Cytotherapy 24 (2022) 818–826

Contents lists available at ScienceDirect

CYTOTHERAPY

journal homepage: www.isct-cytotherapy.org

International Society ISCT Cell & Gene Therapy®

FULL-LENGTH ARTICLE
Basic Research

# Rapid single-cell identification of Epstein–Barr virus-specific T-cell receptors for cellular therapy

María Fernanda Lammoglia Cobo[1], Carlotta Welters[1], Leonie Rosenberger[2], Matthias Leisegang[2,3,4], Kerstin Dietze[1], Christian Pircher[2], Livius Penter[1,5], Regina Gary[6], Lars Bullinger[1,4], Anna Takvorian[1], Andreas Moosmann[7,8], Klaus Dornmair[9], Thomas Blankenstein[10], Thomas Kammertöns[2], Armin Gerbitz[11], Leo Hansmann[1,4,*]

[1] *Department of Hematology, Oncology and Tumor Immunology, Charité–Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany*
[2] *Institute of Immunology, Charité–Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany*
[3] *David and Etta Jonas Center for Cellular Therapy, The University of Chicago, Chicago, Illinois, USA*
[4] *German Cancer Consortium (DKTK), partner site Berlin, German Cancer Research Center (DKFZ), Heidelberg, Germany*
[5] *Dana-Farber Cancer Institute, Boston, Massachusetts, USA*
[6] *Department of Internal Medicine 5–Hematology/Oncology, University Hospital Erlangen, Erlangen, Germany*
[7] *Department of Medicine III, Klinikum der Universität München, Munich, Germany*
[8] *German Center for Infection Research (DZIF), Munich, Germany*
[9] *Institute of Clinical Neuroimmunology, University Hospital and Biomedical Center, Ludwig Maximilian University of Munich, Munich, Germany*
[10] *Molecular Immunology and Gene Therapy, Max-Delbrück-Center for Molecular Medicine (MDC), Berlin, Germany*
[11] *Hans Messner Allogeneic Stem Cell Transplant Program, Princess Margaret Cancer Centre, Toronto, Canada*

ARTICLE INFO

*Article History:*
Received 11 December 2021
Accepted 10 March 2022

*Key Words:*
adoptive T-cell therapy
allogeneic stem cell transplantation
Epstein–Barr virus
single-cell technologies
virus-associated malignancies

ABSTRACT

*Background and aims:* Epstein–Barr virus (EBV) is associated with solid and hematopoietic malignancies. After allogeneic stem cell transplantation, EBV infection or reactivation represents a potentially life-threatening condition with no specific treatment available in clinical routine. *In vitro* expansion of naturally occurring EBV-specific T cells for adoptive transfer is time-consuming and influenced by the donor's T-cell receptor (TCR) repertoire and requires a specific memory compartment that is non-existent in seronegative individuals.
The authors present highly efficient identification of EBV-specific TCRs that can be expressed on human T cells and recognize EBV-infected cells.
*Methods and Results:* Mononuclear cells from six stem cell grafts were expanded *in vitro* with three HLA-B*35:01- or four HLA-A*02:01-presented peptides derived from six EBV proteins expressed during latent and lytic infection. Epitope-specific T cells expanded on average 42-fold and were single-cell-sorted and TCR$\alpha\beta$-sequenced. To confirm specificity, 11 HLA-B*35:01- and six HLA-A*02:01-restricted dominant TCRs were expressed on reporter cell lines, and 16 of 17 TCRs recognized their presumed target peptides. To confirm recognition of virus-infected cells and assess their value for adoptive therapy, three selected HLA-B*35:01- and four HLA-A*02:01-restricted TCRs were expressed on human peripheral blood lymphocytes. All TCR-transduced cells recognized EBV-infected lymphoblastoid cell lines.
*Conclusions:* The authors' approach provides sets of EBV epitope-specific TCRs in two different HLA contexts. Resulting cellular products do not require EBV-seropositive donors, can be adjusted to cell subsets of choice with exactly defined proportions of target-specific T cells, can be tracked *in vivo* and will help to overcome unmet clinical needs in the treatment and prophylaxis of EBV reactivation and associated malignancies.


## Introduction

Epstein–Barr virus (EBV) belongs to the family of gamma-herpesviruses, and more than 80% of humans over the age of 20 are infected [1]. EBV predominantly infects B cells, resulting in different forms of latent (non-productive) and lytic (virus producing)

* Correspondence: Leo Hansmann, MD, Department of Hematology, Oncology and Tumor Immunology, Charité–Universitätsmedizin Berlin, Augustenburger Platz 1, Berlin 13353, Germany.
*E-mail address:* leo.hansmann@charite.de (L. Hansmann).

https://doi.org/10.1016/j.jcyt.2022.03.005

infection. Primary EBV infection of a human being is usually self-limiting and controlled by T cell-dominated immune responses, leading to latent virus persistence [2]. Infected cells present characteristic sets of EBV peptides on HLA that can be recognized by EBV-specific T cells [3,4]. In the proliferative latency III program of B-cell infection, six EBV nuclear antigens and three latent membrane proteins (LMPs), among others, are expressed. EBV nuclear antigens regulate replication of the viral genome and are involved in B-cell transformation, disruption of cell cycle checkpoints and lymphoma development [5–8]. Although LMP1 is a major transforming protein, LMP2 can drive proliferation in the absence of B-cell receptor stimulation and is involved in the induction of lymphoma-like phenotypes in B cells [9–11]. During the lytic phase, approximately 70 EBV proteins are expressed, including transcription factors BRLF1 and BZLF1, messenger RNA export factor BMLF1 and DNA polymerase processivity factor BMRF1, which contain peptides that can be presented on HLA [3].

Clinical observations and experiences from adoptive transfer of EBV-specific T-cell products suggest that T-cell responses are critical for controlling EBV infection and maintaining the latent phase [12–16]. Immunodominant EBV epitopes that drive substantial $CD8^+$ T-cell expansion have been identified in a variety of HLA contexts [17–20]. Expanded EBV epitope-specific T cells persist after acute infection [21] and can constitute up to 5% of circulating $CD8^+$ T cells in asymptomatic immunocompetent individuals [3].

Apart from often inapparent primary infection, EBV can cause life-threatening complications, including post-transplantation lymphoproliferative disorders (PTLDs), in states of severe immunosuppression associated with solid organ or allogeneic stem cell transplantation (allo-SCT). During the first 100 days after allo-SCT, T cells are typically substantially reduced in numbers and functionally inhibited, allowing EBV reactivation in approximately 30% of patients, with limited, non-specific treatment options available in clinical routine [22]. Especially at risk are EBV-seropositive patients who receive stem cell grafts from seronegative donors—a constellation of increasing relevance with rising numbers of younger haploidentical stem cell donors [23]. PTLDs occur in 1–8% of patients after allo-SCT [24], and close to 100% are EBV-associated when they develop within the first 6 months [25]. In summary, it would be beneficial if clinical conditions demonstrating impaired T-cell immunity and high risk of EBV-associated complications could be bridged with easily accessible, highly specific cellular products.

EBV-specific T-cell products have been shown to be effective in controlling infections and associated malignancies [13,26,27]. Current strategies for the generation of virus-specific T-cell products include *in vitro* expansion of epitope-specific (third-party) T cells from peripheral blood or stem cell grafts [27–29]. However, the following technical and clinical obstacles have prevented broad translation of such products into clinical routine: (i) *in vitro* expansion requires an antigen-experienced memory compartment, (ii) frequencies of epitope-specific T cells can be variable between individuals and products, (iii) donor selection and HLA allotypes are likely to influence functional capacities of the product and (iv) such products are laborious to produce and only directly available at a few specialized centers.

The authors hypothesized that the T-cell compartment of allogeneic stem cell grafts could be used to identify sets of T-cell receptors (TCRs) specific for carefully selected latent and lytic EBV epitopes in the context of pre-defined HLA backgrounds. These TCRs would be available "off-the-shelf" for production of EBV-specific T-cell products within minimum amounts of time. The authors' approach allows the use of T-cell sources of choice independent of EBV serostatus, guarantees target epitope specificity with clearly defined frequencies of EBV-specific T cells, results in a product that can be tracked *in vivo* by specific antibodies and can be expanded to other HLA allotypes for prophylaxis or treatment of EBV and associated malignancies.

## Methods

### *Stem cell grafts*

The authors collected leftover material from six granulocyte colony-stimulating factor-mobilized stem cell grafts of EBV-seropositive donors who expressed either HLA-B*35:01 or HLA-A*02:01. Mononuclear cells were isolated using Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, USA) and cryopreserved in human serum albumin (Grifols, Barcelona, Spain) supplemented with 10% dimethyl sulfoxide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The study was approved by the local institutional review board (Ethikkommission der Charité–Universitätsmedizin Berlin; approval no. EA2/197/18), all participants gave written informed consent and the entire study was conducted in accordance with the principles of the Declaration of Helsinki.

### *Peptide-specific* in vitro *expansion*

Mononuclear cells were thawed, washed twice with CellGro DC medium (Sartorius CellGenix GmbH, Freiburg, Germany) and rested for 16 h at 37°C and 5% carbon dioxide ($CO_2$). Subsequently, $5 \times 10^7$ to $3 \times 10^8$ cells were stimulated for 2 h with synthetic peptides (JPT Peptide Technologies, Berlin, Germany) at 1 $\mu$g/mL per peptide. Cells were washed twice and expanded for 9 days at $2.5 \times 10^6$ cells/mL in CellGro DC medium, 1% GlutaMAX (Life Technologies, Carlsbad, CA, USA), 1% donor serum and 50 IU/mL IL-2 (aldesleukin; Novartis Pharma GmbH, Fehrbellin, Germany) at 37°C and 5% $CO_2$. Fresh medium was supplied on day 5. After expansion, cells were cryopreserved.

### *Flow cytometry*

All flow cytometry reagents, including monoclonal antibodies and live/dead dyes, were titrated and used according to the manufacturers' instructions. Phycoerythrin- and allophycocyanin-labeled peptide major histocompatibility complex (pMHC) tetramers (National Institutes of Health Tetramer Core Facility, Atlanta, GA, USA) were provided at 1.1–1.5 mg/mL in water and diluted as 20% glycerol (SERVA Electrophoresis GmbH, Heidelberg, Germany) stocks. Per stain, the authors used 0.63 $\mu$L of pMHC tetramer stock solution in 150 $\mu$L phosphate-buffered saline (Life Technologies) supplemented with 2% fetal bovine serum (FBS) (Life Technologies). Flow cytometry data were acquired on Navios (Beckman Coulter, Brea, CA, USA), LSRFortessa (BD Biosciences, Franklin Lakes, NJ, USA) and Aurora (Cytek Biosciences, Fremont, CA, USA) instruments.

### *Fluorescence-activated cell sorting*

Cells were thawed, rested in Roswell Park Memorial Institute (RPMI) 1640 with 10% FBS for 1 h at 37°C and 5% $CO_2$ and stained with monoclonal antibodies. Single cells were index-sorted into 96-well plates pre-filled with OneStep reverse transcription polymerase chain reaction buffer (QIAGEN, Hilden, Germany) using a FACSAria Fusion cell sorter (BD Biosciences) as described previously [30].

### *Single-cell TCR$\alpha\beta$ sequencing*

Polymerase chain reaction amplification, molecular barcoding, library preparation and MiSeq (Illumina, San Diego, CA, USA) sequencing were carried out as previously described [31,32]. Clonal expansion was defined as two or more cells with identical TCR$\alpha$ and TCR$\beta$ CDR3 amino acid sequences. Cells that expressed two TCR$\alpha$

chains in combination with the same TCR$\beta$ chain were defined as one clone if TCR$\alpha$ chains were identical; cells in which only one of these TCR$\alpha$ chains was identified were also included in the clone.

*TCR expression on 58$\alpha^-\beta^-$ cell lines*

Missing sequence parts of leader, variable and constant regions of selected TCRs were completed with data downloaded from the international ImMunoGeneTics information system. Reconstructed TCR sequences were synthesized (Thermo Fisher Scientific, Waltham, MA, USA) and expressed in 58$\alpha^-\beta^-$ cell lines as previously described [32,33]. The 58$\alpha^-\beta^-$ cells expressed human CD8$\alpha\beta$ chains and green fluorescent protein (GFP) under the control of the nuclear factor of activated T-cell promoter [33], thus indicating T-cell activation by GFP expression. TCR expression was confirmed by mouse CD3 staining and detection by flow cytometry. As positive control for TCR activation, TCR-recombinant cell lines were stimulated with plate-bound anti-mouse CD3 for 16 h at 37°C and 5% $CO_2$. GFP expression was measured with flow cytometry and IL-2 production was detected in cell culture supernatants using the DuoSet enzyme-linked immunosorbent assay (ELISA) ancillary reagent kit 2 (R&D Systems, Minneapolis, MN, USA).

*TCR expression on third-party human T lymphocytes*

TCRs were expressed on T cells of a healthy female, EBV-seropositive donor that expressed HLA-A*02:01 and HLA-B*35:01. TCR inserts were constructed as described earlier and human TCR constant regions were replaced with mouse constant region sequences to minimize mispairing with endogenous TCR chains. All TCR constructs were codon-optimized for expression in human cells. To generate retroviral vector particles to transduce human cells, 18 $\mu$g MP71 vector, including the TCR insert, was diluted in 150 $\mu$L water and 250 mM calcium dichloride and combined with 150 $\mu$L transfection buffer, comprising 1.6 g sodium chloride (Sigma-Aldrich, Burlington, MA, USA), 74 mg potassium chloride (Sigma-Aldrich), 50 mg disodium hydrogen phosphate (Sigma-Aldrich) and 1 g 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Sigma-Aldrich), and 100 mL water adjusted to pH 6.76. The mixture was added dropwise to 8.5 $\times$ $10^5$ 293Vec-RD114 producer cells (BioVec Pharma, Québec, Canada). Cells were cultured at 37°C and 5% $CO_2$ for 6 h, and medium was changed afterward.

For transduction, 1.5 $\times$ $10^6$ human lymphocytes were stimulated with 400 IU/mL IL-2 (Chiron Corporation, Emeryville, CA, USA) in a 24-well plate pre-coated with 5 $\mu$g/mL anti-CD3 (BD Pharmingen, San Diego, CA, USA) and 1 $\mu$g/mL anti-CD28 (BD Pharmingen) for 2 days. Afterward, cells were spinoculated on two consecutive days for 90 min at 800 $\times$ g and 32°C with 1 mL filtered (0.45 $\mu$m pore size) RD114 retroviral vector supernatant, 400 IU/mL IL-2 and 8 $\mu$g/mL protamine sulfate (Sigma-Aldrich). Spinoculated cells were expanded in cell culture medium supplemented with 400 IU/mL IL-2 for 10 days and rested for 2 days with 40 IU/mL IL-2 before cryopreservation. Efficiency of TCR transduction was determined with flow cytometry by mouse TCR$\beta$ constant region staining.

*Lymphoblastoid and mini-lymphoblastoid cell lines*

Lymphoblastoid cell lines (LCLs) were generated by transformation of peripheral blood mononuclear cells with supernatant of the EBV strain B95.8 as previously described [34]. Mini-LCLs were prepared by immortalizing HLA-B*35:01$^+$ or HLA-A*02:01$^+$ B cells with the recombinant mini-EBV plasmid p1495.4 [35,36]. Mini-EBV plasmids contained less than half of the EBV genome, and mini-LCLs could not produce infectious particles [37]. Detailed HLA class I data of all LCLs and mini-LCLs used in this study are included in the supplementary material.

*Co-culture of TCR-recombinant cells with target cells*

A total of 60 000 TCR-recombinant 58$\alpha^-\beta^-$ cells were cultured with 100 000 antigen-presenting cells. Cells were co-cultured in 150 $\mu$L RPMI 1640 and 10% FBS in 96-well plates for 16 h at 37°C and 5% $CO_2$. For target peptide loading, 3 $\times$ $10^6$ antigen-presenting cells were incubated with the respective target peptide at 7.5 $\mu$mol/L for 30 min prior to co-culture.

TCR-transduced human lymphocytes were cultured at 50 000 T cells with 10 000 potential target cells in 200 $\mu$L RPMI 1640 and 10% FBS in 96-well plates at 37°C and 5% $CO_2$ for 20 h. Exact effector-to-target ratios of individual co-cultures depended on frequencies of CD8$^+$ and TCR-transduced T cells within individual T-cell preparations and can be found in the supplementary material. Interferon gamma (IFN-$\gamma$), granzyme B and tumor necrosis factor alpha (TNF-$\alpha$) were determined in cell culture supernatants using a human IFN-$\gamma$ ELISA set (BD Biosciences), human granzyme B DuoSet ELISA kit (R&D Systems) and human TNF-$\alpha$ DuoSet ELISA kit (R&D Systems).

## Results

*Expansion of EBV epitope-specific T cells from stem cell grafts*

Efficient identification of EBV epitope-specific TCRs requires sufficient frequencies of specific T-cell clones. Therefore, the authors used *in vitro* expansion of 5 $\times$ $10^7$ to 3 $\times$ $10^8$ mononuclear cells from five EBV-seropositive allogeneic stem cell grafts in the presence of three synthetic EBV-derived peptides presented on HLA-B*35:01 and one additional graft with four synthetic peptides presented on HLA-A*02:01 (Table 1). The peptides used were selected immunodominant epitopes expressed during lytic and latent infection phases, and frequencies of specific CD8$^+$ T cells were determined by flow cytometry using pMHC tetramer staining.

During *in vitro* expansion, absolute numbers of CD8$^+$ T cells increased (Figure 1A), and peptide-specific CD8$^+$ T cells expanded on average 42-fold (range, 1–228). Degrees of expansion varied between stem cell grafts and individual peptides (Figure 1B,C). Frequencies of HPV-, YPL- and EPL-specific HLA-B*35:01-restricted CD8$^+$ T cells increased on average 25-, 10- and 108-fold, respectively (Figure 1B). Frequencies of GLC-, CLG-, FLY- and YVL-specific HLA-A*02:01-restricted CD8$^+$ T cells increased 14-, 8-, 26- and 27-fold, respectively (Figure 1C). Detailed cell numbers for each stem cell graft before and after expansion can be found in supplementary Table 1. Representative pMHC tetramer staining is shown in Figure 1D (see supplementary Figure 1; see supplementary Table 2).

*Single-cell identification of EBV epitope-specific TCRs*

Reliable and efficient identification of paired TCR$\alpha\beta$ sequences from complex T-cell populations requires single-cell resolution. The authors isolated epitope-specific CD8$^+$ T cells by pMHC tetramer staining of stem cell grafts expanded *in vitro* and subsequent fluorescence-activated cell sorting (see supplementary Table 2). Gating for single-cell sorting is illustrated in Figure 2A. TCR$\alpha\beta$ genes of every

**Table 1**
Peptides for EBV epitope-specific *in vitro* expansion.

| Label | Amino acid sequence | Protein | Virus phase | Presented on HLA |
|---|---|---|---|---|
| HPV | HPVGEADYFEY | EBNA1 | Latency I, II, III | B*35:01 |
| YPL | YPLHEQHGM | EBNA3A | Latency III | B*35:01 |
| EPL | EPLPQGQLTAY | BZLF1 | Lytic | B*35:01 |
| GLC | GLCTLVAML | BMLF1 | Lytic | A*02:01 |
| CLG | CLGGLLTMV | LMP2A | Latency II, III | A*02:01 |
| FLY | FLYALALLL | LMP2A | Latency II, III | A*02:01 |
| YVL | YVLDHLIVV | BRLF1 | Lytic | A*02:01 |

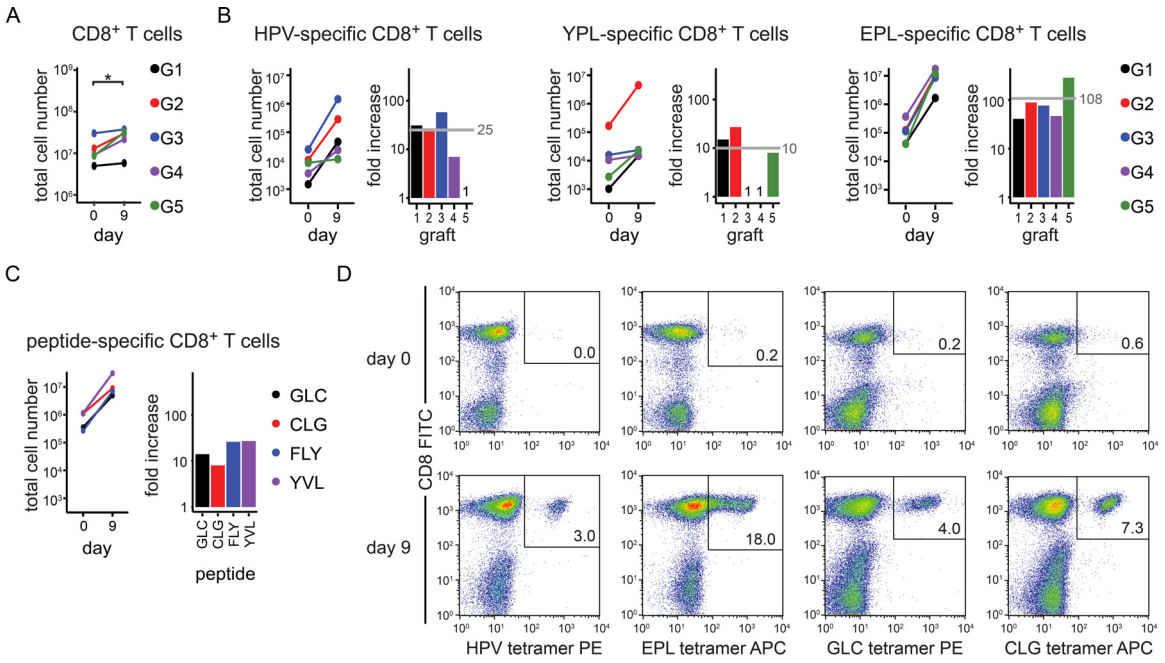


**Figure 1.** Expansion of EBV peptide-specific T cells from stem cell grafts. Mononuclear cells from allogeneic stem cell grafts were expanded *in vitro* in the presence of EBV-derived peptides for 9 days. (A) Total numbers of CD8$^+$ T cells from five stem cell grafts (G1–5). (B) Total numbers and fold expansion of HLA-B*35:01-restricted peptide-specific T cells from five stem cell grafts (G1–5). Gray lines and gray numbers indicate averages. (C) Total cell number and fold expansion of HLA-A*02:01-restricted peptide-specific T cells from stem cell graft G6. (D) Frequencies of peptide-specific CD8$^+$ T cells before (day 0) and after (day 9) expansion as determined by pMHC tetramer staining. HPV- and EPL (presented on HLA-B*35:01)-specific expansions from stem cell graft G3 are shown as an example along with GLC- and CLG (presented on HLA-A*02:01)-specific expansions from stem cell graft G6. Plots are pre-gated on live single T cells. Numbers within gates indicate percentages. Significance determined by two-sided paired sample *t*-test. *$P < 0.05$. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

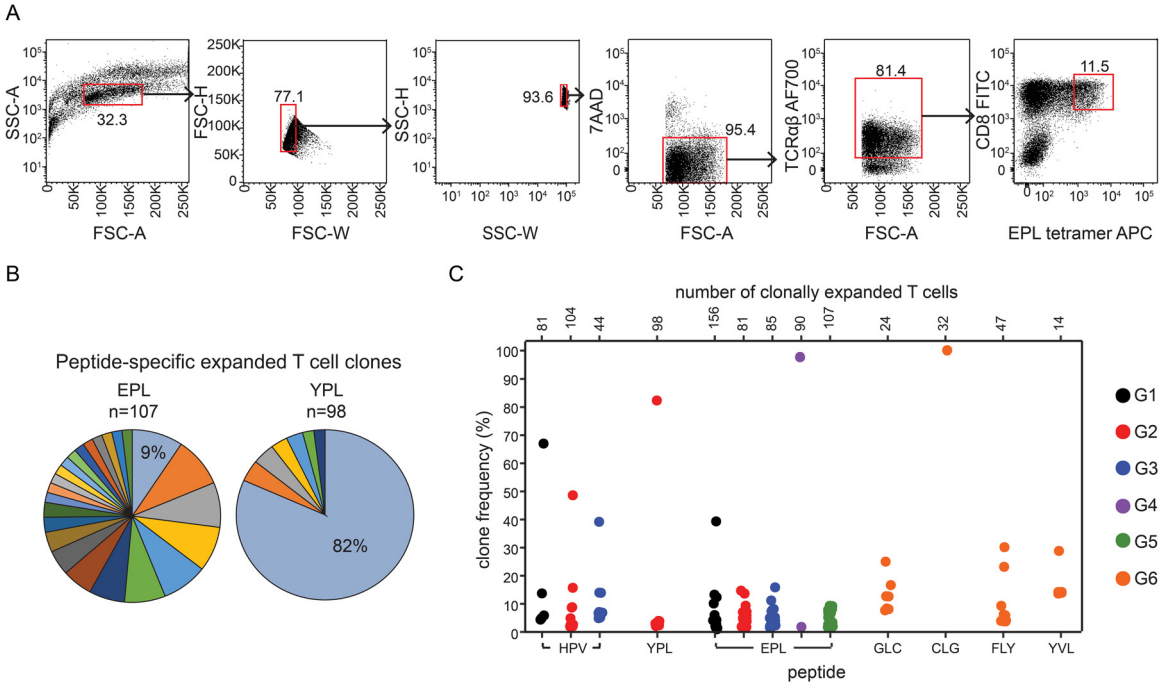


**Figure 2.** Identification of EBV epitope-specific TCRs. (A) Gates for single-cell sorting are shown in red. Selection of peptide-specific CD8$^+$TCR$\alpha\beta^+$ cells after gating on single lymphocytes and exclusion of dead cells. Data for single-cell sorting of EPL-specific cells from stem cell graft G3 are shown as a representative example of all sorts (n = 13). Numbers adjacent to gates indicate percentages. (B) Frequencies of T-cell clones after pMHC tetramer-specific single-cell sorting and sequencing. Expanded clones share identical TCR$\alpha\beta$ CDR3 amino acid sequences. Results from EPL-specific clonal expansion of stem cell graft G5 and YPL-specific clonal expansion of stem cell graft G2 are shown as examples. Numbers of clonally expanded cells are indicated above each chart. Percentages indicate percentages of clonally expanded cells. (C) Frequencies of expanded epitope-specific T-cell clones of all expansions (n = 13). Data points indicate individual expanded clones. Frequencies represent frequencies within clonally expanded cells for each peptide specificity. Numbers above the plot indicate total numbers of clonally expanded T cells. APC, allophycocyanin; FITC, fluorescein isothiocyanate; FSC-A, forward scatter area; FSC-H, forward scatter height; FSC-W, forward scatter width; 7AAD, 7-aminoactinomycin D; SSC-A, side scatter area; SSC-H, side scatter height; SSC-W, side scatter width.

single sorted cell were sequenced using next-generation sequencing (see supplementary Table 3), and clonal expansion was defined as detection of identical TCR$\alpha\beta$ CDR3 amino acid sequences in at least two cells.

Numbers and sizes of expanded T-cell clones varied between stem cell grafts and between epitope specificities (Figure 2B,C). Although *in vitro* expansion resulted in, for example, 24 different EPL-specific T-cell clones, with dominant clones accounting for only 9% of clonally expanded cells (EPL-specific expansion of stem cell graft G5), another expansion contained seven different YPL-specific clones, with the dominant clone comprising 82% of clonally expanded cells (Figure 2B). Frequencies of epitope-specific T-cell clones from all *in vitro* expansions are summarized in Figure 2C. The strongest clonal expansion was observed for CLG-specific T cells from stem cell graft G6, where only one expanded clone could be detected.

When comparing TCR sequences of epitope-specific clones between individual grafts, five TCRs (two HPV- and three EPL-specific) were found in more than one stem cell graft, and their degree of clonal expansion did not exceed 11% of clonally expanded T cells (see supplementary Table 4). In summary, clonal expansion was stem cell graft- and peptide-dependent and showed two patterns: (i) expansion of a few dominant clones comprising almost the entire clonally expanded T-cell compartment and (ii) expansion of a variety of less dominant clones, each accounting for less than approximately 35% of clonally expanded T cells.

### *Confirmation of target epitope specificity of expanded T-cell clones*

Although identification of largely expanded dominant clones within pMHC tetramer-sorted T cells suggested target peptide specificity, specificities of smaller size clones were less clear. To confirm target peptide specificity, the authors expressed TCRs of 17 expanded T-cell clones covering specificities for all peptides that had been used for *in vitro* expansion on 58$\alpha^-\beta^-$ reporter T cells with nuclear factor of activated T-cell-driven GFP expression (Table 2, Figure 3A). TCR-recombinant cell lines were named "58-[name of the TCR]" and incubated with antigen-presenting cells loaded with the respective peptides. GFP expression and IL-2 production were measured as indicators of T-cell activation. Mini-LCLs were used as antigen-presenting cells and loaded with peptides of choice.

All TCR-recombinant cell lines produced GFP and IL-2 upon stimulation with plate-bound anti-CD3 (see supplementary Figure 2). Upon co-incubation with target peptide-loaded antigen-presenting cells, 16 of 17 TCRs were activated, and no activation could be detected upon incubation with non-target peptide-loaded antigen-presenting cells (Figure 3B,C; also see supplementary Figure 3). TCR EPL7A4 could not be activated by its presumed target peptide and was excluded from further analysis. Notably, 58-GLC1B11 and 58-GLC1B4 shared an identical TCR$\beta$ chain but expressed different alpha chains (Table 2). The authors expressed both alpha chains individually together with the corresponding TCR$\beta$ chain, and both combinations resulted in productive TCRs specific for the same target peptide. In summary, the authors confirmed specificity for a panel of 16 TCRs targeting EBV epitopes presented during the latent and lytic infection phase.

### *TCR-transduced third-party human lymphocytes recognize EBV-infected cell lines*

To determine their translational potential, the authors selected three HLA-B*35:01- and four HLA-A*02:01-restricted TCRs, confirmed that they were not broadly cross-reactive with HLA other than the target HLA (see supplementary Figure 4; see supplementary Table 5), expressed them in human lymphocytes and tested their reactivity with EBV-infected cells. TCRs were expressed on CD4-depleted human lymphocytes, and TCR-transduced T cells were co-cultured with four EBV-infected LCLs (named B01, JY, B03 and DJS). TCR-transduced lymphocytes were named "hL-[name of the TCR]," and recombinant TCR expression was detectable on average on 34% of $CD8^+$ T cells (see supplementary Figure 5). CD137 expression on $CD8^+$ T cells and IFN-$\gamma$ in cell culture supernatants were measured as readouts for T-cell activation, and activation was assumed if either of them was detectable.

TCR-transduced T cells of all six epitope specificities significantly upregulated CD137 expression when incubated with at least one of the corresponding LCLs (Figure 4A; also see supplementary Figure 6). T cells specific for EPL, GLC, CLG, YVL and FLY also produced significant amounts of IFN-$\gamma$ in comparison with non-transduced T cells (Figure 4B). T cells expressing the HPV-specific TCR (hL-HPV13A10) were activated, as indicated by CD137 expression; however, IFN-$\gamma$ production was low and did not reach statistical significance because of relatively high background IFN-$\gamma$ levels of non-transduced T cells, which varied between different LCL and co-incubation experiments.

To further characterize the activation response of TCR-transduced human T cells, the authors selected hL-EPL11A7 as an example and

**Table 2**
Epitope-specific, recombinantly expressed TCRs.

| Label | HLA restriction | G | TRAV | CDR3$\alpha$ AA sequence | TRAJ | TRBV | CDR3$\beta$ AA sequence | TRBJ | % cf[a] |
|---|---|---|---|---|---|---|---|---|---|
| HPV13A10 | B*35:01 | G1 | 5*01 | CAESYTGGFKTIF | 9*01 | 6-1*01 | CASGTEAFF | 1-1*01 | 67 |
| HPV13B12 | B*35:01 | G1 | 10*01 | CVVSEEGGFKTIF | 9*01 | 12-5*01 | CASGLGGSNEQFF | 2-1*01 | 14 |
| HPV9A2 | B*35:01 | G3 | 20*01 | CAVQELVTSGSRLTF | 58:01 | 9*01 | CASTGAGEGPFF | 1-1*01 | 39 |
| HPV9C10 | B*35:01 | G3 | 20*01 | CAVQAMTSSNYKLTF | 53*01 | 9*01 | CASSARTGELFF | 2-2*01 | 14 |
| YPL3D3 | B*35:01 | G2 | 19*01 | CALSEAGGFGNEKLTF | 48*01 | 10-3*01 | CAISDPRDSYEQYF | 2-7*01 | 82 |
| EPL11A7 | B*35:01 | G1 | 1-2*01 | CAVMSSGGSYIPTF | 6*01 | 10-3*01 | CAISTGDSNQPQHF | 1-5*01 | 13 |
| EPL11A12 | B*35:01 | G1 | 24*01 | CAFPGGNKLVF | 47*01 | 10-3*01 | CAISEWDSPTLNSPLHF | 1-6*01 | 10 |
| EPL7A4 | B*35:01 | G3 | 19*01 | CALSRNYGQNFVF | 26*01 | 12-3*01 | CASSLLAATYNEQFF | 2-1*01 | 5 |
| EPL7A10 | B*35:01 | G3 | 1-2*01 | CAVRGSGGSYIPTF | 6*01 | 10-3*01 | CATGTGDSNQPQHF | 1-5*01 | 11 |
| EPL11A10 | B*35:01 | G4 | 24*01 | CALNAGGTSYGKLTF | 52*01 | 7-3*01 | CASSRDFYAYNEQFF | 2-1*01 | 98 |
| EPL13B9 | B*35:01 | G5 | 2*01 | CAVEDMNSGGYQKVTF | 13*02 | 28*01 | CASKRTATYEQYF | 2-7*01 | 8 |
| GLC1B11 | A*02:01 | G6 | 5*01 | CAESTGKLIF | 37*01 | 29-1*01 | CSVGTGGTNEKLFF | 1-4*01 | 17 |
| GLC1B4 | A*02:01 | G6 | 5*01 | CAESTSWGKLQF | 24*02 | 29-1*01 | CSVGTGGTNEKLFF | 1-4*01 | 25 |
| CLG3A10 | A*02:01 | G6 | 21*01 | CAILMDSNYQLIW | 33*01 | 10-2*02 | CASSEDGMNTEAFF | 1-1*01 | 100 |
| FLY5D11 | A*02:01 | G6 | 17*01 | CATEGDSGYSTLTF | 11*01 | 6-5*01 | CASSYQGGNYGYTF | 1-2*01 | 30 |
| FLY5B5 | A*02:01 | G6 | 17*01 | CATVGNSGYSTLTF | 11*01 | 6-5*01 | CASSKQGGNIQYF | 2-4*01 | 23 |
| YVL16D1 | A*02:01 | G6 | 38-2/DV8*01 | CAYRSAFKLTF | 48*01 | 30*01 | CAWSVPLGRREKLFF | 1-4*01 | 14 |

AA, amino acid; cf, clone frequency; G, stem cell graft; TRAJ, TCR$\alpha$ J-gene and allele; TRAV, TCR$\alpha$ V-gene and allele; TRBJ, TCR$\beta$ J-gene and allele; TRBV, TCR$\beta$ V-gene and allele.

[a] Among clonally expanded cells specific for the respective epitope.

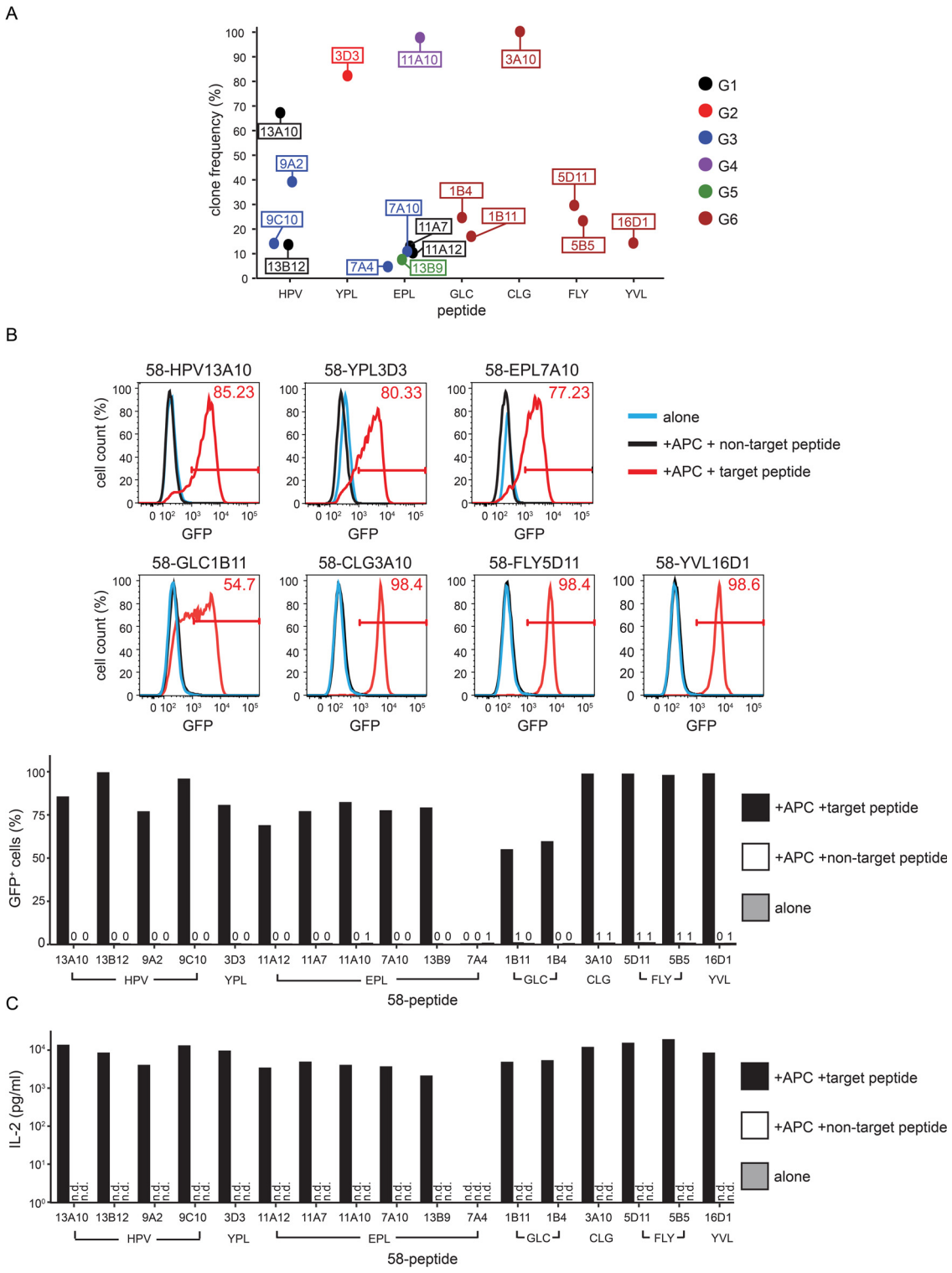


**Figure 3.** Confirmation of target peptide specificity of expanded T-cell clones. (A) TCRs selected for expression in 58$\alpha^-\beta^-$ reporter T cells. Data points indicate individual T-cell clones. Clone frequencies are indicated as frequencies of each individual clone within clonally expanded pMHC tetramer-sorted cells. (B) TCR-recombinant cell lines were co-incubated with peptide-loaded antigen-presenting cells, and GFP expression was measured by flow cytometry as an indicator for T-cell activation. "Alone" refers to TCR-recombinant 58$\alpha^-\beta^-$ reporter cell lines alone. All histograms are pre-gated on live TCR$\alpha\beta^+$CD8$^+$ cells. One co-culture per peptide specificity is shown as an example. (C) IL-2 production as measured by ELISA in cell culture supernatants corresponding to (B) TCR-recombinant data. APC, allophycocyanin; n.d., not detectable.

additionally determined CD107a expression and granzyme B and TNF-$\alpha$ secretion after stimulation with two HLA-A*02:01-matched LCLs (B01 and DJS) and one HLA-A*02:01-mismatched LCL (JY) in the presence of increasing target peptide concentrations. As expected, responses were substantially stronger when LCLs were artificially loaded with the target peptide. However, significant CD137 and CD107a expression as well as IFN-$\gamma$, granzyme B and TNF-$\alpha$ secretion could already be detected without target peptide loading, and T-cell activation was detectable only upon co-culture with HLA-matched LCLs (see supplementary Figure 7).

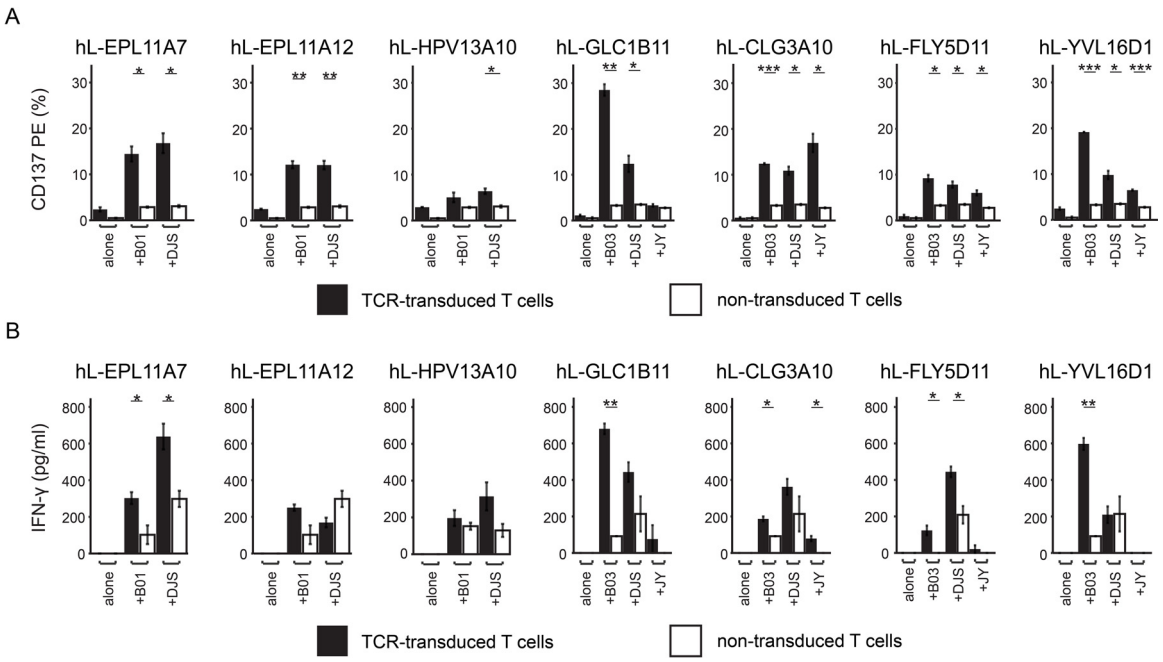


**Figure 4.** Human T cells transduced with EBV epitope-specific TCRs recognize EBV-infected cells Three HLA-B*35:01-restricted (EPL11A7, EPL11A12, HPV13A10) and four HLA-A*02:01-restricted (GLC1B11, CLG3A10, FLY5D11, YVL16D1) TCRs were expressed in human lymphocytes (named "hL-[name of the TCR]") and cultured with LCLs (B01, B03, DJS, JY). HLA-B*35:01-restricted TCRs were cultured with HLA-B*35:01-expressing B01 and DJS LCLs. HLA-A*02:01-restricted TCRs were cultured with HLA-A*02:01-expressing B03, DJS and JY LCLs. Non-transduced T cells were used as negative controls. (A) CD137 expression determined by flow cytometry. Plots are pre-gated on live $CD8^+$ lymphocytes. Data show three replicates from one experiment. (B) IFN-$\gamma$ in cell culture supernatants was measured by ELISA. Data are representative of independent experiments (n = 3). Co-cultures of each experiment were done in triplicate. All bars represent mean values ± standard error. Significance determined by Welch two-sample *t*-test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. PE, phycoerythrin.

## Discussion

The authors' research addresses the unmet clinical need of availability of highly specific T-cell products with reliable and reproducible characteristics for prophylaxis and treatment of EBV infection and associated malignancies within minimum amounts of time. The authors defined sets of TCRs that guarantee EBV epitope specificity, recognize EBV-infected cells in two different HLA contexts and can be expressed in T-cell sources of choice.

There are a variety of elegant methodologies for identification of virus-specific TCRs and in-depth characterization of their immune phenotypes [38–40]. The authors decided to use stem cell grafts for epitope-specific T-cell expansion, which was especially helpful for identification of otherwise potentially low-frequency T-cell clones against target antigens (e.g., derived from LMP2A). Although in theory any T-cell source, including peripheral blood, may be sufficient, stem cell grafts have considerable advantages: (i) detailed HLA typing is readily available, (ii) EBV serostatus is provided, (iii) they are characterized with regard to T-cell content and (iv) leftover material from one routine stem cell transplantation is sufficient for epitope-specific T-cell expansion, circumventing otherwise unnecessary higher volume blood draws from healthy individuals. Access to already HLA-typed stem cell donors can be especially helpful for identification of epitope-specific TCRs in the context of uncommon HLA types. In addition to these rather technical advantages, virus-specific T cells generated from stem cell donor specimens have already been used for clinically effective treatment [41–43], making them T-cell sources of choice for the authors' purposes.

To increase the chances of successful epitope-specific T-cell expansion and broad applicability of potentially resulting T-cell products, the authors chose target epitopes that had previously been well characterized, are known to strongly contribute to life-long EBV-specific T-cell memory and effector repertoires in infected individuals [3,20,44–46] and are presented on HLA types covering approximately 30–40% of the population (HLA-A*02: 29%, HLA-B*35: 6%) [47].

Reliable identification of EBV-specific TCRs required identification of epitope-specific T cells and highly efficient TCR$\alpha\beta$ sequencing at the single-cell level. The authors used pMHC tetramer staining for fluorescence-activated cell sorting index sorting of single peptide-specific T cells and subsequent paired TCR$\alpha\beta$ single-cell sequencing [30,48]. The authors have previously demonstrated that the combination of these technologies represents one of the most reliable and efficient approaches for identification of paired TCR$\alpha\beta$ sequences and associated immune phenotypes of single cells [31, 32]. In theory, epitope-specific T cells could have been sorted without prior *in vitro* expansion; however, frequencies of epitope-specific T cells were low before expansion (<1% of T cells), and accuracy and efficiency of single-cell sorting and TCR$\alpha\beta$ sequencing increase substantially with higher frequencies of target populations [49]. Degrees of clonal expansion varied between stem cell grafts and peptides used for *in vitro* expansion, yet epitope-specific TCRs could be successfully identified even in cases of oligoclonal expansions, in which clones of interest occupied less than 35% of $CD8^+$ clonally expanded T cells.

Five TCRs were expanded across different stem cell grafts. In the setting of only partially matched HLA types and peptide-driven *in vitro* expansion followed by pMHC tetramer-specific sorting, overlap of TCR repertoires of sorted populations is difficult to predict and will be influenced by the diversity of TCR repertoires and limited overlap between individuals. For nine of the re-expressed TCRs, the TCR$\alpha$ and/or TCR$\beta$ chains had already been deposited in the public database VDJdb; however, paired TCR$\alpha\beta$ information, which is critical for specificity, was available only for TCRs GLC1B11 and FLY5D11 [50]. For example, the TCR$\beta$ chain of TCR HPV13A10 has been described as part of a Melan A-specific TCR, whereas the alpha chain of the same TCR can be part of a cytomegalovirus IE1-specific TCR. The authors proved experimentally that, in combination, these alpha and beta chains compose the EBV epitope-specific TCR HPV13A10,

underlining the importance of paired TCR$\alpha\beta$ single-cell sequencing. Furthermore, among pMHC tetramer-sorted cells, the authors identified TCR$\beta$ chains that paired with two different TCR$\alpha$ chains. For one of these TCR$\beta$ chains, the authors showed experimentally that combining with either TCR$\alpha$ chain resulted in the productive TCRs GLC1B4 and GLC1B11, which were specific for the same epitope.

To confirm epitope specificity of selected TCRs, the authors used modified 58$\alpha^-\beta^-$ cells as reporter cells and mini-LCLs as antigen-presenting cells. Mini-LCLs contain a selected set of latent EBV genes [35]; however, none of the TCR-recombinant 58$\alpha^-\beta^-$ cell lines were activated by mini-LCLs, most likely due to low target antigen expression/presentation. Nevertheless, mini-LCLs could efficiently present artificially loaded peptides.

EBV epitope specificity for a variety of publicly available TCRs has already been demonstrated using artificially peptide-loaded antigen-presenting cells; however, data on TCRs that recognize EBV-infected cells without additional peptide loading are limited. The single-cell resolution of the authors' approach yielded sets of candidate TCRs specific for the target peptides of choice. To demonstrate that the identified TCRs could indeed recognize virus-infected cells, seven TCRs against latent and lytic phase epitopes were expressed on human lymphocytes and incubated with LCLs that expressed the required HLA-A*02:01 or B*35:01 allele. LCLs show a latency III EBV gene expression pattern and a general cellular phenotype that closely correspond to PTLDs [51]. All tested TCRs showed *in vitro* reactivity with LCLs by CD137 upregulation and/or IFN-$\gamma$ production, making them promising candidates for translation into highly specific T-cell products for adoptive transfer. The authors chose IFN-$\gamma$ secretion as the readout for T-cell activation because it requires triggering of at least 20–50% of TCRs on a T cell, and cytotoxic activity can be assumed if IFN-$\gamma$ secretion is detectable [52]. As an example, for one TCR, the authors showed that in case of target antigen recognition, both CD107a expression and granzyme B and TNF-$\alpha$ secretion were also detectable. Although the majority of LCLs are not in the lytic infection phase, it has already been shown that LCLs can efficiently activate T cells recognizing lytic phase epitopes [53].

With respect to potential TCR cross-reactivity with HLAs other than the target HLA, the authors could not detect T-cell activation upon incubation with HLA-mismatched mini-LCLs for all seven TCRs that were transduced into human peripheral blood lymphocytes. However, more detailed studies of HLA cross-reactivity are likely required before therapeutic application can be implemented. For TCR expression in human peripheral blood lymphocytes, the authors replaced the human TCR constant regions with their murine counterparts to (i) avoid mispairing of TCR$\alpha\beta$ chains [54] and (ii) allow staining with mouse TCR$\beta$ constant region antibodies. Whether expression of the murine constant regions could result in therapeutically relevant immunogenicity has to be determined in further studies; however, TCR$\alpha\beta$ mispairing could also be avoided by using minimally murinized TCR constant regions, reducing the risk of immunogenicity [55].

In addition to EBV infection and PTLDs, there are a variety of EBV-associated solid malignancies in which the pathophysiological role of EBV is still a matter of debate. Especially in Hodgkin lymphoma, natural killer/T-cell lymphoma and nasopharyngeal carcinoma, not all EBV antigens can be assumed to be equally expressed and presented [40,56]. Nevertheless EBV-directed T-cell therapy might represent a targeted therapeutic option with tolerable side effects and promising results in (pre-)clinical applications [57,58].

*Conclusions*

The authors present efficient identification of EBV-specific TCRs for translation into highly specific cellular therapeutics that can be available within minimum amounts of time. T-cell products will have exactly defined EBV epitope-specific T-cell content and can be tracked *in vivo* by mouse TCR$\beta$ constant region staining. T-cell sources for TCR expression and compositions of T-cell subsets are the investigator's choice and can potentially be adjusted and functionally manipulated before adoptive transfer. The authors' methodologies can be expanded to other epitopes and HLA types and might be successfully applied beyond EBV and other viral infections.

## Funding

LH received major financial support from Deutsche Krebshilfe e.V. (70113355), Berliner Krebsgesellschaft e.V. (HAFF202013 MM) and the German Cancer Consortium. MFLC was supported by scholarships from the Berlin School of Integrative Oncology, Charité–Universitätsmedizin Berlin and the Mexican National Council of Science and Technology. LP is supported by a research fellowship from the German Research Foundation (PE 3127/1-1) and is a scholar of the American Society of Hematology. KDo was supported by SyNergy (EXC 2145 SyNergy, 390857198).

## Declaration of Competing Interest

LB is on the advisory committees of AbbVie, Amgen, Astellas, Bristol Myers Squibb, Celgene, Daiichi Sankyo, Gilead, Hexal, Janssen, Jazz Pharmaceuticals, Menarini, Novartis, Pfizer, Sanofi and Seattle Genetics and supports research at Bayer and Jazz Pharmazeuticals.

## Author Contributions

Conception and design of the study: MFLC, AG and LH. Acquisition of data: MFLC, CW, LR, KDi, CP, AT and LH. Analysis and interpretation of data: MFLC, CW, LR, ML, CP, LP, RG, LB, JM, AM, KDo, TB, TK, AG and LH. Drafting or revising the manuscript: MFLC, AM, TB and LH. All authors have approved the final article.

## Acknowledgments

The authors thank Kirstin Rautenberg and Hans-Peter Rahn at the preparative flow cytometry unit at the Max Delbrück Center for Molecular Medicine, Berlin, Germany, for expert fluorescence-activated cell sorting support. The authors also thank the National Institutes of Health Tetramer Core Facility for providing the pMHC tetramers.

## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2022.03.005.

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