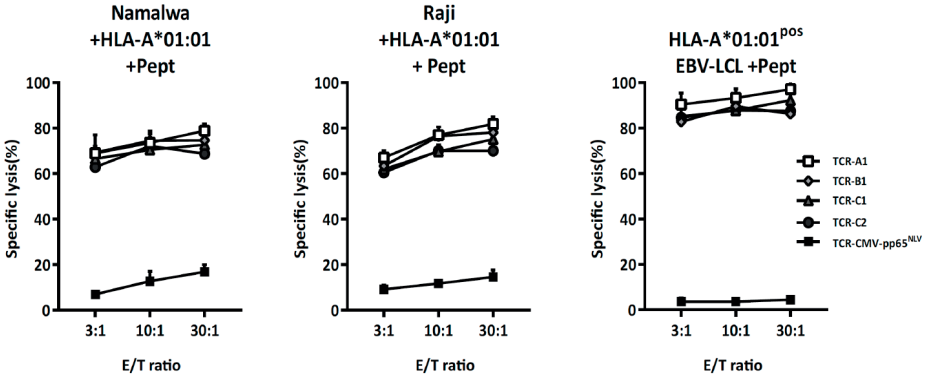


Supplementary Figure 4. pMHC-EBV-LMP2^{ESE} tetramer staining of CD8^{pos} and CD4^{pos} primary T cells with and without endogenous TCR knock-out(CRISPR/Cas9).

Shown are histograms of specific HLA-A*01:01/pMHC-LMP2^{ESE} tetramer (black-line) and irrelevant HLA-A*01:01 restricted pMHC-pp50^{VTE} tetramer (grey) stainings of CD8^{pos} and CD8$^{pos/\Delta TCR}$ T cells (upper panels) or CD4^{pos} and CD4$^{pos/\Delta TCR}$ T cells (lower panels) transduced and enriched for EBV-LMP2^{ESE}-specific TCRs. Numbers in the middle-right represent percentage of EBV-LMP2^{ESE}-specific T cells.



Supplementary Figure 5. LMP2 expression of EBV-associated malignant cell-lines
LMP2 mRNA expression was determined by qRT-PCR. LMP2 expression of EBV-associated malignant cell-lines Namalwa, Raji, EBV-LCLs and mutu-III are shown. Expression shown was calculated as relative to the household genes VSP29 and GUSB, which was set to 1.
Shown are representative data of one experiment carried out in triplicate



Supplementary Figure 6. CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs lyse target cells pulsed with LMP2 peptide.
CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs were tested for their lytic capacity to target malignant cells and EBV-LCLs pulsed with LMP2^{ESE} peptide. CD8$^{pos/\Delta TCR}$ T cells transduced with a TCR targeting HLA-A*02:01/CMV-pp65^{NLV} were used as control.
Data are shown from one experiment carried out in triplicate