Generation of Epstein-Barr Virus-specific T Cell Receptor-engineered T Cells for Cancer Treatment

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von

M. S c. K r y s t y n a D u d a n i e c

Präsidentin der Humboldt-Universität zu Berlin

Prof. Dr.-Ing. Dr. Sabine Kunst

Dekan der Lebenswissenschaftlichen Fakultät
der Humboldt-Universität zu Berlin

Prof. Dr. Bernhard Grimm

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Vorsitzender:
Prof. Dr. Thomas Sommer

Gutachterinnen/Gutachter:
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Prof. a. D. Dr. Thomas F. Meyer
Prof. a. D. Dr. Wolfgang Uckert

Weitere/s Mitglied/er:
Prof. Dr. Christian Schmitz-Linneweber

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1. Zusammenfassung

Die adoptive T-Zell-Therapie (ATT) ist eine sich schnell entwickelnde Immuntherapie, die bei Patienten, die an verschiedenen Krebsarten leiden, eine positive klinische Reaktion anzeigt. Eine Variante der ATT ist eine T-Zellen-Rezeptor (TCR)-Gentherapie, bei der Patienten-T-Zellen mit krebsspezifischen TCRs ausgestattet werden.

Die Herstellung der TCR-erzeugten T-Zellen ist schnell und robust und erfordert eine geringe Anfangsmenge an Patienten-T-Zellen. Der Mangel an verfügbaren krebsspezifischen TCRs, die auf verschiedene Moleküle des menschlichen Leukozytenantigens (HLA) der Klasse I beschränkt sind, schließt jedoch viele Patienten von der Krebsbehandlung aus. Die Generierung einer krebsspezifischen TCR-Bibliothek, die aus gut definierten TCRs besteht, könnte die Zahl der Patienten, die an klinischen Studien teilnehmen, erhöhen.

Das Ziel dieser Doktorarbeit war es, Epstein-Barr-Virus (EBV)-spezifische TCRs zu identifizieren und zu isolieren, um eine EBV-spezifische TCR-Bibliothek als ein nützliches Werkzeug der TCR-Gentherapie bei der Behandlung von EBV-bedingten Krebserkrankungen zu generieren.

Zur Identifizierung EBV-spezifischer TCRs wurde die in unserem Labor entwickelte TCR-Nachweis- und Isolationsplattform eingesetzt. Diese Plattform, die auf der Single-HLA K562-Zellbibliothek basiert, ermöglicht die Identifizierung krebsspezifischer TCRs, die auf beliebige HLA-Klasse-I-Moleküle beschränkt sind. Autologe T-Zellen wurden mit EBV-Antigen-exprimierenden dendritischen Zellen (DCs) stimuliert und expandiert. EBV-Antigen-spezifische T-Zellen wurden als Reaktion auf CD137-Überexpression und IFNγ-Sekretion nach Kokultur mit EBV-positiven Single-HLA K562-Zelllinien identifiziert und sortiert. Die Next-Generation-Sequenzierung (NGS) wurde angewandt, um dominante TRA- und TRB-Ketten-Gensequenzen zu identifizieren. Die Funktionalität der TCRs wurde durch Messung der IFNγ-Sekretion nach Kokultur mit EBV-positiven Krebszelllinien und LCLs analysiert. Die Empfindlichkeit der TCRs gegenüber den Peptid/HLA (pHLA)-Komplexen wurde durch Messung der halb-maximalen IFNγ-Freisetzung bestimmt.

Insgesamt wurden neun EBV-spezifische TCRs von EBV-positiven Spendern isoliert und charakterisiert, die verschiedene pHLA-Komplexe von EBV-Latentmembranproteinen (LMP1, LMP2A) und Kernprotein (EBNA3C) erkannten. Zusätzlich wurde ein neuartiges immunogenes LMP1-Epitop (QQNWWTLLV)

entdeckt, das auf HLA-C*15:02 beschränkt ist.

Definierte EBV-spezifische TCRs können als Grundlage für die EBV-spezifische TCR-Bibliothek verwendet werden, die eine wertvolle Quelle von TCRs für die schnelle Generierung von EBV-spezifischen T-Zellen zur Behandlung von Krebspatienten mit verschiedenen HLA-Typen darstellt.

1. Summary

Adoptive T cell therapy (ATT) is a fast developing immunotherapy indicating positive clinical response in patients suffering from different type of cancers. One type of the ATT is a T cell receptor (TCR) gene therapy, which involves endowing patient T cells with cancer-specific TCRs.

Manufacturing of the TCR-engineered T cells is fast and robust, requiring small initial amount of patient T cells. However, lack of available cancer-specific TCRs restricted to various human leukocyte antigen (HLA) class I molecules eliminates many patients from cancer treatment. Generation of a cancer-specific TCR library consisting of well-defined TCRs could increase the number of patients enrolled in clinical trials.

The aim of this PhD thesis was to identify and isolate Epstein-Barr virus (EBV)-specific TCRs in order to generate the EBV-specific TCR library as a useful tool of the TCR gene therapy for treatment of EBV-related malignancies.

To identify EBV-specific TCRs, the TCR detection and isolation platform, developed in our laboratory, was applied. This platform, based on the single-HLA K562 cell library, allows identifying cancer-specific TCRs restricted to any HLA class I molecules. Autologous T cells were stimulated and expanded using EBV-antigen expressing dendritic cells (DCs). EBV-antigen specific T cells were identified and sorted in response to CD137 overexpression and IFNγ secretion after coculture with EBV-positive single-HLA K562 cell lines. Next generation sequencing (NGS) was applied to identify dominant TRA and TRB chain gene sequences. Functionality of TCRs was analyzed by measuring the IFNγ secretion after coculture with EBV-positive cancer derived cell lines and LCLs. TCRs sensitivity to the peptide/HLA (pHLA) complexes was estimated by measuring the half-maximum IFNγ release.

In total, nine EBV-specific TCRs of EBV-positive donors that recognized various pHLA complexes of EBV latent membrane proteins (LMP1, LMP2A) and nuclear protein (EBNA3C) were isolated and characterized. Additionally, a novel immunogenic LMP1 epitope (QQNWWTLLV) restricted to a HLA-C*15:02 was discovered.

Defined EBV-specific TCRs can be used as a basis for the EBV-specific TCR library, which provides a valuable source of TCRs for rapid generation of EBV-specific T cells to treat cancer patients with different HLA types.

2. Introduction

2.1. T cell receptor (TCR)

2.1.1. TCR structure

T cell receptor (TCR) is a disulfide-linked transmembrane heterodimer expressed on a T cell surface. Each T cell carries approximately 30 000 identical TCR copies. In humans, most of T cells carry TCRs composed of an alpha (α) and a beta (β) chain (TRA and TRB, respectively) and some of T cells carry TCRs composed of gamma (γ) and delta (δ) chains (TRG and TRD, respectively). However, due to targeting a different type of antigen and a distinct approach to their recognition, the TCR γ/δ will not be included in this thesis.

The structure of the α/β TCR resembles single structures of a Fab antibody fragment. Each of the chains consists of a constant region (C region) with a homology to an immunoglobulin C domain and a variable region (V region) with a homology to an immunoglobulin V domain.

The complete V region of the TRA (TRAV) chain is encoded by two separate gene segments that were linked by alternative splicing, namely: variable (V; TRAV locus consists of about 70 variants) gene and joining (J; TRAV locus consists of about 61 variants) gene. The V region of the TRB (TRBV) chain, besides V gene (TRBV locus consist of 52 variants) and J gene (TRBV locus consists of about 13 variants), contains additionally the diversity gene (D; TRBV locus consists of 2 variants), which is located between the V and J gene segments (Fig. 1.).

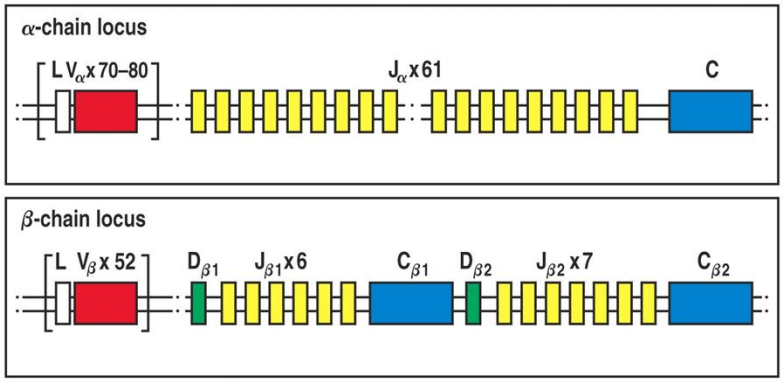


Fig. 1. Illustration of the germline arrangement of the TCR α and TCR β loci. The TCR α locus (top line) is located on chromosome 14 and consists of 70 -80 V gene segments, each preceded by an exon encoding the leader sequence (L). The V gene segments are followed by 61 J gene segments. At the end of the TCR α locus is a single C gene. The TCR β locus (bottom line) is located on chromosome 7 and consists of 52 V gene segments, each preceded by an L sequence. The V gene

segments are followed by two separate clusters, each containing a single D gene, six or seven J gene segments and a single C gene. Source of figure: (Paul 2003).

Both TRAV and TRBV chains contain an antigen-binding site with three hypervariable loops, also called the complementarity-determining regions (CDRs), namely; CDR1, CDR2 and CDR3. In both TRAV and TRBV chains, CDR1 and CDR2 are located in V segments. In the TRAV chain, the CDR3 region consists partly of V and J segments. In the TRBV chain, the CDR3 region consists partly of V and J segments and the full D segment. The center of the antigen-binding site forms the CDR3 region, which is the most variable TCR region and unique for each T cell. Thus, the CDR3 region will mainly interact with unique peptides, while the CDR1 and CDR2 regions will mainly interact with HLA molecules (Fig. 2A); (K. M. Murphy 2011).

2.1.2. TCR complex

The intracellular part of the α/β TCR heterodimer is very short and unable to provide an intracellular signal that leads to the activation of T cells. Thus, the α/β TCR heterodimer is accompanied by four signaling chains (two ε, one δ, and one γ), which together form a CD3 complex and one disulfide-linked homodimer ζ chain. All the chains together create a TCR complex (Fig. 2B). The CD3 complex is needed to stabilize the α/β TCR heterodimer during transportation to the surface of the T cell and for signal transmission (K. M. Murphy 2011).

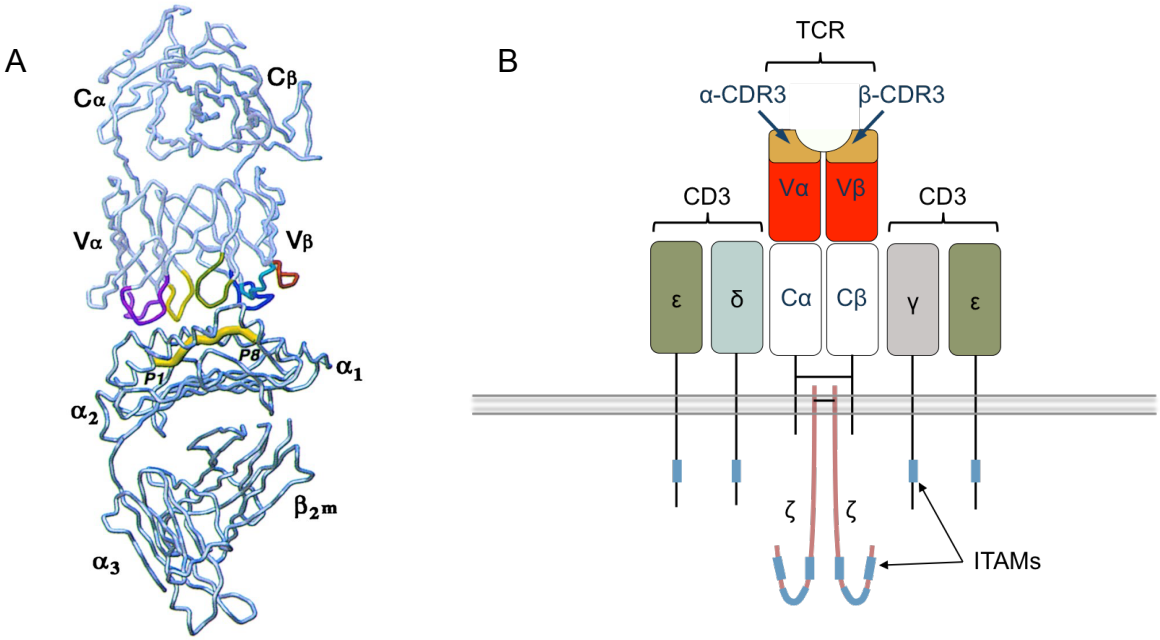


Fig. 2. Antigen-binding site of the TCR complex. (A) Illustration of TCR binding to the peptide/HLA complex. In the TCR (protein above epitope) distinguished are variable (V) and constant (C) regions of α and β chains. CDR loops of the TCR are colored: the CDR1 and CDR2 loops of α chain are in light and dark purple, respectively, and the CDR1 and CDR2 loops of β chain are in light and dark blue, respectively. The CDR3 region of α chains is in yellow and CDR3 region of β chains is in green. The thick yellow line P1-P8 is an epitope buried within a groove of the HLA class I molecule. In the HLA class I molecule (protein below epitope) distinguished are α chain consisting of three domain (α_1, α_2 and α_3) and β_2-microglobilin (β_2m). Source of figure B: (Paul 2003). (B) Illustration of the TCR complex consisting of the antigen binding α (TRA) and β (TRB) chains and signaling chains composed of CD3 complex (two ε, one δ, and one γ) and one disulfide-linked homodimer ζ. Each CD3 chain has one immunoreceptor tyrosine-based activation motif (ITAM) and each ζ chain has three ITAMs that are important for signal transduction when the TCR is ligated with antigen. The ITAMs are shown as blue segments

2.1.3. TCR recognition of peptide/HLA complex

TCR, in contrast to the B cell receptor (BCR) or antibody, does not recognize an intact antigen. The TCR recognizes an unfolded part of the antigen, called an epitope (here also called peptide), which was previously processed inside the cell and presented on the major histocompatibility complex (MHC), in humans also called the human leukocyte antigen (HLA).

TCR expressed on CD8-positive T cell recognizes an 8-10 amino acid long peptide buried within a groove of the HLA class I molecule. The peptide binding is stabilized at both sides of the groove by interaction between atoms in the free amino and carboxy termini of the peptide and amino acid residues of the groove. Longer peptides may also bind to the groove, especially when they can bind at their carboxy terminus, however, the flanking regions are subsequently cleaved by exopeptidases, present in the endoplasmic reticulum, which is the peptide binding site with HLA class I molecules (Fig. 3).

Both TRAV and TRBV chains interact with the peptide/HLA complex. The TRAV chain interacts with the amino-terminal half of the epitope and the TRBV interacts with the carboxy-terminal half of the epitope. In addition, the TRAV and TRBV chains also interact with the HLA class I molecule (Fig. 2A); (K. M. Murphy 2011).

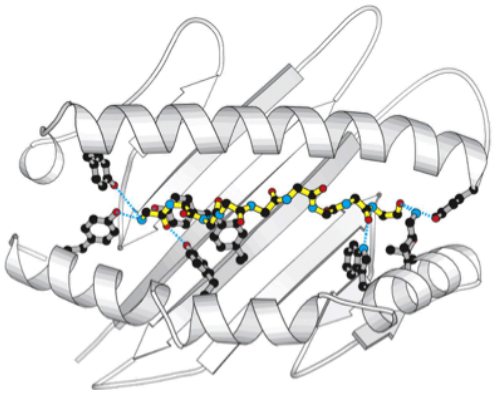


Fig. 3. Peptide binding to the HLA class I molecule. Peptide (shown in yellow) interacts with both ends of the groove of the HLA class I molecule through hydrogen bonds (shown as dashed blue line). Black circles are carbon atoms, red are oxygen and blue are nitrogen. The side chains of the HLA class I molecule that interact with peptide are shown in gray. Source of figure: (Paul 2003).

2.2. Adoptive T cell therapy

Adaptive T cell therapy (ATT) has become a promising breakthrough in cancer treatment, providing a significant alternative to standard treatments such as oncological surgery, chemotherapy and radiotherapy. The ATT is a type of immunotherapy involving isolation of T cells from a cancer patient, *ex vivo* stimulation and expansion of cancer-specific T cell clones, followed by infusion back into the patient to eliminate cancer cells.

Compared to the standard treatments, the ATT has several advantages that make this therapy attractive for cancer treatment. First, the cancer-specific T cell clones have the ability to recognize and eliminate cancer cells, without harming healthy tissues. The *in vitro* expansion of cancer-specific T cell clones is robust, providing a large number of T cells to enhance cancer regression, especially in the immunosuppressed patients (Rosenberg and Restifo 2015). Furthermore, infused T cells can prevent the development of distant metastasis due to their natural traffic ability to the cancer site (Slaney, Kershaw, and Darcy 2014). Finally, memory T cells, established after priming of naïve T cells, can provide control of cancer growth for many years after initial treatment (Mami-Chouaib et al. 2018).

Due to the generation methods of cancer-specific T cells, the ATT can be divided into two groups. The first group consists of the therapies using unmodified T cells, such as tumor infiltrating lymphocytes (TILs) or *in vivo* stimulated and expanded antigen-specific cytotoxic T cell lines (CTLs). The second group consists of therapies using genetically modified T cells, such as chimeric antigen receptor T cells (CAR-T cells)

and T cell receptor-engineered T cells (TCR-engineered T cells)

Recent research and successful clinical trials provide incontestable evidence that the ATT is the future-oriented method of cancer treatment. However, each of the ATT methods contains imperfections that require further developments to fast and reliable fight against cancer. The results of individual clinical trials are indicated in the following subsections.

2.2.1. Tumor-infiltrating lymphocyte therapy

Tumor-infiltrating lymphocytes (TILs) are T cells that naturally recognize antigens presented on cancer cells. However, due to the immunosuppressive tumor microenvironment (TME), the TILs were trapped, either inside or in the periphery of the cancer cells, preventing them from transmitting the T cell activation signal.

The general method of TIL generation for the ATT involves T cell isolation from a fresh patient biopsy sample by digesting the sample into a single-cell suspension and culturing them with a high dose of interleukin (IL)-2. Isolated T cell clones are then cultured with established autologous cancer cell lines or with generated antigen-presenting cell lines (APCs) to select and grow the best cancer-specific T cell clones (Rosenberg and Restifo 2015).

A significant breakthrough in the TIL therapy followed two major discoveries. The first occurred when Rosenberg et al. (1988) found that *ex vivo* culturing TILs with a high dose of IL-2 improves their expansion, without loosing anticancer reactivity. The second occurred when the same group indicated that lymphodepletion of patients receiving chemotherapy prior to TIL infusion enhanced cancer regression and maintenance of TILs in the host (Dudley et al. 2005). The clinical trial conducted by Dudley et al. (2015) shows an objective clinical response in 50% (18 out of 35) of the treated melanoma patients with a mean duration of 11.5 ± 2.2 months at the time of publication.

However, TIL generation is time consuming and requires about six weeks before TILs are ready to be infused back into patients. Furthermore, in many cases the biopsy samples are too small to isolate and/or generate cancer-specific T cell clones (Kong et al. 2018). These two obstacles eliminate many patients from TIL therapies.

2.2.2. Tumor-specific cytotoxic T lymphocyte therapy

Tumor-specific cytotoxic lymphocyte (CTL) therapy was best described for cancers

associated with viruses, such as Epstein-Barr virus (EBV), (Roskrow et al. 1998), and (Haque et al. 2002). The entire EBV-infected population that developed lifelong immunity to this virus becomes suitable EBV-specific T cell donors.

The CTL therapy involves isolation of EBV-specific T cells from EBV-positive donors, *ex vivo* stimulation and expansion of these T cells using APCs, such as autologous lymphoblastoid cell lines (LCLs), followed by infusion to patients.

One of the first successful clinical trials with EBV-specific CTLs was the treatment of patients who developed post-transplant lymphoproliferative disorder (PTLD) (see: EBV-associated cancers) after hematopoietic stem cell transplantation (HSCT). Clinical trials conducted on 13 PTLD patients, enrolled between 1993 and 2005, showed a complete response in 85% of patients after infusion of EBV-specific CTLs, with a mean duration of six years at the time of publication (Heslop et al. 2010). However, a clinical trial using EBV-specific CTLs in patients with Hodgkin's lymphoma (HL) showed less efficacy. Objective clinical response was observed in 43% (3 out of 7) of the treated patients (Bollard et al. 2018). The lower efficiency of the CTL therapy among these patients might be due to the fact that the HL expressed a limited number of EBV antigens that are less immunogenic compared to EBV antigens expressed in the PTLD (see: EBV-associated cancers).

EBV-specific CTLs cannot be isolated from each patient due to their previous lymphodepletion or immunosuppression. Hence, Rooney et al. developed the off-the-shelf virus-specific T cell (VSTs) bank, which consists of the ready-to-use EBV-specific T cells, generated from healthy EBV-positive donors (Tzannou et al. 2017). However, finding perfectly matched HLA donors is very difficult. Thus, the method assumes the use of VST cell lines that matched with the patient's HLA type as close as possible. Still, using HLA-mismatched VST cell lines may cause development of the graft-versus-host disease (GVHD), which can lead to death of patients (Heslop et al. 2010). In addition, this method does not provide EBV-specific T cells with the best TCR affinity for the peptide/HLA complex, which may affect the patient's response to the therapy.

2.2.3. Chimeric antigen receptor T cell therapy

Chimeric antigen receptor T cells (CAR-T cells) are lymphocytes that were genetically endowed with chimeric antigen receptors, enabling them to recognize cell surface antigens in an HLA-independent manner. The CAR is an artificial fusion

protein consisting of two regions, namely extracellular and intracellular. The extracellular region consists of a single-chain variable fragment (scFv) derived from an antibody as the antigen-binding domain. The intracellular region consists of a combination of signal proteins derived from T cell, such as CD3ζ chain, CD28, OX40, and 4-1BB (CD137) molecules, responsible for signal transmission (Eshhar et al. 1993), and (June et al. 2018).

CAR-T cell therapy involves isolation of T cells from a patient and their *ex vivo* expansion. The expanded T cells are then genetically modified with antigen-specific CARs. The CAR-encoding gene sequences can be introduced into the T cells using retroviral vectors, such as gammaretroviruses (RV) or lentiviruses (LV) or non-viral methods, such as transposons or Crispr/Cas9-based technology (Rohaan, Wilgenhof, and Haanen 2019). Thoroughly characterized CAR-T cells are then infused back to the patients (Levine et al. 2017).

CARs recognize molecules that are expressed on the surface of cancer cells. Because these molecules are also often expressed on healthy cells they are called tumor-associated antigens (TAAs; see: TCR targets/cancer antigens classification). Targeting TAAs can lead to the off tumor/on target toxicity, resulting in patient death. (June et al. 2018). Nevertheless, the CD19 molecule, which is only expressed on the B cell lineage, proved to be an optimal target for CAR-T cells in the treatment of B cell leukemia. The clinical trial showed an objective clinical response in 60% (15 out of 25) of the treated B cell lymphoma patients, with a mean duration of six months at the time of publication (Ying et al. 2019).

CARs recognize antigens independently of HLA complexes, which is an advantage compared to other ATTs, because cancer cells tend to lose their HLA surface expression (Garrido et al. 2016). However, many cancer antigens come from inside the cells, such as intracellular antigens or viral antigens, where they are processed and presented on the HLA complexes. Thus, the inability of CARs to recognize endogenous antigens limits the use of CAR-T cell therapy in cancer treatment.

2.2.4. TCR gene therapy

In T cell receptor (TCR) gene therapy, autologous T cells are genetically endowed with antigen-specific TCRs, enabling them recognition of cancer cells that were not recognized by the repertoire of endogenous T cells.

The TCR gene therapy involves identification and isolation of cancer-specific TCRs from the cancer-reactive T cell clones (see: Source of antigen-specific TCRs). TCR-encoding genes are then *in vitro* transferred to the patient's T cells using, as with CARs, viral or non-viral methods. The expanded TCR-engineered T cells are then infused back to the patients (Morgan et al. 2006), and (Park, Rosenberg, Morgan 2011).

The first successful clinical trial of TCR gene therapy targeted the MART-1 melanoma differentiation antigen, which is expressed on healthy melanocytes and melanomas. In the clinical trial, 13% (2 out of 15) of melanoma patients experienced tumor regression in liver and lung hilum and showed complete clinical response two years after injection of MART-1-specific T cells (Morgan et al. 2006).

The MART-1-specific TCR was isolated from TILs of cancer patient who experienced complete clinical response of metastatic melanoma after injection of autologous cancer-specific TILs. Thus, the TILs expressed optimal affinity TCRs for MART-1 antigen, recognizing cancer cells but not affecting healthy tissue. Indeed, none of the patients developed the off tumor/on target toxicity. However, in another clinical trial using MART-1-specific TCR with very high affinity to the MART-1 antigen isolated from transgenic mice, toxic side effects occurred damaging skin, eye and ear of treated patients (Johnson et al. 2009). Hence, the TCR gene therapy requires careful selection of cancer-specific TCRs and target tumor-antigens before use in the clinic.

Nevertheless, another successful clinical trial of TCR gene therapy was also observed for the cancer-testis antigen NY-ESO-1, which is expressed in 25% of melanoma patients and 80% of synovial cell sarcoma patients. The clinical trial, using NY-ESO-1-specific TCR-engineered T cells, showed objective clinical response in 67% (4 out of 6) of the treated synovial cell sarcoma patients and in 45% (5 out of 11) of the treated melanoma patients (Robbins et al. 2011).

Manufacturing of TCR-engineered T cells is rapid and capable of generating large quantities of engineered T cells from a small initial amount of patient T cells. However, the bottleneck of this therapy is lack of available cancer-specific TCRs that would be restricted not only to the cancer-specific antigens but also to the various HLA class I molecules. So far most of the available TCRs are restricted to the most common HLAs, such as HLA-A*02:01 (found in about 50% of the world population), eliminating many patients from cancer treatment (Morgan et al. 2006), (Robbins et al. 2011), (Morgan et al. 2013), and (Chapuis et al. 2019). One of the reasons for this

problem are the current methods of TCR identification and isolation, which are very laborious and time-consuming, indicating the clear need for development of new methods.

2.2.4.1. TCR gene therapy for EBV-associated cancers

TCR gene therapy is an alternative approach for the treatment of EBV-associated cancers by using EBV-specific engineered T cells that indicate a more precise targeting of cancer cells without harming healthy tissues.

Preclinical studies using LMP1- and LMP2A-specific TCR-engineered T cells indicate a significant increased survival of mice compared to mice treated with untransduced T cells (Cho et al. 2018), and (Yang et al. 2011). However, so far no TCR gene therapy using EBV-specific TCR-engineered T cells was reported. This is due to the lack of available cancer-specific TCRs for use in clinical trials. To date, only a few EBV-antigen specific TCRs were generated. Most of them are restricted to the LMP2A antigen and to the most common HLAs of the world (HLA-A*02:01) and Asian (HLA-A*11:01) population, which eliminates many patients from cancer treatment (Cho et al. 2018), (Frumento et al. 2013), (Xue et al. 2013), and (Zheng et al. 2015). Thus, generation of an EBV-specific TCR library consisting of well-defined TCRs restricted to various EBV antigens and HLA class I molecules could increase the number of patients enrolled in clinical trials. In addition, collected patient data could provide more information required to analyze the reliability and effectiveness of therapy.

2.3. TCR targets/ cancer antigens classification

T cells can differentiate infected and/or mutated cells from the healthy tissue by recognition of pathogenic or cancer antigens presented on malignant cells. Identification of the antigens, which are recognized by TCRs, is one of the most important keys for the TCR-engineered T cell therapy in the fight against cancer. The antigens can be identified by transfecting a cDNA library established from cancer cells into antigen-loss cancer cell clones and testing their ability to activate autologous T cells (van der Bruggen et al. 1991). More recently, researchers focus on identification of epitopes (short peptides presented on the HLA molecules) derived from cancer-specific antigens as targets for TCRs. The epitopes can be identified by two basic methods. The first, called “reverse immunology” involves predicting

immunogenic epitopes from the gene sequence of a cancer-specific antigen (Viatte, Alves, and Romero 2006). The second approach involves elution of immunogenic epitopes from HLA molecules from the surface of cancer cells by mass spectrometry (Hunt et al. 1992).

Cancer antigens can be divided into two main groups. The first group contains antigens that are also expressed on healthy tissues. This group includes overexpressed, differentiated and cancer-testis antigens that are commonly named tumor-associated antigens (TAAs). The second group called tumor-specific antigens (TSAs or neoantigens) includes antigens that are not naturally expressed on healthy cells and are either formed by mutations or derived from external organisms such as viruses (Vigneron 2015).

2.3.1. Overexpressed antigens

This group of antigens represents any proteins that are overexpressed on the cancer cells compared with cells of healthy tissues (Bright, Bright, and Byrne 2014). Thus, targeting this group of antigens is very difficult and requires identification of TCRs with relatively low affinity sufficient to recognize the overexpression of antigens on cancer cells without eliminating healthy cells. To the most studied overexpressed antigens, among others, belongs the Wilms tumor 1 (WT1), a transcription factor, which expression was indicated at least 10-fold higher in the human leukemias such as acute myeloid leukemia (AML), chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (ALL) compared with normal hematopoietic cells (Inoue et al. 1997). Next example is the growth factor ERBB2 (HER2/NEU), which expression was indicated 100 to 200-fold higher in breast and ovarian cancer epithelial cells compared with healthy epithelial cells (Fisk et al. 1995). However, besides this large difference in ERBB2 expression between healthy and cancer cells, ERBB2-specific CAR T cells, used in clinical trial, recognized low levels of ERBB2 expressed on normal lung epithelial cells, resulting in the death of patient due to cytokine release syndrome (CRS) (Morgan et al. 2010).

2.3.2. Differentiation antigens

Differentiation antigens are expressed only on the given type of cells. An example is CD19 molecule, which expression is restricted to the B cells including B cell lymphomas such as chronic lymphocytic leukemia (CLL) or acute lymphocytic

leukemia (ALL) (Nobles et al. 2019). In this case, CD19-specific T cell toxicity towards healthy B cells is acceptable due to the low impact on patient life after B cell depletion (Brentjens et al. 2011). In contrast, a MART-1-specific antigen is expressed on healthy melanocytes and melanomas. Clinical trial targeting the MART-1 antigen using a high affinity TCR caused a toxic side effect damaging skin, eye and ear of treated patients (Johnson et al. 2009).

2.3.3. Cancer-testis antigens

Cancer-testis (CT) antigens are a group of antigens normally expressed in male germ cells in testis. However, these antigens can also be expressed in the fetal ovary and placenta trophoblast. To the best described CT antigens belong MAGE-A1 and NY-ESO-1. (Simpson et al. 2005) and ("Cancer-Testis (CT) Antigens - Holland-Frei Cancer Medicine - NCBI Bookshelf" n.d.). Expression of the CT antigens were also indicated in various types of cancer such as melanoma, breast cancer, bladder cancer, prostate cancer, and hepatocellular carcinoma (Chen et al. 1997). The germ cells do not express HLA class I molecules and therefore cannot present antigens on the surface, making them invisible to T cells. Thus, targeting this group of antigens is safe for healthy cells (Haas, D'Cruz, and De Bault 1988).

2.3.4. Tumor-specific antigens

To the group of tumor-specific antigens (TSAs, or neoantigens) belong foreign proteins, which expression do not exist in cells of healthy tissue but is present in cancer cells. These antigens derived either from virus genes, expressed in infected cells or from non-synonymous genetic mutations (Jiang et al. 2019). TSAs, which are essential for carcinogenesis, known as drive-mutations, are an ideal target for T cells because their expression is less prone to get lost during the cancer development. Potentially this means that targeting drive-mutations can lead to the complete elimination of cancer cells. Moreover, the exclusive expression of TSAs in cancer cells minimizes the risk of autoimmune toxicity (Schietinger, Philip, and Schreiber 2008).

The disadvantage of neoantigens derived from non-synonymic genetic mutations is their rare repetition among individuals, which requires personalized treatment of patients, increasing the cost of therapy and waiting time. The exception are Ki-RAS point mutations, which are found in about 90% of pancreatic adenocarcinomas and

40% of colorectal adenocarcinomas (Shono et al. 2003).

Viral antigens, which contribute to the uncontrolled proliferation and immortalization of cancer cells, are also ideal targets for T cells. However, the ability of viruses to avoid the immune system is a matter of their effectiveness in immunotherapy. The evolution of viruses has endowed them with many mechanisms to escape from immune control (LUCAS et al. 2001).

To hinder the recognition of cancer cells by T cells, the oncogenic viruses, such as EBV, human papillomavirus (HPV) or cytomegalovirus (CMV) established latency stages, where only small numbers of low immunogenic antigens are expressed (Dugan, Coleman, and Haverkos 2019), (Pinidis et al. 2016) and (Beltran and Cristea 2014). Oncogenic viruses can interfere with antigen processing and presentation in cancer cells. An example is the EBV nuclear antigen 1 (EBNA1), which amino acid sequence contains a Gly-Ala repeat domain preventing digestion by the proteasome and thus presentation on HLA class I molecules (Levitskaya et al. 1997). Intracellular processing of HPV antigens (E6 and E7) is interrupted by downregulation or loss of the transporter proteins TAP1 and TAP2 in the cervical carcinoma (Evans et al. 2001, 6; Cromme et al. 1994). CMV uses four glycoproteins US2, US3, US6 and US11 to downregulate HLA class I expression in infected cells, impairing antigen presentation (Jones et al. 1996). Another viral immune avoidance mechanism involves modification of the composition of the tumor microenvironment (TME). In EBV-positive HLs, the EBNA 1 antigen induces an expression of CCL20 chemokines, which are responsible for attracting immunosuppressive Th2 and Treg cells (Tan et al. 2018).

2.4. Source of antigen-specific TCRs

Source of antigen-specific TCRs are CD8-positive T cells that previously encountered and responded to the target antigens presented on cancer cells. In most cases, the TCRs are isolated from activated T cells that in response to APCs express activation markers, such as the CD137 or interferon gamma (IFNγ) molecule. These markers are then used to sort T cells by e.g. flow cytometry sorting.

The TCRs can be obtained from T cells activated in three different manners. To the first group belong T cells that naturally responded to the cancer-specific antigen in the human body, e.g. TILs (Lu et al. 2014). The second group are T cells that were previously isolated and *in vitro* primed (in case on using naïve T cells) or stimulated

(in case of using memory T cells) with the antigen, e.g. allo-restricted T cells and antigen-stimulated T cells (Wilde et al. 2012), and (Lorenz et al. 2017). Finally, TCRs can be isolated from the non-tolerant T cell repertoire of the transgenic mice with the humanized T cell recognition system (Obenaus et al. 2015).

2.4.1. Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TILs) are T cells that *in vivo* recognize antigens presented on cancer cells. However, due to the immunosuppressive cancer microenvironment, these T cells are often incapable to expand and eliminate cancer cells. Isolation and *ex vivo* expansion of the TILs reactivate their antitumor capability (June 2007). The TILs are the source of cancer-specific TCRs that can recognize an individual and unknown cancer mutation within the patients suffering from the same type of cancer. Research analysis of two TIL clones from two melanoma patients indicate that each of the TIL clones recognize a unique mutation derived from different proteins (Lu et al. 2014).

Some of the TILs indicate anticancer reactivity to antigens derived from proteins that are irrelevant for maintaining the cancer phenotype, hence their expression in cancer is gradually downregulated. It is therefore important to analyze each new antigen recognized by isolated cancer-specific TILs before use in clinical trials (Lu et al. 2014).

2.4.2. Allo-restricted T cells

Identification of TCRs with optimal affinity to the TAAs may be most effective for use in TCR gene therapy (Obenaus et al. 2015). TCRs isolated from TILs, which recognize TAAs presented on the self-HLA molecules usually are of low affinity. This is due to a thymus negative selection that eliminates T cells with high affinity to the self-antigen/HLA complexes preventing development of autoimmune diseases. Whereas, the thymus selection does not exclude T cells that recognize self-antigens presented on the allogeneic HLA molecules (allo-HLA). Thus, potentially, a part of the T cell repertoire contains allo-restricted T cells, indicating high affinity to the TAAs presented on the allo-HLA molecules (de Visser, Schumacher, and Kruisbeek 2003). Allo-restricted T cells are generated using autologous DCs electroporated with *in vitro* transcribed (ivt)-RNA encoding an allo-HLA molecule and a TAA. Stimulation of autologous T cells with the generated DCs activates and expands allo-restricted

antigen-specific T cells, which are a source of TCRs (Wilde et al. 2009).
The allo-HLA molecule as a foreign protein can activate a positive response in autologous T cells. Therefore, it is important to select only those allo-restricted T cells, which recognize allogeneic antigen/HLA complexes, but not allo-HLA molecule itself (Wilde et al. 2012).

2.4.3. Antigen-stimulated T cells

Each person has an unique T cell repertoire capable to generate up to 10^{13} different TCRs, which is crucial to recognize and eliminate pathogens and cancer cells (Nikolich-Žugich, Slifka, and Messaoudi 2004). Thus, cancer-specific TCRs can be isolated from the T cell repertoire of healthy donors or cancer patients by *in vitro* priming or stimulation of the PBMCs with tumor-specific antigens (TSAs) presented on established cancer cell lines (Khalaf et al. 2019), organoids (Dijkstra et al. 2018) or generated APCs (Wilde et al. 2012). The APCs can be transfected with a whole length antigen or loaded with peptides. There are three main advantages of transfecting APCs with the whole length antigen. First, the antigen is naturally processed and presented by HLA molecules on the APCs, which means that the same epitope/HLA combination will be presented on the cancer cells. Second, stimulated T cells with the generated APCs have an opportunity to detect the most favorable epitope. Finally, the APCs are generated from naïve DCs, so the transfected antigen is the only one processed and presented by these cells.
However, generated APCs with either whole length antigen or peptides present an enormous amount of epitopes on their surface, which might lead to activation of low-affinity T cells, incapable of recognizing cancer cells (Kunkel et al. 2003).

2.4.4. Human TCR/HLA-A*02:01 transgenic mice

Isolation of high affinity TCRs against the self-TAAs from autologous repertoires is difficult due to the negative selection of T cell in the thymus. Transgenic mice with the humanized T cell recognition system are, next to the allo-restricted T cells, a tool to identify high affinity TCRs. The human transgenic mice, called ABabDII, are a result of crossing two mouse strains. First one is the HHDII mouse, which expresses a single-chain construct of the human HLA-A*02:01 molecule fused to mouse H-$2D^b$ α3 transmembrane domain (to allow binding to mouse CD8 molecules) and human β2-microglobulin (β2m) gene. The HHDII mice were, additionally, double knocked-out for

murine MHC class I molecules (H-2D^b) and β2m (Pascolo et al. 1997). The second is the ABab mouse, which expresses human □□ TCRs and were knocked-out for murine □□ TCRs (Li et al. 2010). Finally, the generated ABabDII mouse expresses human □□ TCR repertoire and human HLA-A*02:01 without expression of mouse □□ TCR repertoire and mouse MHC class I molecules.

Human TAAs are different from their analogous versions expressed in mice. Therefore, injected human TAAs into transgenic ABabDII mice are recognize as a foreign molecules and can activate high affinity T cells in the mouse autologous repertoire, because the mouse T cells were not deleted during the thymic selection (Obenaus et al. 2015). The limitation of the ABabDII mouse model is its restriction to the HLA-A*02:01 molecule, which eliminate many potential high affinity TCRs restricted to other HLA molecules. Generation of the humanized transgenic mice with different HLA molecules is very time consuming (it is takes years to generate such a transgenic mouse model), laborious and expensive. Moreover, the transgene of human □□ TCR gene loci in the ABabDII mouse model lacks a few □□ TCR chains, which may be crucial to generate cancer-specific TCRs.

2.5. Epstein-Barr virus

Herpesviruses are large double-stranded DNA viruses that infect both animals and humans. Nine herpesviruses were identified in humans and classified into three subfamilies, which are alpha, beta and gamma. To the alpha subfamily belongs herpes simplex virus 1 and 2 (HSV-1 and HSV-2) known also as human herpesvirus (HHV-1) and (HHV-2), and varicella-zoster virus (VZV or HHV-3). To the beta subfamily belongs human cytomegalovirus (HCMV or HHV-5) and human herpesviruses 6A-B and 7 (HHV-6A-B and HHV-7). To the gamma subfamily belong Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) and the Epstein–Barr virus (EBV) also known as human herpesvirus 4 (HHV-4) (Damania 2004).

The EBV has a diameter of about 122–180 nm and its DNA contains about 172,000 base pairs that encode about 85 genes. The viral genome is enveloped by multiple layers. The outer envelope contains glycoproteins that are necessary to infect host cells (Fig. 4A); (Amon and Farrell 2005).

EBV is ubiquitous and infects over 90% of the entire population. The infection usually occurs in childhood and in most cases is asymptomatic. However, primary EBV

infection in adolescence in 30-50% of cases causes infectious mononucleosis that manifests as fever, sore throat, enlarged lymph nodes in the neck, and fatigue. In some cases, such as in immunocompromised or immunosuppressed individuals, EBV can lead to tumor development in B-, T- and NK-cells as well as in epithelial cells. The EBV after primary infection establishes lifelong latency stage in the host B cells (Shannon-Lowe and Rickinson 2019).

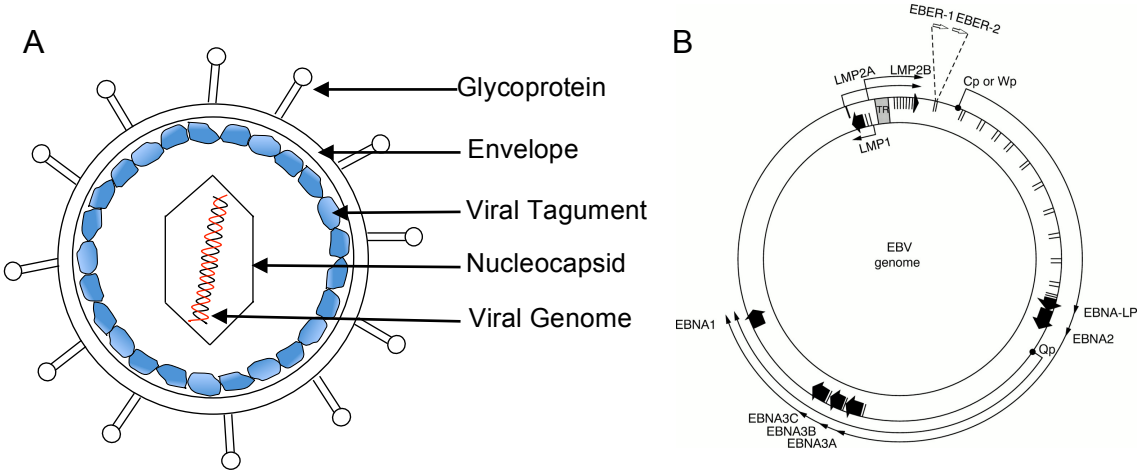


Fig. 4. (A) Simplified EBV structure. (B) Diagram of the location of EBV latent genes within the double stranded DNA episome. The outer long arrow represents EBV gene transcription in latency type III, where all EBNAs are transcribed from either Cp or Wp promoter. The inner shorter arrow represents the EBNA1 gene transcription from the Qp promoter, which is activated during latency types I and II. The thick, short arrows represent direction of gene transcription and exons for each LMPs and EBNAs. The dashed arrows represent the highly transcribed non-polyadenylated RNAs, EBER1 and EBER2. Source of the figure: (L S Young, Dawson, and Eliopoulos 2000).

2.5.1 EBV life cycle

The EBV infects both B cells and epithelial cells. The primary EBV infection is transmitted from host to host via saliva. First, the EBV entrance tonsil epithelial cells by binding viral glycoprotein BMRF-2 to the host β1 integrin. Next, viral glycoprotein gH/gL interacts with host αvβ6/αvβ8 integrin, triggering fusion with the epithelial cell membrane. After passing through tonsil epithelial cells, the EBV infects naïve B cells by binding viral glycoprotein gp350/220 to the host receptor type 2 (CR2), also known as CD21. Then viral glycoprotein gp42 interacts with cellular HLA class II molecules, triggering fusion with B cell membrane. In most cases, B cells are the final destination of the virus (Odumade, Hogquist, and Balfour 2011)

The EBV infection cycle can be divided into two phases, latent and lytic. The main

role of the lytic phase is rebuilding of virion and escape from the host cell. The lytic replication can be divided into immediate-early, early, and late stages. During the immediate-early stage are expresses proteins (e.g., BZLF1 and BRLF1) that enhance expression of early and late proteins. The early stage expresses proteins (e.g., BNLF2a) involved in viral DNA replication, host metabolism modulation and blockade of antigen processing. Finally the late stage expresses proteins necessary to rebuild virion, such as the viral capsid antigens (VCA).

During latency, the EBV genome is hidden in the nucleus as an episom or integrates with the host genome. In this phase, only a limited number of antigens are expressed, depending on the stage of latency, from 10 to zero antigens (see: EBV latent antigens). The latency can be divided into four stages, which are III, II, I and 0. Latency III expresses all 9 antigens, which are EBV nuclear antigens (EBNA1, EBNA2, EBNAs-3A, -3B, -3C, and EBNA-LP), latent membrane proteins (LMP1, LMPs-2A, and -2B). Latency II expresses EBNA1, LMP1 and LMP2A-B antigens. During latency I only EBNA1 is expressed and during latency 0 none of the antigens are expressed. In addition, in all EBV three latency stages the small non-coding RNAs called EBV-encoded small RNAs (EBERs) 1 and 2 are expressed (Fig. 4B) (Odumade, Hogquist, and Balfour 2011).

Upon infection of naïve B cells, the EBV genome is transported to the nucleus, where EBV latent III genes undergo expression. Those genes activate the B cell growth program, leading to the proliferation of blasting B cells. After initial clonal expansion, the blasting B cells enter to the germinal center, where the EBV changes the latency stage from III to I/II. In the germinal center B cells differentiate into memory B cells, where the EBV change latency stage from I/II to 0. Infected memory B cells enter the peripheral blood, where the EBV spontaneously turns into the lytic cycle, leading to the apoptosis of B cells and the release of viruses (Fig. 5) (Küppers 2003), and (Shannon-Lowe and Rickinson 2019).

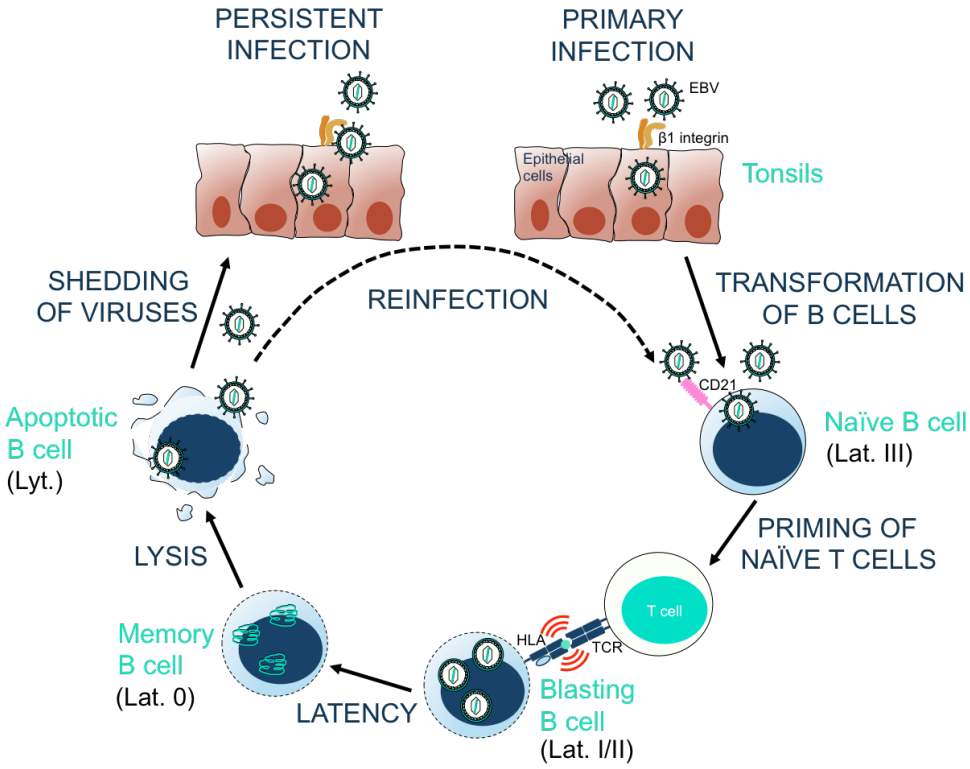


Fig. 5. The EBV life cycle. The EBV infection is transmitted from host to host via saliva. The virus passes thought the tonsil epithelium to the naïve B cells where is transported to a nucleus and expresses the antigens from latency stage III. In the nucleus of naïve B cells, EBV gene products activate the B cell growth program, resulting in the proliferation of blasting B cells and transition of EBV latency from stage III to I/II. Priming of naïve T cells by antigen-presenting cells occurs in parallel. To attain long-term persistence and avoid destruction of the host cell by the immune system, EBV establishes a 0 latency stage in memory B cells where no EBV antigen expression occurs. In memory B cells, EBV can spontaneously activate a lytic cycle leading to apoptosis of B cells and release of viruses. Lat: latency; lyt: lytic.

2.5.2. EBV latent antigens

2.5.2.1. EBV nuclear antigen 1

EBV nuclear antigen 1 (EBNA1) is the only EBV antigen expressed in both lytic and latent phases as well as in all EBV-associated malignancies. EBNA1 has multiple functions in infected cells. First, EBNA1 is required to replicate EBV genomes in dividing cells by interacting with the origin of viral replication (oriP) within the viral episome, allowing EBV DNA to replicate once per cell cycle. Secondly, EBV episomes in cells are present at low copy numbers, and therefore maintaining them at a stable copy number in dividing cells requires equal mitotic segregation during cell division. EBNA 1 ensures equal segregation by binding episomes to the cellular chromosomes in mitosis (Frappier 2012), and (Kang and Kieff 2015). Finally, EBNA1

modulates several signaling pathways essential for cancer proliferation. EBNA 1 downregulates TGFβ1 expression, inhibiting B cell apoptosis. Moreover, TGFβ1 is involved in controlling expression of antibody and HLA class II molecules on immature and mature B cells (Wood et al. 2007) (Lebman and Edmiston 1999). Next, EBNA1 inhibits the NF-κB pathway, which is a regulator of genes responsible for cell proliferation, differentiation and cell apoptosis. Inhibition of NF-κB pathway leads to development of NPC by inducing tissue hyperplasia (Valentine et al. 2010).

2.5.2.2. EBV nuclear antigen 2 and leader protein

EBV nuclear antigen 2 (EBNA2) and leader protein (EBNA LP) are the first two antigens expressed shortly after primary B cell infection. Both antigens are required to transform B cells into LCLs (Kang and Kieff 2015).

EBNA2 acts as a transcription activator that regulates expression of EBV latency genes (EBNAs and LMPs) and modified expression of cellular genes (e.g., Cyclin D2 or c-myc oncogene), leading to cell immortalization (Sinclair et al. 1994), and (Kaiser et al. 1999). EBNA2 mimics a cellular Notch pathway. EBNA2 and Notch-IC (an intracellular domain transferred to the nucleus after Notch pathway activation) activate expression of other proteins by binding to the RBP-Jκ. The RBP-Jκ acts as a transcriptional repressor that binds to the consensus sequence GTGGGAA and blocks transcription by recruiting a corepressor complex. EBNA2 and Notch-IC activate the RBP-Jκ-inhibited promoters by displacing the corepressive complex, thus masking the repression domain of RBP-Jκ (Ansieau, Strobl, and Leutz 2001).

EBNA LP antigen acts as a transcriptional coactivator that enhance transcription of EBNA2-dependent viral and cellular genes by removing corepressor proteins that inhibits EBNA2 activity (Portal, Rosendorff, and Kieff 2006, 4), and (Han et al. 2001, 95).

2.5.2.3. EBV nuclear antigen 3 family

EBV nuclear antigen 3 (EBNA3) family consists of three adjacent antigens namely EBNA-3A, -3B, and -3C. All three antigens share similar gene structure, possess a domain for binding to RBPJ and are transcribed from the same C promoter (Cp) that is regulated by EBNA2. Although all three antigens regulate transcription of vial and host genes, in vitro data showed that only EBNA-3A and -3C are necessary for B cell transformation, where EBNA 3B is redundant (Kang and Kieff 2015).

EBNA-3A and -3C share many similar functions. Both of them cooperate to downregulate expression of Bcl-2-interacting mediator of cell death (Bim), which is main regulator of B-cell survival during lymphopoiesis (Anderton et al. 2008). EBNA3A and -3C suppress the EBNA2 transcriptional activity and cMyc transcription by binding to the RBPJ proteins (Kang and Kieff 2015). Moreover, both of the EBV antigens cooperate to suppress a transcription of the cyclin dependent kinase inhibitors (CDKI) p16INK4A and p14ARF, which are tumor suppressors, allowing cell proliferation (Maruo et al. 2011). Additionally, EBNA-3A and -3C interacts with many cellular proteins, among others, PU.1, p300, SUMO-1, SCF-Skp2, Nm23-H1 (Kang and Kieff 2015).
EBNA3B acts as tumor suppressor whose inactivation in B cell lymphoma leads to the lymphomagenesis and immune evasion (White et al. 2012).

2.5.2.4. Latent membrane protein 1

Latent membrane protein 1 (LMP1) is a transmembrane protein that mimics function of the CD40 receptor, which belongs to the tumor necrosis factor receptor family (TNFRSF), expressed mainly on B cells. Both LMP1 and CD40 activate NF-κB, JNK and JAK/STAT pathways, leading to the B cell activation, proliferation and differentiation (Graham, Arcipowski, and Bishop 2010). LMP1 was shown to upregulates surface molecules, such as ICAM1, LFA1, CD40, CD21, CD23 and HLA class II as well as to downregulates CD10 expression and membrane adhesion. LMP1 upregulates expression of the proapoptotic Bim in Hodgkin's lymphoma, which is downregulated by EBNA-3A and -3C. (Kang and Kieff 2015). In addition, LMP1 is necessary to convert the latent phase into the lytic phase and release the virus from infected cells (Ahsan et al. 2005).

2.5.2.5. Latent membrane protein 2A and 2B

Latent membrane protein 2A (LMP2A) is a transmembrane protein that mimics function of B cell receptor (BCR), which is necessary for B cell activation, survival, and development. LMP2A inhibits BCR expression on B cells developing in the bone marrow by blocking the immunoglobulin (Ig) heavy chain expression while allowing immunoglobulin light-chain rearrangement. Consequently, BCR negative B cells leave the bone marrow and survive in the blood periphery. This indicates that LMP2A signaling bypasses the requirement of Ig recombination and provides a

developmental and survival signal (Caldwell, Brown, and Longnecker 2000). In addition, Longnecker et al. indicated that LMP2A provides survival signal and resistance to apoptosis by activation of the Ras/PI3K/Akt pathway (Portis and Longnecker 2004). Furthermore, LMP2A provides long-term persistence of EBV-transformed B cells in the blood periphery by blocking the BCR signal transduction, preventing B cell activation and thus reactivation of the lytic phase (Caldwell et al. 1998).

LMP2B is a transmembrane protein that differs from LMP2A only in the first exon. LMP2A encodes 119 amino acid N-terminal tail that is missing in LMP2B. The role of LMP2B is to inhibit LMP2A activity by blocking LMP2A phosphorylation (Rovedo and Longnecker 2007).

2.5.3. EBV-associated cancers

Infections contribute to 13% of all cancers worldwide (de Martel et al. 2020). To date, eight human tumor-associated viruses have been identified, namely hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV), human herpes virus 8 (HHV8), Merkel cell polyomavirus (MCPyV), Human T-lymphotropic virus 1 (HTLV-1), Cytomegalovirus (CMV) and Epstein Barr virus (EBV); (Chang, Moore, and Weiss 2017), and (Michaelis, Doerr, and Cinatl 2009).

In 1964, EBV was identified as a first human cancer-associated virus by three scientists; Anthony Epstein, Bert Achong and Yvonne Barr, who isolated the virus from Burkitt's lymphoma cells (Epstein, Achong, and Barr 1964).

EBV is associated with malignant and non-malignant diseases, such as infectious mononucleosis or multiple sclerosis (Ebell 2004), and (Moreno et al. 2018). Most of the EBV-associated malignancies derive from B cells, while the others are derived from T cells, NK cells, as well as nasopharyngeal and gastric epithelial cells. The B cell lymphomas include post-transplant lymphoproliferative disorders (PTLD), Hodgkin lymphoma (HL), Burkitt lymphoma (BL) and Diffuse large B cell lymphoma (DLBCL). According to the above-mentioned types of infected cells, EBV contributes to the development of T/NK lymphomas/leukemias, nasopharyngeal carcinoma (NPC) and gastric carcinoma. Additionally, the EBV-associated cancers can be classified using viral latency phases, e.g., the PTLD expresses EBV antigens activated in the viral latency III, while the HL expresses antigens from viral latency II (Shannon-Lowe and Rickinson 2019).

2.5.3.1. Burkitt's lymphoma

Burkitt's lymphoma (BL) was the first EBV-associated cancer discovered (Epstein, Achong, and Barr 1964). A characteristic feature of BL cells is the chromosomal translocation of the c-myc oncogene to the immunoglobulin loci. Consequently, continuous activation of the c-myc oncogene leads to cancerogenesis by increasing the survival of translocation-positive B cells (Cowling, Turner, and Cole 2014).

BL occurs mainly in children and is an aggressive B cell lymphoma (doubling time of a cancer cell 24–48 h) that can be divided into three forms, namely: endemic (eBL), sporadic (sBL) and HIV-associated BL. eBL (also known as "African variant") is the most frequent form and mostly occurs in children living in malaria regions such as equatorial Africa, Brazil, and Papua New Guinea (Brady, MacArthur, and Farrell 2007). Over 95% of eBL cases are associated with EBV expressing antigen pattern from latency I. In the years 2002–2006, eBL was reported in 3.1 per 100,000 children, annually. (Ogwang et al. 2008). eBL mainly presents in the jaw and the abdomen. In contrast, sBL (also known as "non-African variant") and HIV-associated BL occur in all age groups and are rarely associated with EBV (Brady, MacArthur, and Farrell 2007).

2.5.3.2. Hodgkin's lymphoma

Hodgkin's lymphoma (HL) is a B cell lymphoma, which is characterized by presence of abnormal, multinucleated B cells, known as Reed-Sternberg cells (RS cells). According to the current World Health Organization (WHO) classification, HL was categorized into classical HL (cHL), and less common nodular lymphocyte predominant HL (NLPHL). Based on the morphology of the RS cells and the tumor microenvironment composition, cHL was divided into four subtypes, namely: lymphocyterich cHL (LRCHL), nodular sclerosis (NS) cHL, mixed cellularity (MC) cHL, and lymphocyte depletion (LD) cHL. All cHL subtypes share a common immunophenotype (CD30+, CD40+, CD15+, IRF4/MUM1+) and approximately 50% of all cHL incidences are associated with EBV expressing antigen pattern from latency II (Carbone and Gloghini 2018). cHL is ubiquitous and occurs in all genders and age groups, with two distinct peaks in cancer development. First, in people aged 20-39 and second above 60 years (Zhou et al. 2019). Worldwide in 2018, 79,990 new cases (constituting 0.4% of all new cancers) and 26,167 deaths (constituting 0.3% of all cancer deaths) were diagnosed (Bray et al. 2018).

2.5.3.3. EBV-post-transplant lymphoproliferative disorder

EBV-post-transplant lymphoproliferative disorder (EBV-PTLD) is B cell lymphoma that develops in immunosuppressed patients after hematopoietic stem cell transplant (HSC) and solid organ transplant (SOT). Statistically, PTLD occurs in over 1% of patients after allogeneic HSCT. Depending on the type of organ transplant, the EBV-PTLD occurs in 1 to 10% of patients after lung, heart, liver and kidney transplantation, and in 20% of patients after small bowel transplantation. Over 90% of the EBV-PTLD cases are associated with EBV, expressing antigen pattern from latency III (Gottschalk, Rooney, and Heslop 2004).

2.5.3.4. EBV-positive diffuse large B cell lymphoma, not otherwise specified

EBV-positive diffuse large B cell lymphoma (DLBCL), not otherwise specified (NOS) is a type of DLBCL, classified by the WHO in 2018. This cancer is a rare and aggressive B cell lymphoma that mainly affects nodal and extranodal sites, such as gastrointestinal tract, skin and bone marrow. The EBV-positive DLBCL, NOS is characterized by presence of a homogeneous population of large B cells, appearing as large centro-blasts, immunoblasts or Hodgkin and Reed-Sternberg (HRS)-like cells. The lymphoma cells demonstrate necrosis and apoptosis. More than 90% of cases are associated with EBV, expressing latency III or latency II antigen pattern. The EBV-positive DLBCL, NOS is ubiquitous, however, more cases have been reported in Asia and South America, compared to Europe and North America. The average age of cancer development is 70 years (Murthy et al. 2017), and (Castillo et al. 2018).

2.5.3.5. Nasopharyngeal carcinoma

WHO classified three types of the Nasopharyngeal carcinoma (NPC), of which type II (differentiated non-keratinized cancer) and III (undifferentiated cancer) are associated with EBV, expressing latency I or latency II antigen pattern (Shannon-Lowe and Rickinson 2019). These type II and III cancers account for 80% of all NPC cases worldwide (Lawrence S. Young and Dawson 2014). Nasopharyngeal carcinoma is a squamous cell carcinoma that occurs in the nasopharyngeal epithelium. The characteristics of the cells are different for each type. Type II shows no clear differences from healthy epithelial cell types on light microscopy. In contrast, type III cancer cells have oval or circular follicular nuclei with prominent nucleoli. In

addition, cell margins are blurred. The NPC is present in all genders and age groups, with two distinct peaks in cancer development. First peak occur in people aged 15-24 and second peak in people aged 65-79. The prevalence of NPC in Western countries is sporadic, with less than one case per 100 000 individuals, annually. However, the NPC is prevalent in Asia, especially in southern China, with 20-30 new cases per 100 000 individuals, annually (Ji et al. 2011).

2.5.3.6. EBV-associated gastric carcinoma

EBV-associated gastric carcinoma (EBVaGC) is a gastric epithelial cancer, which accounts for 10% (up to 75,000 new cases per year) of all gastric cancers worldwide (Nishikawa et al. 2018). EBVaGC is characterized as monoclonal proliferation of EBV-positive cancer cells expressing pattern of latency I. In addition, increased lymphocytic infiltration in EBVaGC samples was detected in histological studies. EBVaGC is ubiquitous, but is twice as common in men as it is in women (G. Murphy et al. 2009).

2.5.3.7. EBV-associated T/NK-Cell lymphoproliferative diseases

EBV-associated T/NK-Cell Lymphoproliferative diseases (EBV-T/NK LPDs) are a group of rare cancers of T cells (CD8- and CD4-positive) and NK cells that develop after an acute primary EBV infection or a chronic active EBV infection. In 2016, the World Health Organization classified the EBV-T/NK LPDs into fife types, namely: chronic active EBV infection (CAEBV) of T- and NK-cell type (cutaneous and systemic forms), systemic EBV-positive T-cell lymphoma of childhood, aggressive NK-cell leukemia, extranodal NK/T-cell lymphoma, nasal type, and primary EBV-positive nodal T/NK-cell lymphoma. Over 90% of LPD EBV-T/NK types are EBV positive and express the antigen pattern of latency I and/or latency II. The EBV-T/NK LPDs occur mainly in south-east Asia and are common for all genders and age groups (Kim et al. 2019), (Lawrence S. Young and Rickinson 2004), and (Kimura and Fujiwara 2019).

Tab. 1. Summary of EBV-associated malignancies and their classification according to the EBV latency stage

Diseases	**% of EBV positive**	**Latency**
Endemic Burkitt's lymphoma	>95%	I
Hodgkin's lymphoma	50%	II
EBV-post-transplant lymphoproliferative disorder	>90%	III
EBV-positive diffuse large B cell lymphoma, not otherwise specified	>90%	II or III
Nasopharyngeal carcinoma	100%	I or II
EBV-associated gastric carcinoma	90%	I
EBV-associated T/NK-Cell lymphoproliferative diseases	>90%	I or II

3. AIM

Manufacturing of EBV-specific TCR-engineered T cells for TCR gene therapy is fast requiring small initial amounts of patient T cells. However, lack of available EBV-specific TCRs impairs the application of this method in the clinic.

Thus, the aim of this PhD thesis was to identify and isolate EBV-specific TCRs targeting epitopes of EBV antigens presented on various types of HLA class I molecules and their re-expression in T cells. The determined TCRs can serve as a basis for creating an EBV-specific TCR library providing an useful tool of TCR gene therapy in the treatment of EBV-associated cancers. To this end, the following steps were taken:

1. EBV-specific T cells were identified and isolated from PBMCs of EBV-positive donors using the single-HLA K562 cell library.
2. EBV-specific TCRs were isolated upon CD137 expression and analyzed using the Next Generation Sequencing (NGS) method.
3. Functional EBV-specific TCRs were reconstructed and re-expressed in T cells.
4. Functional characteristics of TCR-engineered T cell were performed by coculture with EBV-positive cancer-derived cell lines and measuring TCRs sensitivity to the peptide/HLA (pHLA) complexes.
5. Identification of TCR binding sites was performed by functional analysis of cocultured EBV-specific TCR-engineered T cell with truncated versions of EBV antigens (epitope identification).

4. Material and methods

All procedures were performed according to the manufacturer's protocols and instructions, unless otherwise stated.

The supplemented RPMI 1640 GlutaMAX medium term, which can be found in the text, stands for the RPMI 1640 GlutaMAX medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal calf serum (FCS; Biochrom, Germany), 1x MEM Non-Essential Amino Acids Solution (100X), 1mM Sodium Pyruvate (100 mM) and 100 U/ml Penicillin-Streptomycin (10,000 U/mL) (all Gibco, Thermo Fisher Scientific, USA).

4.1. Material

4.1.1. Primer list

Primers were synthetized by the Eurofins Genomics (Gernamy). All forward antigen-specific primers were additionally flanked with the *NotI* restriction site at the 5' ends (GCGGCCGCC). The *NotI* restriction site sequence contains the Kozak sequence. All revers antigen-specific primers were additionally flanked with the *EcoRI* restriction site (GAATTC) and codon stop (TAA or TGA) at the 3' ends. This approach facilitated cloning transgenes to the vectors.

Name	Sequence	Purpose
Sequencing primers		
T7 forward promoter	TAATACGACTCACTATAGGG	Sanger Sequencing of pcDNA3.1(-)
BHG reverse	TAGAAGGCACAGTCGAGG	Sanger Sequencing of pcDNA3.1(-)
SeqLEADERfwd	CAGCATCGTTCTGTGTTGTCT	Sanger Sequencing of pMP71
Seqrev	CATTTAAATGTATACCCAAATCAA	Sanger Sequencing of pMP71
T3 forward promoter	ATTAACCCTCACTAAAGGGA	Sanger Sequencing of pCR 4Blunt-TOPO vector
Antigen-specific primers		
EBNA3C_not_fwd	ATAGCGGCCGCCATGGAATCATTTGAAGG	EBV EBNA3Cwt forward for amplification from LCL cDNA
EBNA3C_eco_rev	ATAGAATTCTTAATCTAGCTCACTTTCAGTGG	EBV EBNA3Cwt revers for amplification from LCL cDNA
LMP2A_not_fwd	ATAGCGGCCGCCATGGGGTCCCTAGAAATG	EBV LMP2Awt forward for amplification from LCL cDNA
LMP2A_eco_rev	ATAGAATTCTTATACAGTGTTGCGATATGGG	EBV LMP2Awt revers for amplification from LCL cDNA

EBNA1_not_fwd	ATAGCGGCCGCCATGTCTGACGAGGGGC	EBV EBNA1wt forward for amplification from LCL cDNA
EBNA1_eco_rev	ATAGAATTCTCACTCCTGCCCTTCC	EBV EBNA1wt revers for amplification from LCL cDNA
dLMP1_not_fwd	ATAGCGGCCGCCATGAGTGACTGGACTGGAGG	EBV dLMP1wt forward for amplification from LCL cDNA
LMP1_eco_rev	ATAGAATTCTTAGTCATAGTAGCTTAGCTGAACTG	EBV dLMP1wt revers for amplification from LCL cDNA
EBNA3C.1_not_fwd	ATAGCGGCCGCCATGCCATCAGTCTGCGCCCT	EBV EBNA3Cwt forward for gene truncation
EBNA3C.6_not_fwd	ATAGCGGCCGCCATGCCCATACGCCCCATT	EBV EBNA3Cwt forward for gene truncation
EBNA3C.7co_not_fwd	ATAGCGGCCGCCATGCCTCCCCCTATGCCG	EBV EBNA3Cwt forward for gene truncation
EBNA3C.2co_eco_rev	CGCGAATTCTTATGAATCTTCGGTGGTTTCAAC	EBV EBNA3Cwt revers for gene truncation
EBNA3C.3co_eco_rev	CGCGAATTCTTAGAGGGCGCAGACTGATG	EBV EBNA3Cwt revers for gene truncation
EBNA3C.4co_eco_rev	ATAGAATTCTTAGAGATGTGGTGCCCTGGGAT	EBV EBNA3Cwt revers for gene truncation
EBNA3C.5co_eco_rev	ATAGAATTCTTAAATGGGGCGTATGGGGGCTC	EBV EBNA3Cwt revers for gene truncation
EBNA3C.8co_eco_rev	ATAGAATTCTTAGGGGAATCTTGTTGGAATG	EBV EBNA3Cwt revers for gene truncation
LMP2A_2_not_fwd	ATAGCGGCCGCCATGCTGATTTTGGGCACACT	EBV LMP2Awt forward for gene truncation
LMP2A.1co_eco_rev	CGCGAATTCTTACACTGCTGCCAAGAGTAGAAGT	EBV LMP2Awt revers for gene truncation
LMP2A.2co_eco_rev	CGCGAATTCTTACACAAGTGTCCATAGGAGCATGA	EBV LMP2Awt revers for gene truncation
LMP2A.3co_eco_rev	CGCGAATTCTTATACAGTGTTGCGATATGGGGTCGGT	EBV LMP2Awt revers for gene truncation
LMP2A_4_eco_rev	CGCGAATTCTTAAGCATATAGGAACAGTCGTG	EBV LMP2Awt revers for gene truncation
LMP2A_5_eco_rev	CGCGAATTCTTATAATAACATGCAGAACAAAT	EBV LMP2Awt revers for gene truncation
LMP2A_6_eco_rev	CGCGAATTCTTAGGCTACCATGGTGAGCAGGC	EBV LMP2Awt revers for gene truncation
LMP2A_7_eco_rev	CGCGAATTCTTAGCAGCATCTAATGACCCCAA	EBV LMP2Awt revers for gene truncation
dLMP1_1co_eco_rev	ATAGAATTCTTAGAAGGCTAGGAAGAAGGCCAAAAGC	EBV dLMP1wt revers for gene truncation
dLMP1_2co_eco_rev	ATAGAATTCTTAGTCAGGACCACCTCCAGGTG	EBV dLMP1wt revers for gene truncation
TCR RACE primers		
TRAC_RACE	CGGCCACTTTCAGGAGGAGGATTCGGAAC	Gene-specific revers for 5' RACE PCR-mTRAC for TOPO cloning
TRBC_RACE	CCGTAGAACTGGACTTGACAGCGGAAGTGG	Gene-specific revers for 5' RACE PCR-mTRBC for TOPO cloning
TRAC_RACE_deep seq	TGGTACACGGCAGGGTCAGGGTTCTGG	Gene-specific revers for 5' RACE PCR-mTRAC for TOPO cloning

TRBC_RACE_deep seq	CAAACACAGCGACCTCGGGTGGGAACAC	Gene-specific revers for 5' RACE PCR-mTRBC for TOPO cloning

4.1.2. Vector list

Vector (name)	Source of vectors	Transgenes of vectors	Annotation
pcDNA3.1 (-) mammalian expression vector	(Invitrogen, Thermo Fisher Scientific, USA)	- EBV antigens (LMP1, LMP2A, EBNA1 and EBNA3C) - CD80 gene - GFP gene	
Retroviral MP71 vector	Generated in the group of Prof. Dr. Wolfgang Uckert by Dr. Boris Engels (MDC, Berlin, Germany)	- TCR cassettes (see: Construction of TCR cassettes) - HLA-class I genes	(Engels et al. 2003)
pMP71-TCRva15con	Kind gift from Vasiliki Anastasopoulou (MDC, Berlin, Germany)	TRAV chains*	DNA fragment of mTRAC (from 3' end to the XmaJI unique restriction site, upstream) was fused to the 5' end of PRE in the MP71 vector. The XmaJI was inserted into mTRAC by silent mutation*
pMP71-TCRvb12con	Kind gift from Vasiliki Anastasopoulou (MDC, Berlin, Germany)	TRBV chains*	DNA fragment of mTRBC (from 3' end to the Eco72I unique restriction site, upstream) was fused to the 5' end of PRE in the MP71 vector*

MDC: Max Delbrück Center for Molecular Medicine, Berlin, Germany.
mTRAC: mouse TCR constant alpha region. mTRBC: mouse TCR constant beta region.
PRE: Posttranscriptional Regulatory Element
* see: Construction of the murinized TRAV and TRBV chains (mTRA and mTRB) for functional analysis of mTRA and mTRB chain combinations

4.1.3. EBV antigens

EBV antigen (dLMP1, LMP2A, EBNA1 and EBNA3C) sequences for analysis and synthesis were obtained from the UniProt Database (https://www.uniprot.org) and/or the RefSeq: NCBI Reference Sequence Database (https://www.ncbi.nlm.nih.gov/refseq/).

Antigens	Sequence information source	Generation source
EBNA1	UniProtKB: P03211 RefSeq: NC_007605.1	GeneArt (Invitrogen, Thermo Fisher Scientific, USA).
EBNA3C	UniProtKB: A0A0B6VJ05 RefSeq: NC_007605.1	GeneArt (Invitrogen, Thermo Fisher Scientific, USA).
dLMP1	UniProtKB: P03230 RefSeq: NC_007605.1	LCL WIN (IHW: 9095)
LMP2A	UniProtKB: P13285 RefSeq: NC_007605.1	LCL WIN (IHW: 9095)

LCL: lymphoblastoid cell line. EBNA1: EBV nuclear antigen 1. EBNA3C: EBV nuclear antigen 3C. dLMP1: delta latent membrane protein1. LMP2A; latent membrane protein 2A

4.1.4. Peptides

Preselected peptides (see: Prediction of immunogenic epitope sequences for EBV-specific TCRs) were synthetize by the PepTrack™ Peptide Libraries service (JPT Peptide Technologies, Germany) providing peptides with a purity of 30-50%. Epitopes recognized by isolated EBV-specific TCRs (see: Table 13) for peptide titration analysis were synthetize by Custom Peptide Synthesis service (JPT Peptide Technologies, Germany) providing peptides with the purity above 95%.

4.1.5. Antibodies for staining

Specificity	Conjugate		Clone/Cat. #	Manufacturer
T cells				
CD4	PE, APC		RPA-T4	BD Biosciences
CD8	PE		RPA-T8	BioLegend
CD8	APC		HIT8a	BioLegend
CD137	PE, APC		4B4-1	BioLegend
mTRBC	PE, APC		H57-597	BioLegend
DCs				
CD80	PE	iso mIgG1, κ	557227	BD Biosciences
iso mIgG1, κ	PE		MOCP-21	BioLegend

CD83	PE-Cy7	iso mIgG1, κ	HB15e	BioLegend
iso mIgG1, κ	PE-Cy7		MOCP-21	BioLegend
CD86	APC	iso mIgG1, κ	555660	BD Biosciences
iso mIgG1, κ	APC		550826	BD Biosciences
HLA-DR, -DP, -DQ	FITC	iso mIgG2a, κ	555558	BD Biosciences
iso mIgG2a, κ	FITC		555573	BD Biosciences

4.1.6. Cell lines

Cell line (name)	Type of cell line	Source of cell line	Annotation
Buffy Coats	PBLs	- The German Red Cross (DRK-Kreisverband Berlin-Nordost, Germany) - Donors	Blood samples were drawn with written and informed consent of healthy donors
BSM (IHW: 9032), EK (IHW: 9054), WIN (IHW: 9095), DEM (IHW: 9007), KASOII (IHW: 9009), SA (IHW: 9001), HO104 (IHW: 9082)	LCLs	Kind gift from Prof. Dr. Dolores Schendel, Medigene, Munich, Germany) and Prof. Dr. Elfriede Nößner (Helmholtz Zentrum München, Germany)	
L591	B lymphoblastoid cells (from patient with Hodgkin's lymphoma)	Kind gift from Dr. Uta Höpken (MDC, Berlin, Germany)	
RPMI 6666 (ATCC CCL-113)	Hodgkin's lymphoma derived cell line	American Type Culture Collection (ATCC), USA	
K562 cell line (ATCC CCL-243)	Chronic myelogenous leukemia (CML) derived cell line	American Type Culture Collection (ATCC), USA	The single-HLA-cell library was generated in the group of Prof. Dr. Wolfgang Uckert by Dr. Felix Lorenz using this cell line.

MDC: Max Delbrück Center for Molecular Medicine, Berlin, Germany.
PBLs: peripheral blood lymphocytes
LCLs: lymphoblastoid cell lines

4.2. Methods

4.2.1. TCR detection and isolation platform

To identify EBV-specific TCRs, the recently in our group developed TCR detection and isolation platform based on a single-HLA cell library was applied (Lorenz et al. 2017). This platform allows identifying TCRs that recognize cancer-specific antigens presented on any HLA class I molecules. The TCR platform consists of multiple steps depicted in Figure 6.

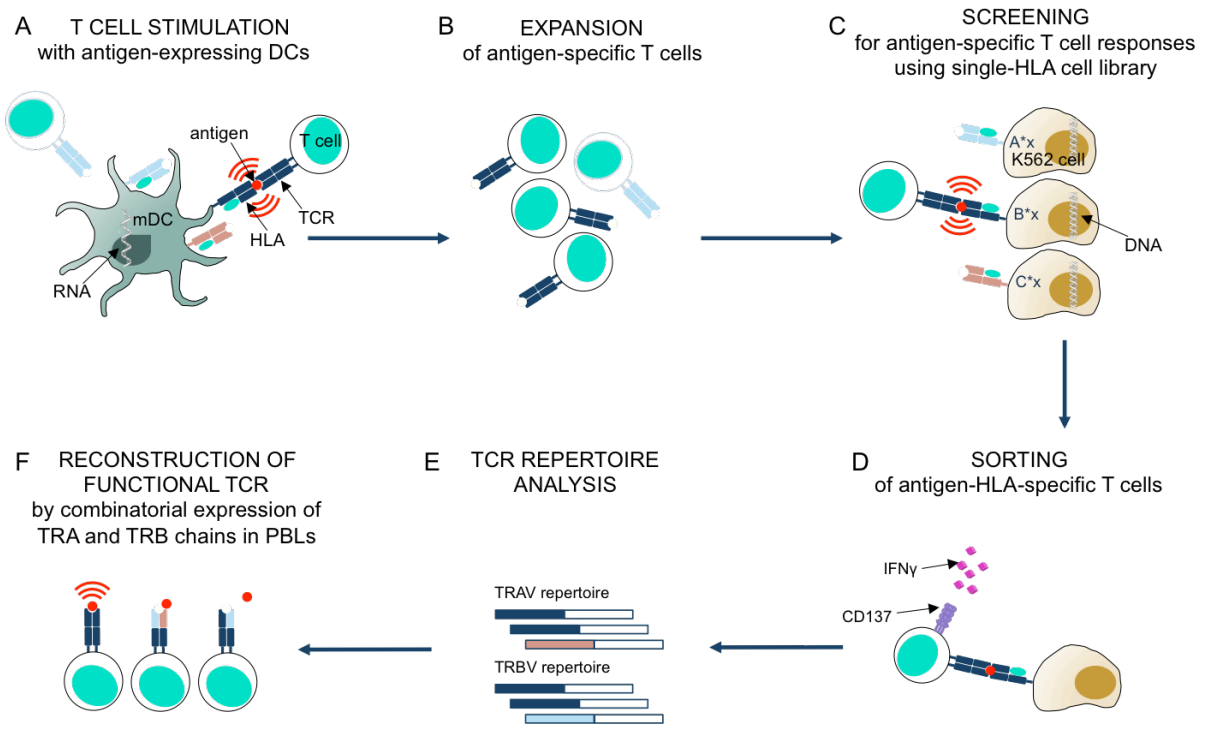


Fig. 6. Illustration of the multi-step TCR platform for detection and isolation of cancer-specific TCRs. (A-B) Autologous T cells were stimulated and expanded using antigen-expressing dendritic cells (DCs). (C) Antigen- and single-HLA-expressing K562 cells were cocultured with expanded T cells. After overnight coculture (>16 h) the positive T cell response was analyzed by measuring the CD137 expression by FACS and IFNγ secretion by ELISA. (D) Antigen-specific T cells were identified and sorted in response to the overexpression of CD137 (E) Next generation sequencing was applied to identify dominant TRA and TRB chain gene sequences from the antigen-specific T cell population. (F) Dominant TRA and TRB chains were combined and expressed in PBLs to identify the cancer-specific TCRs.

4.2.2. Molecular biology

4.2.2.1. EBV antigen isolation

LMP1 and LMP2A were isolated from the EBV (B95-8 strain)-transformed B lymphoblastoid cell line WIN (IHW: 9095), as follows: Total RNA was extracted using

the RNeasy Mini Kit (Qiagen, Germany) and transcribed into a cDNA using the SuperScript II Reverse Transcriptase and the Oligo(dT)$_{12-18}$ Primer (Invitrogen, Thermo Fisher Scientific, USA). PCR reaction was performed using generated cDNA as a template, the Phusion High-Fidelity DNA Polymerase (New England Biolabs, USA) and EBV-antigen specific primers (see: Primer list). PCR products were isolated from an agarose gel using the Invisorb Spin DNA Extraction Kit (Stratec, Germany) and cloned to the vectors (see: Molecular cloning). EBNA1 and EBNA3C were synthetized by the GeneArt Gene Synthesis (Invitrogen, Thermo Fisher Scientific, USA).

4.2.2.2. Molecular cloning

Transgenes were cloned to the vectors as follow: pcDNA3.1(-), MP71 vectors and their corresponding transgenes (see: Vector list) were digested with *NotI* and *EcoRI* restriction enzymes (Fermentas, USA and New England Biolabs, USA). The pMP71-TCRva15con and murinized TRAV chains were digested with *NotI* and *XmaJI* restriction enzymes (Fermentas, USA; New England Biolabs, USA). The pMP71-TCRvb12con and murinized TRBV chains were digested with *NotI* and *Eco72I* restriction enzymes (Fermentas, USA; New England Biolabs, USA). Digested vectors were dephosphorylated using the alkaline phosphatase, (Roche, Switzerland). Desired DNA fragments of vectors and transgenes were separated by an agarose gel electrophoresis and purified from gel slices using the Invisorb Spin DNA Extraction Kit (Stratec, Germany). The purified DNA fragments were ligated at a molar ratio of transgene to vector of 3:1 using the Rapid DNA ligation Kit (Roche, Switzerland). Ligated vectors were transformed to the chemo-competent bacteria MACH-1 (Invitrogen, Thermo Fisher Scientific, USA) using heat-shock method (4 μl ligation mix, 50 μl MACH-1 bacteria, 10 min on ice, 30 sec in 42^{o}C, 1 min on ice, 1 ml super optimal broth (SOC) medium, 1 h in 37^{o}C, 2000 rpm for 1 min to pellet bacteria, plating on LB-ampicillin agar plate overnight). Ligated vectors were isolated from single MACH-1 colonies using the Plasmid Maxi Kit (Qiagen, Germany). DNA sequence analysis was performed by the Sanger sequencing services of Eurofins (Germany) using sequencing primers (see: Primer list).

4.2.2.3. TRAV and TRBV chains isolation and identification

Next generation sequencing (NGS)

TRAV and TRBV chains from donors 1, 2 and 4 were isolated and sequenced using NGS as follows: Total RNA was directly isolated from sorted $CD8^+/CD137^+$ T cell fraction of EBV-specific T cells using the RNeasy Mini Kit (Qiagen, Germany). Generation of cDNA, TRAV and TRBV-specific chain amplification (RACE PCR) and their labeling with adapters and indexes (included in the Kit), compatible with the Illumina sequencing platform, was performed using the SMARTer Human TCR a/b Profiling Kit (Takara Bio USA, Inc., USA). Samples were processed by Novogene (HK) Company Limited (Wan Chai, Hong Kong, China) using NGS.

TOPO PCR cloning

TRAV and TRBV chains from donor 3 were isolated using TOPO PCR cloning as follows: Total RNA was directly isolated from sorted $CD8^+/CD137^+$ T cell fraction of EBV-specific T cells using the RNeasy Mini Kit (Qiagen, Germany) and transcribed to a cDNA using the SMARTer RACE 5'/3' Kit and the 5'-CDS Primer A (included in the Kit) (Takara Bio USA, Inc., USA). TRAV and TRBV-specific chain amplifications (RACE-PCR) was performed using: 5 µl 5' RACE cDNA reaction mix, 5 µl Universal primer mix (UPM; long for a 1^{st} PCR and short for a 'nested' PCR) (all Takara Bio USA, Inc., USA), 10 µl 5X Phusion GC Buffer, 1 µl dNTP (10mM each), 0.5 µl Phusion DNA Polymerases (all Invitrogen, Thermo Fisher Scientific, USA), 0.5 µl TCR RACE primers: TRAC_RACE, TRBC-RACE (50 pmol/µl) and/or nested-spec. primers: TRAC_RACE_deep seq, TRBC_RACE_deep seq (50 pmol/µl) (see: Primer list), and up to 50 µl H_2O. The RACE PCR conditions were provided in the SMARTer RACE 5'/3' Kit user manual. The RACE PCR products were cloned into the pCR 4Blunt-TOPO vector (included in the Kit) using the Zero Blunt TOPO PCR Cloning Kit and cloned into the One Shot TOP10 chemically competent E. coli (Pub No. MAN0000110; all Invitrogen, Thermo Fisher Scientific, USA) using heat-shock method (see: Molecular cloning). Vectors were isolated from single Top10 colonies using the Plasmid Maxi Kit (Qiagen, Germany). DNA sequence analysis was performed by the Sanger sequencing services of the Eurofins (Germany) using the T3 sequencing primer (see: Primer list).

4.2.3. Cell Biology

4.2.3.1. Conditions for maintenance and cryopreservation of cell lines

Cancer cell lines, LCLs, PBLs (including CD8-positive T cells and transduced T cells), K562 cell lines (including single-HLA cell library) (see: Cell lines) were cultured in suspension, in the supplemented RPMI 1640 GlutaMAX medium. The cell lines were seeded at a concentration of ≥ $0.5x10^6$/ml, passaged in a ratio of 1:2 when the medium turned yellow/orange and incubated at 38°C, in a 6.5% CO_2 and maximum humidity. The cell lines were cryopreserved in FCS (Biochrom, Germany), supplemented with 10% dimethyl sulfoxide (DMSO) (Carl Roth, Germany) and stored at -80°C.

4.2.3.2. Maturation of dendritic cells (DCs) and generation of EBV-antigen presenting cells (EBV-APCs)

DCs maturation

Autologous mature DCs (mDCs) were generated from plate adherent monocytes as follows: 7.5 x 10^7 PBMCs were seeded to a 75 cm^2 cell culture flask (horizontally positioned) in 15 ml of a DC medium (RPMI1640 VLE (Biochrom, Germany), 1.5% human serum (Biochrom, Germany) and incubated for 30 min at 38°C, in a 6.5% CO_2 and maximum humidity. Then, flasks were tapped and incubated for another 30 min. The flasks were tapped again and a supernatant containing suspended cells was collected and stored in -80°C as a source of T cells. Plate-adherent cells were washed three times in phosphate-buffered saline (PBS) and incubated in 15 ml of the DC medium supplemented with IL-4 (20 ng/ml) and GM-CSF (100 ng/ml) (both PeproTech, USA) for 48 h. Then, 3 ml of the DC medium supplemented with a DC maturation cocktail (GM-CSF (100 ng/ml), IL-4 (20 ng/ml), IL-1b (10 ng/ml), TNF-□ (10 ng/ml), IFNγ (250 ng/ml), PGE2 (250 ng/ml), Poly (I:C) (20 ng/ml), R848 (20 ng/ml) (all PeproTech, USA)) was added to the plate-adherent cells for 24 h. The mDCs (detached cells) were harvested and used directly for flow cytometry (see: Flow cytometry analysis) and ivtRNA electroporation (see: EBV-antigen presenting cells (EBV-APCs) generation).

EBV-antigen presenting cells (EBV-APCs) generation

EBV-APCs were generated from mDCs as follow: mDCs were washed, pelleted and resuspended at a concentration of 5-15x10^6/ml in the chilled Opti-MEM reduced serum medium (Gibco, Thermo Fisher Scientific, USA). Then, 200 μl of mDCs and 10-15 μg of ivtRNA encoding EBV antigens (or ivtRNA encoding GFP gene) were mixed in a 4 mm electroporation cuvette (Bio-Rad, USA). Electroporation of mDCs was performed using the GenePulser, at setting 250 uF and 0.35 kV (Bio-Rad, USA). Electroporated mDCs were transferred to a 6-well plate containing 3 ml/well of the original DC medium, supplemented with the maturation cocktail (see: DCs maturation), for 3 h before flow cytometry (see: Flow cytometry analysis) and T cell stimulation (see: CD8-positive T cell stimulation with EBV-APCs).

4.2.3.3. Isolation of autologous CD8-positive T cells and EBV-antigen specific stimulation

CD8-positive T cell isolation

CD8-positive T cells were isolated from a peripheral blood as follow: 50-500 ml of blood was diluted 1:2 with the low endotoxin and w/o $Ca2^+$/$Mg2^+$ PBS Dulbecco (Biochrom, Germany). 35 ml of diluted blood was transferred to the SepMate PBMC Isolation Tubes (STEMCELL Technologies Inc., Canada), previously filled with 15 ml of the Biocoll separating solution (Biochrom, Germany). Tubes were centrifuged at 1200xg for 10 min with the brake on. The white blood cell (WBCs) layer was collected and washed twice in the, above mentioned, PBS. Thrombocytes were eliminated from the WBCs by centrifugation at 140xg for 15 min with the brake on. CD8-positive T cells were isolated by negative selection using the CD8-positive T Cell Isolation Kit, human (Miltenyi Biotec, Germany) and used for flow cytometry (cytometry (see: Flow cytometry analysis) and stimulation with EBV-APCs (see: CD8-positive T cell stimulation with EBV-APCs).

CD8-positive T cell stimulation with EBV-APCs

CD8-positive T cells were stimulated with autologous EBV-APCs as follow: Before coculture, EBV-APCs electroporated with LMP1, LMP2A, EBNA1 and EBNA3C antigens were mixed in a ratio of 1:1. 3 h after electroporation, mixed EBV-APCs were cocultured with autologous CD8-positive T cells in a E:T ratio of 10:4 (1-2x10^6:0.4-0.8x10^6/well), in a 24-well plate containing 2 ml/well of the RPMI 1640 GlutaMAX medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10%

human serum (Biochrom, Germany), 1x MEM non-essential amino acids solution (100X), 1 mM sodium pyruvate (100 mM) and 100 U/ml penicillin-streptomycin (10,000 U/mL) (all Gibco, Thermo Fisher Scientific, USA). The E:T ratio depends on the number of antigens used for CD8-positive T cells stimulation (e.g. E:T ratio for stimulation with one antigen is 10:1). 72 h after stimulation, the CD8-positive T cells were supplemented with IL-7 (5 g/ml; PromoCell, Germany) and IL-2 (25 U/ml; Proleukin, Novartis, Switzerland) every other day and split in a ratio of 1:2, if cell density was too high. 13 days after stimulation, the CD8-positive T cells were restimulated with the mixed autologous EBV-APCs, thawed from the same batch used for the first stimulation. On the 18^{th} day, the stimulated CD8-positive T cells were analyzed (see: Screening of EBV-specific T cells using the single-HLA cell library) and/or stored at -80^{o}C.

4.2.3.4. Screening of EBV-specific T cells using the single-HLA cell library

EBV-specific T cells were screened using the single-HLA cell library as follows: K562 cell lines from the single-HLA cell library were washed, pelleted and resuspended at a concentration of 5-10x10^6/ml in the chilled Opti-MEM reduced serum medium (Gibco, Thermo Fisher Scientific, USA). Then, 200 μl of K562 cells and 5-10 μg of the pcDNA3.1(-) vector encoding one of the EBV antigens (LMP1, LMP2A, EBNA1 or EBNA3C) and CD80 molecule (see: Vector list) were mixed in a 4 mm electroporation cuvette (Bio-Rad, USA). The K562 cell line electroporations were performed using the GenePulser, at setting 250 uF and 0.35 kV (Bio-Rad, USA). Transfected K562 cell lines were transferred to 25 cm^2 cell culture flasks containing 5 ml of the supplemented RPMI 1640 GlutaMAX medium for 48 h. Then, the K562 cell lines transfected with LMP1, LMP2A and EBNA1 antigens were mixed in a ratio of 1:1 and cocultured with EBV-antigen stimulated CD8-positive T cells in an E:T ratio of 5:3 (1x10^5:6x10^4/well), in a 96-well plate containing 200 μl/well of the supplemented RPMI 1640 GlutaMAX medium for 16-20 h. The K562 cell line transfected with EBNA3C antigen was cocultured with EBV-antigen stimulated CD8-positive T cells in a E:T ratio of 5:1 (1x10^5:2x10^4/well) under the above mentioned conditions. After incubation, the supernatant was analyzed for IFNγ secretion and the EBV-antigen stimulated CD8-positive T cells were analyzed for CD137 T cell activation marker expression.

4.2.3.5. Generation of γ-retroviral vector particles

γ-retroviral vector particles were generated as follow: GALV cells (Eufets GmbH, Germany) were seeded at a concentration of $0.85x10^6$ cells in a 6-well plate containing 3 ml/well of the DMEM 1640 GlutaMAX medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% FCS (Biochrom, Germany) and 100 U/ml Penicillin-Streptomycin (10,000 U/mL) (Gibco, Thermo Fisher Scientific, USA). Plates with a cell confluency of 70-80% were transfected with the transfection reagent mix (18 μg of the DNA MP71 plasmid encoding GOI (see: Vector list), 15 µl $CaCl_2$-solution (2.5 M) (Sigma-Aldrich, USA), H_2O up to 150 μl and 150 μl of transfection buffer (16 g NaCl, 740 mg KCl, 500 mg $NaHCO_3$, 10 g Hepes and up to 1000 ml H_2O at a pH 6.75), which was added dropwise to the constantly vortexing transfection reagent mix). Before GALV cells transfection, the transfection reagent mix was incubated 15-20 min at RT. The transfected GALV cells were incubated 6 h at 37°C, in 5% CO_2 and maximum humidity. Then, the supplemented DMEM medium was replaced with the supplemented RPMI 1640 GlutaMAX medium and the transfected GALV cells were incubated another 30-36 h.

4.2.3.6. Retroviral transduction of cell lines

CD8-positive T cells, PBLs, K562 cell lines and cancer cell lines (L591, RPMI666) were transduced as follows: CD8-positive T cells and PBLs were seeded at a concentration of $0.5x10^6$/ml and $1\text{-}1.5x10^6$/ml, respectively, in a precoated non-tissue 24-well plates (1 ml/well of PBS containing anti-CD3 Abs (5 μg/ml OKT3) and anti-CD28 Abs (1 μg/ml) (both Pharmingen, Germany) for 2 h at 37°C in a 5% CO_2, blocked in 0.5 ml of 2% bovine serum albumin (BSA; Biochrom, Germany) diluted in PBS for 30 min at RT, washed twice in 2 ml of PBS) containing 1 ml/well of the supplemented RPMI 1640 GlutaMAX medium and IL-2 (400 U/ml; Proleukin, Novartis, Switzerland) for 36 h at 37°C, in a 5% CO_2. Then, the retroviral supernatant (see: Generation of γ-retroviral vector particles) was filtrated using the 0,45 μm Whatman Syringe Filters (GE Healthcare, Life Sciences, USA) and supplemented with IL-2 (400 U/ml) and protamine sulfate (4 μg/ml). 1 ml of the filtrated retroviral supernatant was added to the cell line in the precoated 24-well plate, and centrifuged at 800xg for 90 min at 32°C. After overnight incubation at 37°C, 1 ml of a cell supernatant was discarded and replaced with 1 ml of the filtrated

retroviral supernatant, supplemented with IL-2 (400 U/ml). Plates were centrifuged at 800xg for 90 min at 32°C and incubated 4-6 h at 37°C. Then, the cell lines were transferred from 24-well plates to 25 cm^2 cell culture flasks containing 4 ml of the supplemented RPMI 1640 GlutaMAX medium with IL-2 (400 U/ml) and incubated at 38°C in a 6.5% CO_2. Cell lines were expanded for nine days. Then, cell were pelleted and resuspended in the supplemented RPMI 1640 GlutaMAX medium containing low concentration of IL-2 (40 U/ml) and incubated at 38°C for 24 h before flow cytometry analysis (see: Flow cytometry analysis) and freezing at -80°C.

K562 cell lines and cancer cell lines were seeded at a concentration of 0.5-x10^5/ml and 1.0x10^5/ml, respectively, in the non-tissue 24-well plates containing 1 ml/well of the supplemented RPMI 1640 GlutaMAX medium for 24 h at 37°C in a 5% CO_2. Then, the retroviral supernatant (see: Generation of γ-retroviral vector particles) was filtrated using the 0,45 μm filter and supplemented with protamine sulfate (4 μg/ml). First and second transductions were performed as described for CD8-positive T cells and PBLs using the supplemented RPMI 1640 GlutaMAX medium without IL-2. The cell lines were expanded for seven days before further use.

4.2.3.7. Sorting of transduced cell lines

K562 and cancer cell lines were sorted using the BD FACSAria II flow cytometer (BD Biosciences, USA) as follow: The cell lines were transduced with MP71 vector encoding HLA class I genes linked with GFP or CFP as selection markers for flow cytometry. Before sorting, the cell lines were pelleted and resuspended in the FACS buffer (PBS supplemented with 1% of FCS (Biochrom, Germany)) and filtrated using the Falcon 5 ml Round-Bottom Tubes with Cell Strainer Cap (STEMCELL Technologies Inc., Canada), Transduced cell lines were sorted twice in bulk to reach at least 90% of cell line purity. The purity of cell lines was checked before each use.

4.2.3.8. Exogenous loading of K562 cell lines with peptides derived from EBV antigens

K562 cell lines were loaded with peptides derived from EBV antigens as follows: Lyophilized peptides (see: Peptides) were dissolved in a dimethyl sulfoxide (DMSO; Carl Roth, Germany) to a concentration of 1x10^{-3} M and titrated to 1x10^{-5}-1x10^{-13} M solutions in the supplemented RPMI 1640 GlutaMAX medium. K562 cell lines were pelleted and resuspended in the, above mentioned, peptide solutions in a

concentration of $1x10^5$ cell/ml for 2 h, at 38°C, in a 5% CO_2. Then, the K562 cell lines were pelleted, resuspended in the supplemented RPMI 1640 GlutaMAX medium and cocultured with TCR-engineered CD8-positive T cells at a E:T ratio of 1:1 ($1x10^4$:$1x10^4$cell/well) in a 96-well plates for 16-20 h, at 38°C. After incubation, the supernatant was collected and used to detect IFNγ secretion.

4.2.4. Functional assays

4.2.4.1. Flow cytometry analysis

Analysis of cell protein expression was performed using the MACSQuant flow cytometer (Miltenyi Biotec, Germany) as follows: 100-200 µl of cells were sampled and washed in 100-200 µl of a FACS buffer (PBS supplemented with 1% of FCS (Biochrom, Germany)). The cells were then pelleted and resuspended in the 50 µl of FACS buffer containing 1:50-1:100 diluted antibodies (see: Antibodies for staining) for 15-20 min, at 4°C. After incubation, the cells were washed in the FACS buffer, resuspended in 100-200 µl of the FACS buffer containing 1% of the SYTOX Green Nucleic Acid Stain (Invitrogen, Thermo Fisher Scientific, USA) and incubated for 5 min, at 4°C before measurement. Analysis of DC protein expression was supplemented with the Fc-receptor blocking step before antibody staining. Thus, the DCs pellet was resuspended in 50 µl of Fc-block (Clone PRP4H11, kind gift of Dr. Elisabeth Kremmer, Helmholtz-Zentrum München, Germany) for 10 min, at 4°C, washed in 200 µl of the FACS buffer and stained as described above.

4.2.4.2. Enzyme-linked immunosorbent assay (ELISA)

Secretion of IFNγ by CD8-positive T cells and PBLs was measured using the OptEIA Human IFNγ ELISA Kit (BD Biosciences, USA) according to the manufacturer's instructions. The reagents of the kit were diluted 1:1000 before use. Standard curves were generated using the lyophilized recombinant human IFNγ (included in the Kit) at a starting concentration of 4000 or 8000 pg/ml.

4.2.5. *In Silico* analysis

4.2.5.1. TRAV and TRBV chain sequence analysis

TRAV and TRBV chain sequences isolated form donor 3 using the Zero Blunt TOPO PCR Cloning Kit (see: TRAV and TRBV chain isolation and identification) were

sequenced by the Sanger sequencing (Eurofins Genomics, Germany) using the T3 sequencing primer (see: Primer list). The sequencing results were then analyzed using the IMGT database (http://www.imgt.org/IMGT_vquest/input).
TRAV and TRBV chain sequences isolated from donors 1, 2 and 4 were sequenced by Novogene (HK) Company Limited (Wan Chai, Hong Kong) using next generation sequencing (NGS). The sequencing results were then analyzed by Julian Gabrysch (AG Uckert) using the MiXCR software (https://mixcr.readthedocs.io/en/master/) and the IMGT database (http://www.imgt.org/IMGT_vquest/input).

4.2.5.2. Construction of the murinized TRAV and TRBV chains (mTRA and mTRB) for functional analysis of mTRA and mTRB chain combinations

Sequence encoding the *NotI* unique restriction site (which contain the Kozak sequence) was fused to the 5' ends of dominant TRAV and TRBV chain sequences. Fragment of a mouse TCR constant alpha region (mTRAC; UniProtKB: A0A075B662), from 5’ end to the *XmaJI* unique restriction site, downstream was fused to the 3’ ends of the dominant TRAV chain sequences. The *XmaJI* was inserted into the mTRAC by silent mutation. Fragment of a mouse TCR constant beta region (mTRBC; UniProtKB: A0A075B5J4), from 5’ end to the *Eco72I* unique restriction site, downstream was fused to the 3’ ends of the dominant TRBV chain sequences. This approach facilitated cloning TRAV and TRBV chains to the pMP71-TCR 15con and pMP71-TCR b12 con, respectively (see: Vector list). The dominant mTRA and mTRB chains were then human codon optimized and synthetized by GeneArt (Invitrogen, Thermo Fisher Scientific, USA).

4.2.5.3. Construction of TCR cassettes

The sequences of the mTRAC and mTRBC were fused to the 3’ ends of the functional TRAV and TRBV chain sequences, respectively. Within the cassettes the mTRA and mTRB chains were fused to the P2A self-cleaving peptide (Szymczak-Workman, Vignali, and Vignali 2012) as follows: mTRB chain-P2A-mTRA chain. The TCR cassettes were then human codon optimized and synthetized by GeneArt (Invitrogen, Thermo Fisher Scientific, USA).

4.2.5.4. Prediction of immunogenic epitope sequences for EBV-specific TCRs

Sequences of immunogenic epitopes for EBV-specific TCRs were determined using the NetMHC 4.0 Server (http://www.cbs.dtu.dk/services/NetMHC/).

5. Results

Results described in this chapter include detection, isolation and characterization of EBV-specific TCRs. In total, nine EBV-specific TCRs of four EBV-positive donors were isolated and analyzed. To preserve the anonymity of donors their names were replaced with numbers from 1 to 4. TCR results isolated from Donor 1 were presented in figures and tables. The results of TCRs isolated from other donors were presented only in tables.

5.1. Maturation of dendritic cells and generation of EBV-antigen presenting cells

DCs are professional antigen-presenting cells (APCs) that have an ability to activate and expand antigen-specific T cells. The DCs develop in a bone marrow. After release, they circulate in a bloodstream in their immature form and remain at this stage until the first contact with a pathogenic antigen. Immature DCs express a very low level of costimulatory molecules (e.g. CD80, -83, -86) and cell surface receptors (e.g. HLA class II), which are too low to initiate T cell activation (Banchereau et al. 2000). Thus, the immature DCs constitute a perfect source of APCs for *in vitro* antigen-specific T cell activation and expansion.

To generate autologous EBV-antigen specific APCs first, immature DCs were isolated from a blood and maturated from plate adherent monocytes. After maturation, the DCs were stained for expression of specific-APC markers (CD80, -83, -86 and HLA class II) to indicate DCs maturation and antigen-presenting capacity. Figure 7A indicates a higher expression of specific-APC markers on mature DCs (mDCs) surface compared to isotype controls. Comparable expression of specific-APC markers was also demonstrated on mDCs surface from other donors (data not shown).

The mDCs were subsequently electroporated with ivtRNA encoding full-length EBV antigens (dLMP1, LMP2A, EBNA1 and EBNA3C). The dLMP1 is a nontoxic and 43 amino acids at the 5 'end truncated variant of wt LMP1. This variant is better expressed in DCs and prevents them from dying after electroporation (Gottschalk et al. 2003). The ivtRNA of EBV antigens was used to ensure endogenous translation, processing and epitope presentation on the cell surface. The second reason for using ivtRNA of EBV antigens was to avoid the presentation of nonspecific antigen on the cell surface in case of electroporation of an expression vector containing other

features, such as selectable markers (Amp, GFP). Transfection efficiency was measured by electroporation of the ivtRNA encoding GFP gene into a separate sample of the mDCs. The GFP expression was measured three hours after electroporation. Figure 7B indicates a high GFP expression in mDCs (87.2%), which proves that transfection was effective. The high GFP expression, above 80%, in mDCs was also demonstrated for other donors and depicted in the Table 2. Autologous EBV-antigen specific DCs were subsequently used to stimulate autologous CD8-positive T cells from given donors.

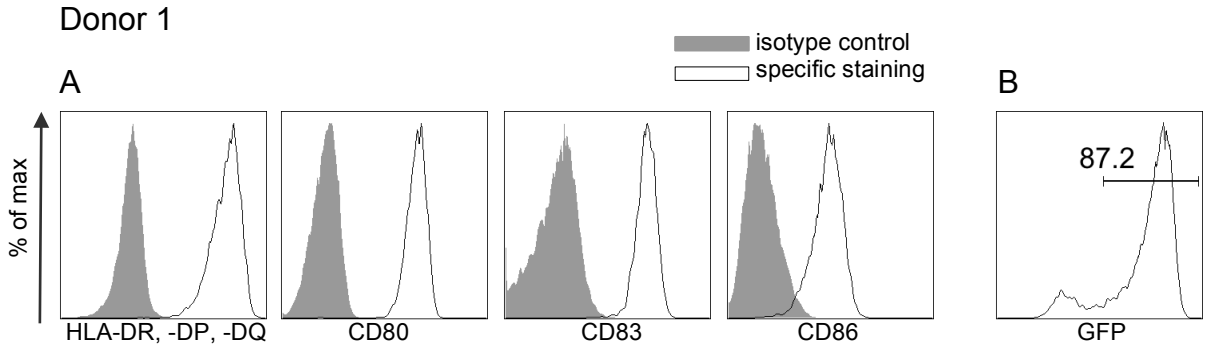


Fig. 7. DCs expressed APC-specific markers after maturation and expressed GFP after electroporation. Adherent monocytes isolated from PBMCs were used to generate mDCs. Adherent monocytes were cultured with GM-CSF and IL-4 for 48 h. After 48 h, the maturation cocktail was added for the next 24 h. (A) The mDCs were stained and analyzed for expression of APC-specific markers (HLA-DR, DP, DQ; CD80; CD83; CD86) (black line) compared to isotype controls (grey area). (B) Three hours after electroporation, the GFP expression was measured. Percentages of GFP positive APCs are indicated.

Tab. 2. Transfection efficiency of mDCs, electropotared with ivtRNA encoding GFP gene.

Donor	Transfection efficiency (% of GFP)
1	87.2
2	87.5
3	81.8
4	-

5.2. Isolation of autologous CD8-positive T cells for EBV-antigen specific stimulation

Primary EBV infection leads to an acute response and expansion of EBV-specific CD8-positive T cells. Upon infection, when the EBV is brought under immunological surveillance, only a small population of these EBV-specific CD8-positive T cells survive, generating long-lived memory T cells (Dunne 2002). Thus, to increase the chances of EBV-specific TCRs isolation, only EBV-positive individuals were selected as blood donors.

To expand EBV-specific T cells, the autologous CD8-positive T cells were isolated from blood by negative selection. After isolation, sorted T cells were stained for CD4 and CD8 to indicate CD8-positive T cell enrichment. Figure 8 indicates 87.1% of CD8-positive T cell enrichment. More than 69% of CD8-positive T cell enrichment was indicated in other donors and depicted in the Table 3. Sorted autologous CD8-positive T cells were subsequently stimulated by autologous EBV-antigen specific DCs

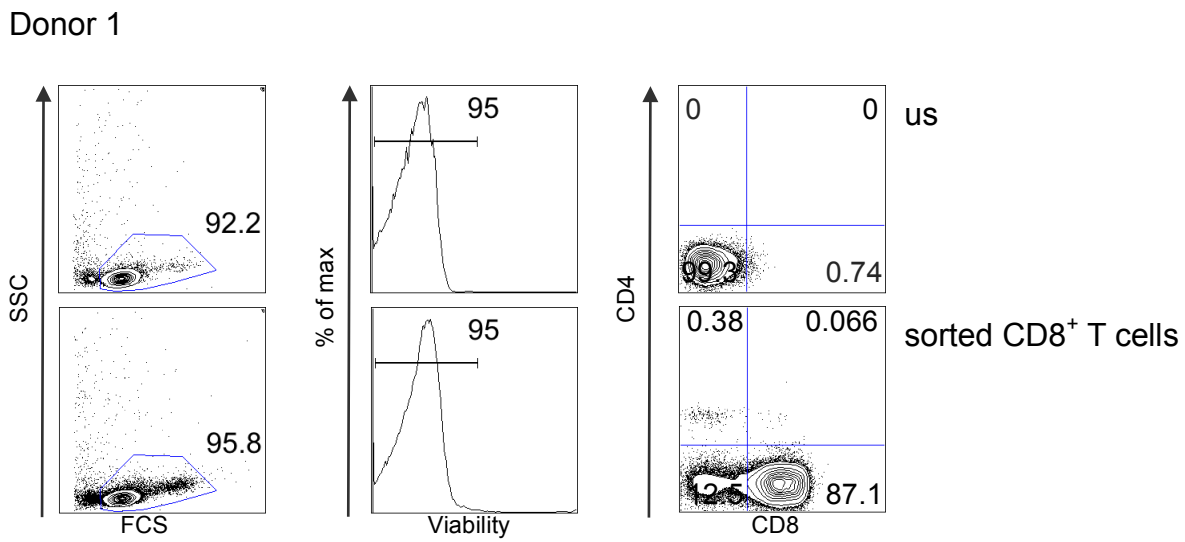


Fig. 8. CD8-positive T cells were isolated from PBMCs before stimulation with EBV-antigen expressing mDCs. CD8-positive T cells were isolated by negative selection and stained with antibodies directed against CD8 and CD4. Unstained (us) CD8-positive T cells were used as a negative control. The T cell viability was determined by the SYTOX Green staining.

Tab. 3. Viability and percentage of sorted CD8-positive T cells.

Donor	Viability of sorted $CD8^+$T cells (%)	$CD8^+$ T cell enrichment (%)
1	95.0	87.1
2	98.9	80.2
3	96.9	69.2
4	-	-

5.3. Screening of EBV-specific T cells using single-HLA cell library

Autologous CD8-positive T cells were stimulated twice with the EBV-antigen specific DCs. Each round of stimulation lasted 14 days. The DCs electroporated with ivtRNA encoding dLMP1, LMP2A and EBNA1 were mixed in a ratio 1:1 before coculture with CD8-positive T cells. This approach increases the chances of isolating T cells specific to different EBV antigens from the entire CD8-positive T cell population of a given donor's blood.

The EBNA3C antigen, compared to dLMP1, LMP2A and EBNA1 antigens, is highly immunogenic (Murray et al. 1992). The rapid expansion of EBNA3C-specific T cells could impede the growth of T cells specific to less immunogenic EBV antigens. Thus, to avoid the expansion of T cells to only the dominant antigen, one part of the CD8-positive T cells was stimulated exclusively with EBNA3C antigen.

To identify EBV-specific T cells for EBV antigen/HLA combinations, the EBV-stimulated T cells were cocultured with K562 cell lines from a single-HLA cell library. The K562 cell lines were selected accordingly to the donor's HLA type. Before coculture, the K562 cell lines were electroporated with all four EBV-encoding antigens and a gene coding for CD80, which were separately cloned into the pcDNA3.1 vector. The CD80 is a costimulatory molecule expressed on the surface of DCs, B cells and other APCs. The CD80 enhancing T cell activation and expansion upon recognition of antigen (Peach et al. 1995). Henceforth, to improve the T cell activation, all K562 cell lines were always be additionally endowed with the CD80 molecule. The electroporated K562 cell lines were then mixed, as in the case of EBV-antigen specific DCs for CD8-positive T cell stimulation.

After overnight coculture, the T cell response was analyzed by measuring expression of CD137 T cell activation marker using FACS (Figure.9.A-B). The second read-out method was measuring the IFNγ secretion by ELISA (Figure.9.C-D).

Figure 9A indicates a significant expression of CD137 on T cells cocultured with A*02:01-K562 cells (11.4%) compared to other HLA-K562 cell lines. Slightly higher expression of CD137 also showed T cells cocultured with B*57:01-K562 cells (6.07%) compared to other HLA-K562 cell lines. Figure 9B indicates a higher expression of CD137 on T cells cocultured with A*02:01- (1.63%), B*57:01- (1.39%) and C*06:02- (1.59%) K562 cell lines compared to other HLA-K562 cell lines. However, the comparison of CD137 expression with IFNγ secretion confirmed the EBV-specific T cell response in only three situations. Figure 9C indicates the correlating IFNγ secretion for T cells cocultured with A*02:01- (2786.12 pg/ml) and B*57:01-(799.71 pg/ml) K562 cell lines. Figure 9D indicates the correlating IFNγ secretion only for T cells cocultured with C*06:02-K562 cells (255.45 pg/ml). Table 4 depicts screening results of all donors. T cells that showed the EBV-specific response were FACS-sorted for further analysis of TRAV and TRBV repertoire.

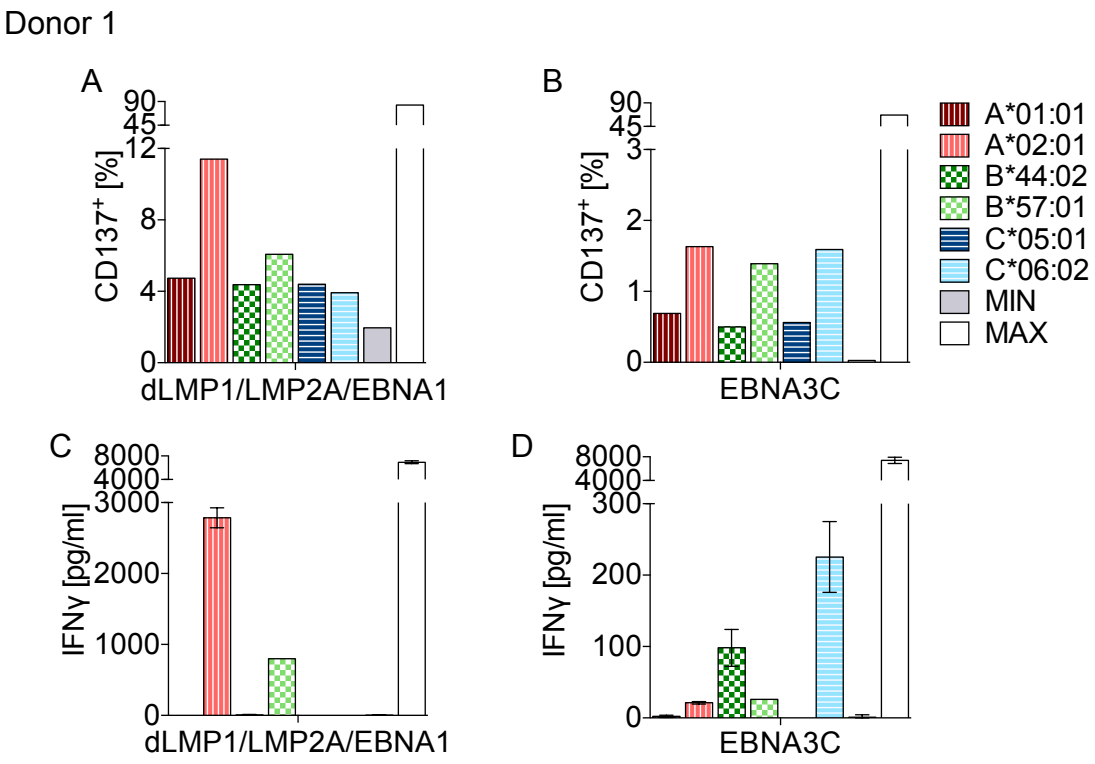


Fig. 9. CD8-positive T cells recognized specific EBV antigen/HLA combinations. CD8-positive T cells stimulated with EBV-antigen expressing mDC were screened for recognition of specific EBV antigen/HLA combinations. The CD8-positive T cells were cocultured with single HLA-expressing

K562 cells from the library, expressing the six HLA alleles of Donor 1. K562 cells were electroporated with 10 µg DNA of EBV-encoding antigens and 10 µg DNA coding for CD80 before coculture. Effector to target cell ratio was 5:1 ($1x10^5$:$2x10^4$). After overnight coculture (>16 h), the IFNγ secretion was measured by ELISA and surface expression of CD137 on T cells was determined by FACS. The data of the IFNγ show means ± s.d.

Tab. 4. CD137 expression and IFNγ secretion by CD8-positive T cells after stimulation with the EBV-antigen expressing mDCs.

Donor	EBV antigen stimulation	HLA (type)	Expression of surface CD137 (%)	Secretion of IFNγ (pg/ml)
1	dLMP1+LMP2A+EBNA1	A*01:01	4.73	0
		A*02:01	**11.40**	**2786.12**
		B*44:02	4.37	7.1
		B*57:01	**6.07**	**799.71**
		C*05:01	4.4	0
		C*06:02	3.92	0
		MIN	1.96	3.55
		MAX	83.4	6926.86
1	EBNA3C	A*01:01	0.69	2.36
		A*02:01	1.63	21.34
		B*44:02	0.5	98.29
		B*57:01	1.39	26.10
		C*05:01	0.56	0
		C*06:02	**1.59**	**255.45**
		MIN	0.02	1.18
		MAX	66.9	7415.04
2	dLMP1+LMP2A+EBNA1	A*24:02	2.03	1411
		A*26:01	6.42	91.17
		B*08:01	5.68	180.44
		B*51:01	3.91	32.61
		C*07:02	1.96	7.94
		C*15:02	**6.88**	**3149.36**
		MIN	1.50	0.91
		MAX	55.8	3386.45
3	LMP2A	**A*02:01**	**9.08**	**1077.69**
		B*07:02	0.78	0
		B*44:02	2.09	0
		C*05:01	0.74	0
		C*07:02	0.52	0
		MIN	0.57	4.85
		MAX	27.4	176.96
3	EBNA3C	A*02:01	3.34	0
		B*07:02	**4.68**	**411.43**
		B*44:02	6.25	188.65
		C*05:01	0.61	0
		C*07:02	0.33	0
		MIN	0.65	0
		MAX	51.6	5323.27

4	EBNA3C	A*02:01	0.25	0
		A*03:01	0.32	0
		B*07:02	0.44	23.58
		B*44:02	**2.48**	**67.83**
		C*05:01	0.30	0
		C*07:02	0.18	0
		MIN	0.22	0
		MAX	62.1	4739.56

The HLAs in bold were considered as positive responses.

5.4. Sorting of CD8$^+$/CD137$^+$ T cell fraction of the EBV-specific T cells

T cells for which CD137 overexpression correlated with IFNγ secretion were considered as EBV specific. CD8$^+$/CD137$^+$ T cell fractions of the EBV-specific T cells were FACS-sorted directly to the lysis buffer.

Figure 10 indicates 18% of sorted CD8$^+$/CD137$^+$ T cells in response to the dLMP1, LMP2A, EBNA1/HLA-A*02:01 combination; 12% of sorted CD8$^+$/CD137$^+$ T cells in response to the dLMP1, LMP2A, EBNA1/HLA-B*57:01 combination and 2.86% of sorted CD8$^+$/CD137$^+$ T cells in response to the EBNA3C/HLA-C*06:02 combination. HPV antigens (E6 and E7) stimulated CD8-positive T cells cocultured with K562 cells expressing E6 and E7 antigen did not respond to any of the HPV antigens presented on the HLA-C*06:02 and were used as a negative control. Table 5 depicts sorting results of CD8$^+$/CD137$^+$ T cell fractions from all donors. Sorted T cells were used to isolate TRAV and TRBV chains and analyze a TCR repertoire using TOPO cloning and NGS.

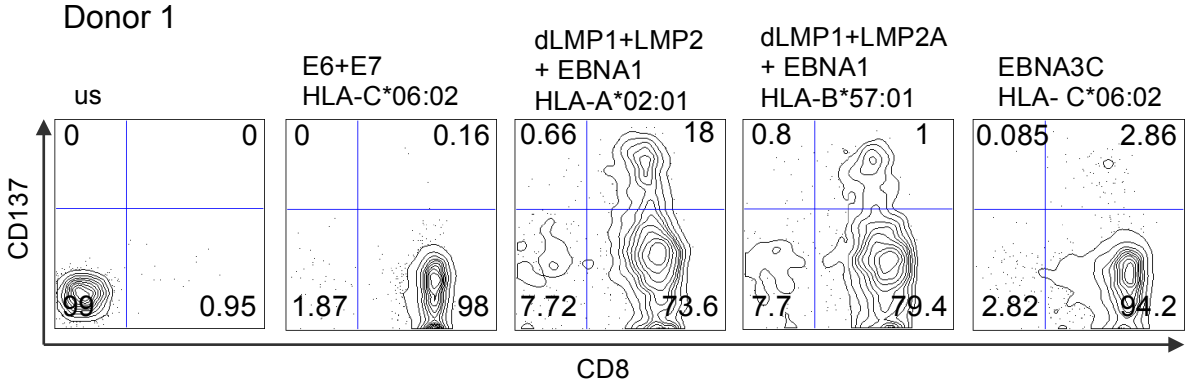


Fig. 10. CD8$^+$/CD137$^+$ T cell fractions were FACS-sorted after response to the EBV antigen/HLA combinations. EBV-specific stimulated CD8-positive T cells were cocultured with K562 cells from single-HLA cell library, expressing all six HLA alleles of Donor 1. K562 cells were electroporated with 10 µg DNA of EBV-encoding antigens and 10 µg DNA coding for CD80 before coculture. Effector to

target cell ratio was 5:1 ($1x10^5$:$2x10^4$). After overnight coculture (>16 h), T cells were stained with antibodies directed against CD8 and CD137. Double-positive T cells were FACS-sorted directly to the lysis buffer. Unstained (us) and E6+E7/C*06:02 samples were used as negative controls.

Tab. 5. Sorting of $CD8^+/CD137^+$ T cell fraction of the EBV-specific T cells after coculture with the single-HLA cell library expressing EBV antigens.

Donor	EBV antigen/HLA combination	$CD8^+/CD137^+$ T cell fraction (%)
1	dLMP1+LMP2A+EBNA1 HLA-A*02:01	18.0
	dLMP1+LMP2A+EBNA1 HLA-B*57:01	12.0
	EBNA3C HLA-C*06:02	2.86
2	dLMP1+LMP2A+EBNA1 HLA-C*15:02	13.5
3	LMP2A HLA-A*02:01	24.8
	EBNA3C HLA-B*07:02	8.23
4	EBNA3C HLA-B*44:02	4.42

5.5. TCR repertoire analysis of sorted $CD8^+/CD137^+$ EBV-specific T cell fractions

To analyze the TCR repertoire, a cDNA library consisting of TRAV and TRBV chains was generated from the sorted EBV-specific $CD8^+/CD137^+$ T cell fractions. For this purpose, two methods were used, namely TOPO cloning and Next-Generation Sequencing (NGS). The TOPO cloning allows identifying dominant TRAV and TRBV chains from a given T cell response. However, the method turned out to be laborious and not efficient. The readout of the V segments is very low (approx. 100 reads per sample), which limits the complete screening of the TRAV and TRBV chain repertoire. To improve the readout of the V segments from other samples, the NGS technology was applied. This technology allows generating many thousands of V segment readings per sample in a shorter time and uses less initial cDNA.

In order to prepare samples for the NGS, the cDNA library of TRAV and TRBV chains were marked with unique adapters and indexes compatible with the Illumina sequencing platform (NGS platform).

Figure 11 indicates the TRAV and TRBV chain repertoires of three EBV-specific T cell responses obtained from the Donor 1. All chains above 10% of threshold (a dashed line) were considered as dominant TRAV and TRBV chains and combined to identify functional TCRs. Table 6 depicts the frequency of the V segments in percent of the dominant TRAV and TRBV chains isolated from all EBV-specific T cell responses from other donors.

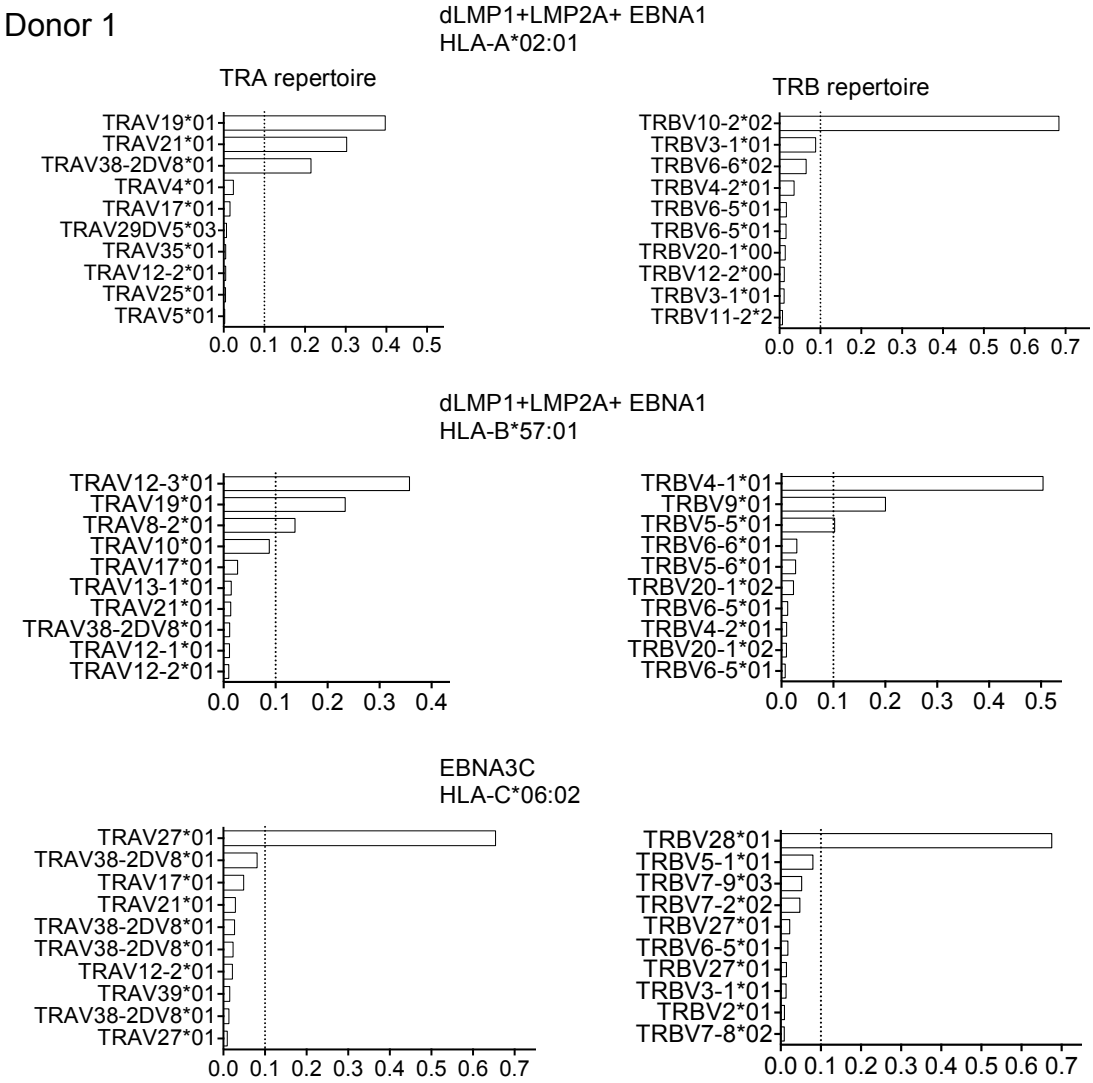


Fig.11. TRAV and TRBV chain repertoires of sorted CD8$^+$/CD137$^+$ T cell fraction of EBV-specific T cells were determined using NGS. TRAV and TRBV chains were directly isolated from CD8$^+$/CD137$^+$ T cell fraction and labeled with indexes compatible with the Illumina sequencing

platform. Samples were processed by the Novogene (HK) Company Limited for the NGS. The x-axis indicates frequency of TRAV and TRBV chains of total sequence reads. The y-axis lists the most frequent TRAV and TRBV chains. All chains above 10% of threshold (dashed line) were used for examination of functional TCRs.

Tab. 6. Dominant TRAV and TRBV chains isolated from $CD8^+/CD137^+$ EBV-specific T cells using NGS.

Donor	EBV antigen/HLA	V segment	V segment frequencies (%)
1	dLMP1+LMP2A+EBNA1 HLA-A*02:01	TRAV19*01 TRAV21*01 TRAV38-2DV8*01	39.7 30.0 21.4
		TRBV10-2*02	68.3
1	dLMP1+LMP2A+ EBNA1 HLA-B*57:01	TRAV12-3*01 TRAV19*01 TRA8-2*01	35.7 23.3 13.7
		TRBV4-1*01 TRBV9*01 TRBV5-5*01	50.3 20.0 10.2
1	EBNA3C HLA-C*06:02	TRAV27*01	65.4
		TRBV28*01	67.5
2	LMP1+LMP2A+ EBNA1 HLA-C*15:02	TRAV5*01 TRAV1-2*01	46.7 18.7
		TRBV4-1*01 TRBV3-1*01	34.2 19.8
4	EBNA3C HLA-B*44:02	TRAV12-1*01 TRAV8-2*01	55.2 15.8
		TRBV2*01 (1) TRBV2*01 (2)	66.3 22.8

To analyze TRAV and TRBV chain repertoire obtained from TOPO cloning, the cDNA library was cloned into the TOP10 chemically competent E. coli. TRAV and TRBV chains were then isolated and (Sanger) sequenced.

Table 7 depicts the TRAV and TRBV chains isolated from EBV-specific T cell responses to the LMP2A/HLA-A*02:01 and EBNA3C/HLA-B*07:02 combinations, both from the Donor 3. The table shows only TCR chains identified more than two times. All of the TCR chains from the given EBV-specific T cell response were combined for identifying functional TCR

Tab. 7. TRAV and TRBV chains isolated from $CD8^+/CD137^+$ EBV-specific T cells using TOPO cloning.

Donor	EBV antigen/HLA	V segment	V segment frequency	% of total reads
3	LMP2A/A*02:01	TRAV20*01	9/112	8.0
		TRAV12-3*01	6/112	5.3
		TRAV19*01	4/112	3.5
		TRBV10-3*02	13/123	10.5
		TRBV5-1*01	9/123	7.3
		TRBV11-2*01	8/123	6.5
		TRBV9*01	5/123	4.0
		TRBV7-9*01	3/123	2.4
		TRBV27*01	3/123	2.4
3	EBNA3C/B*07:02	TRAV26-2*01	8/30	26.7
		TRAV17*01	3/30	10.0
		TRAV17*01	3/30	10.0
		TRAV26-1*01	2/30	6.7
		TRBV27*01 (1)	6/27	22.2
		TRBV27*01 (2)	4/27	17.8

5.6. Functional analysis of dominant TRA and TRB chain combinations

The dominant TRAV and TRBV chains from TOPO and NGS TCR repertoire analysis were cloned separately into a MP71 vector. Within the vector, the TCR chains were fused to mouse constant regions (mTRAC) as shown in Figure 12A (Cohen et al. 2006). This approach increases the probability of pairing and expression of transgenic TRAV and TRBV chains.

To identify functional EBV-antigen specific TCRs, human PBLs were transduced with various combinations of the dominant TRAV and TRBV chains from the given EBV-specific T cell response. After transduction, the PBLs were stained for surface expression of CD8 and mTRAC. The total transduction efficiency was measured by assessing the cell fractions expressing mTRAC. However, for future functional TCR analyses, only CD8 and mTRAC expressing cells were used in the experiments.

Figure 12B indicates the transduction efficiency of three TRAV/TRBV chain combinations isolated from T cells that responded to the dLMP1, LMP2A, EBNA1/HLA-A*02:01; nine TRAV/TRBV chain combinations isolated from T cells that responded to the dLMP1, LMP2A, EBNA1/HLA-B*57:01 and one TRAV/TRBV chain combination isolated from T cells that responded to the EBNA3C/HLA-C*06:02. The lowest transduction efficiency was 6.84% for TRAV27*01/TRBV28*01 chain

combination from T cells that responded to the EBNA3C/HLA-C*06:02. The highest transduction efficiency was 24% for TRAV12-3*01/TRBV9*01 chain combination from T cells that responded to the dLMP1, LMP2A, EBNA1/HLA- B*57:01.

Next, the transduced T cells were cocultured with the corresponding K562 cells from single-HLA cell library. Before coculture, A*02:01-K562 and B*57:01-K562 cell lines were elecroporated with dLMP1, LMP2A and EBNA1 antigens, and the C*06:02-K562 cell line was electroporated only with EBNA3C antigen. The T cell response was analyzed by measuring the IFNγ secretion.

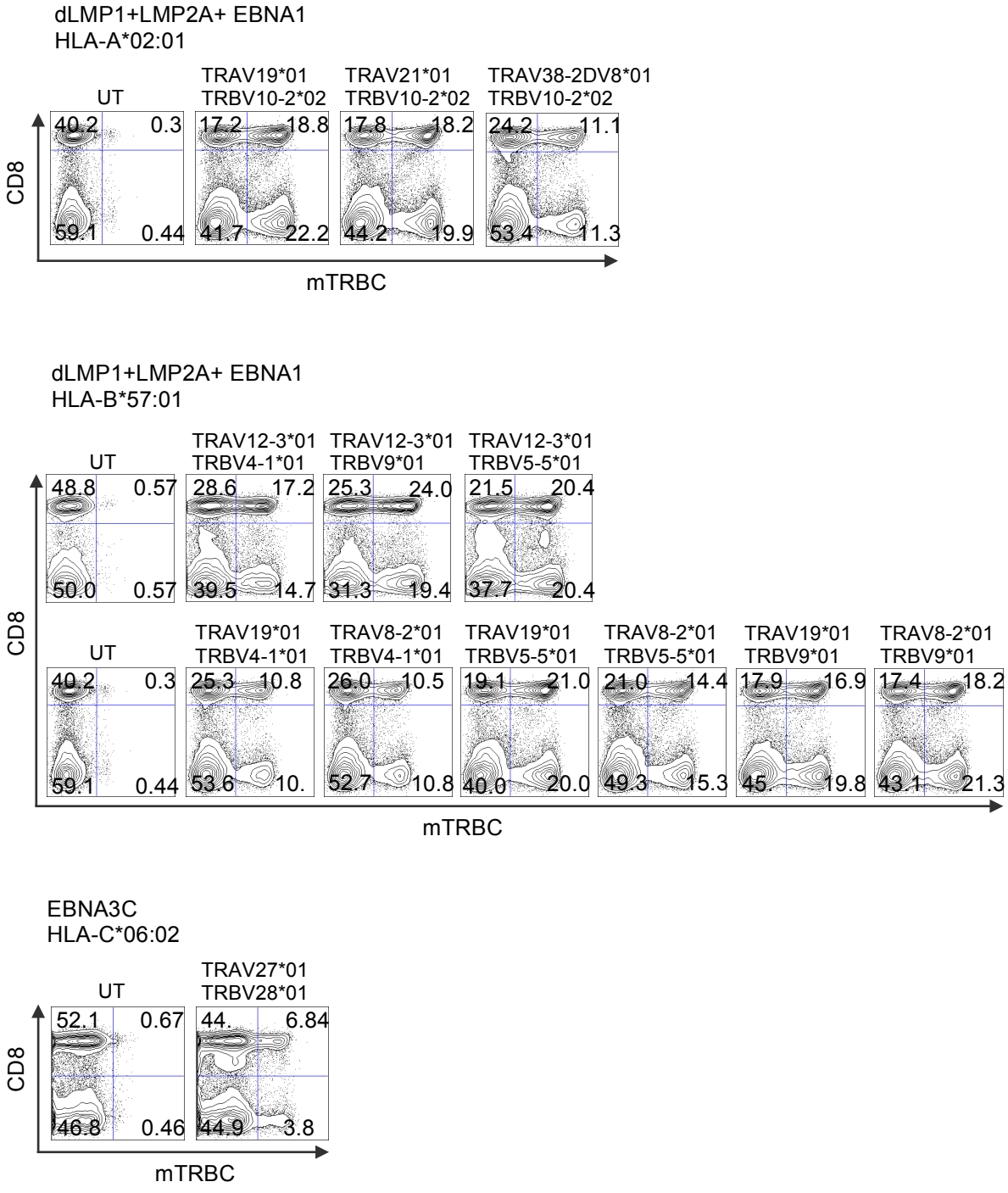
Figure 12C indicates three combinations of TRAV/TRBV chains that responded positively and specifically to the different EBV antigen/HLA complexes. The first response was to the LMP2A/HLA-A*02:01 complex, which was recognized by the TRAV21*01/TRBV10-2*02 chain combination (1218.59 pg/ml).

The second response was the dLMP1/HLA-B*57:01 complex, which was recognized by TRAV8-2*01/TRBV9*01 chain combination (978.02 pg/ml). Here, TRAV/TRBV chain combinations that responded positively but not specifically (recognized more than one EBV antigen) were considered as unspecific and excluded from further study. The last response was to the EBNA3C/HLA-C*06:02 complex that was recognized by TRAV27*01/TRBV28*01 chain combination (2113.80 pg/ml). Functional TRAV/TRBV chain combinations from all donors were depicted in Table 8, where bold numbers indicate positive responses to the given EBV antigen/HLA complexes. Minuses in the table indicate that the given EBV antigens were not use in the functional assay. The functional combinations of TRAV/TRBV chains were used to generate EBV-specific TCR cassettes.

Donor 1

A

B


dLMP1+LMP2A+ EBNA1
HLA-A*02:01
UT
TRAV19*01
TRBV10-2*02
TRAV21*01
TRBV10-2*02
TRAV38-2DV8*01
TRBV10-2*02
40.2
0.3
59.1
0.44
17.2
18.8
41.7
22.2
17.8
18.2
44.2
19.9
24.2
11.1
53.4
11.3
CD8
mTRBC
dLMP1+LMP2A+ EBNA1
HLA-B*57:01
UT
TRAV12-3*01
TRBV4-1*01
TRAV12-3*01
TRBV9*01
TRAV12-3*01
TRBV5-5*01
48.8
0.57
50.0
0.57
28.6
17.2
39.5
14.7
25.3
24.0
31.3
19.4
21.5
20.4
37.7
20.4
UT
TRAV19*01
TRBV4-1*01
TRAV8-2*01
TRBV4-1*01
TRAV19*01
TRBV5-5*01
TRAV8-2*01
TRBV5-5*01
TRAV19*01
TRBV9*01
TRAV8-2*01
TRBV9*01
40.2
0.3
59.1
0.44
25.3
10.8
53.6
10.
26.0
10.5
52.7
10.8
19.1
21.0
40.0
20.0
21.0
14.4
49.3
15.3
17.9
16.9
45.
19.8
17.4
18.2
43.1
21.3
CD8
mTRBC
EBNA3C
HLA-C*06:02
UT
TRAV27*01
TRBV28*01
52.1
0.67
46.8
0.46
44.
6.84
44.9
3.8
CD8
mTRBC

C

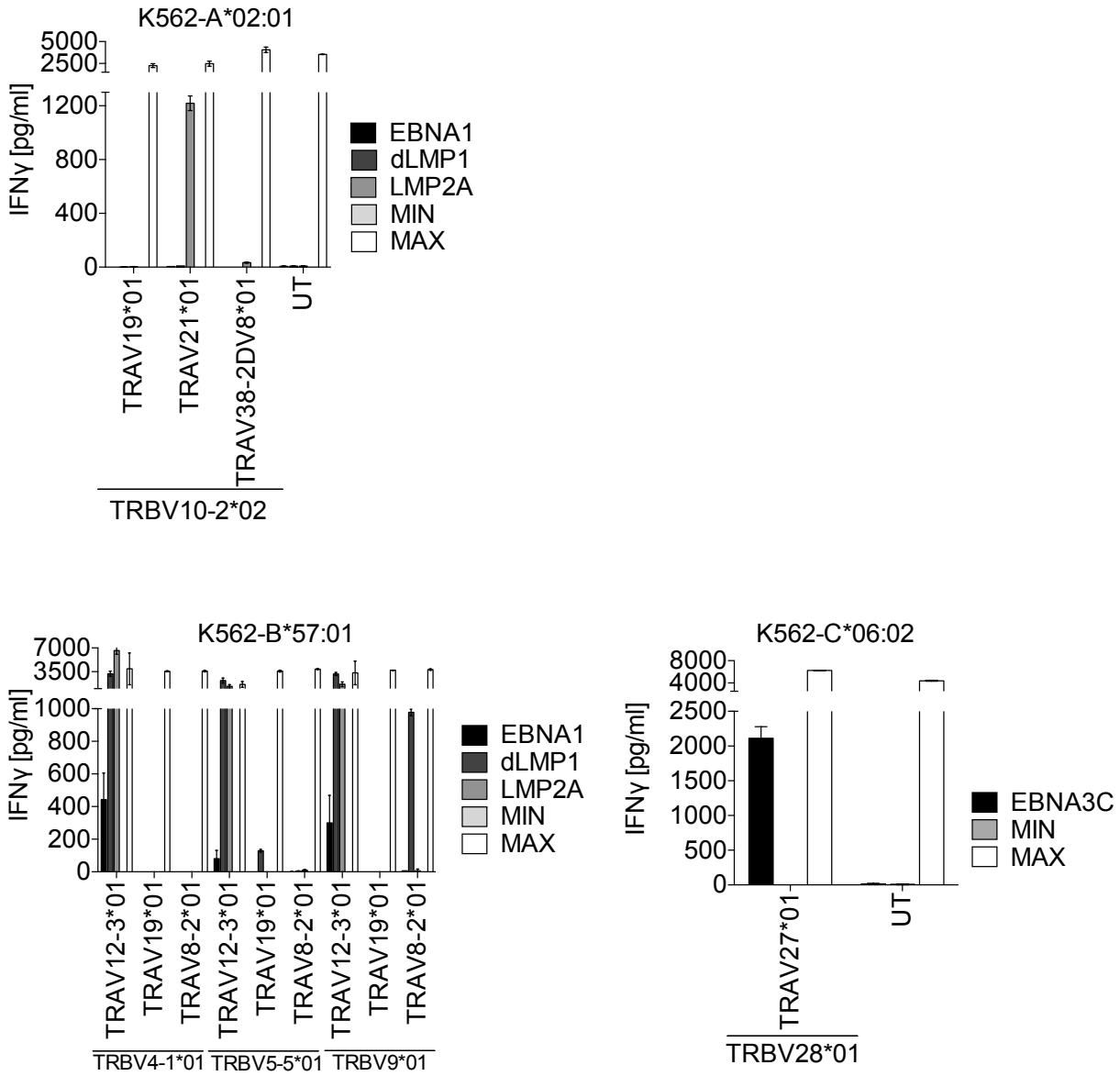


Fig. 12. EBV antigen/HLA combinations were positively and specifically recognized by functional combinations of TRAV/TRBV chains.

PBLs were endowed with different combinations of TRAV and TRBV chains from the NGS analysis. The transduced PBLs were cocultured with corresponding K562 cells from single-HLA cell library. Before coculture, the K562 cells were electroporated with 10 µg DNA of EBV-encoding antigens and 10 µg DNA coding for CD80. Effector to target cell ratio was 2:1 ($5x10^4$:$2.5x10^4$). After overnight coculture (>16 h), IFNγ secretion was measured by ELISA. (A) Dominant TRAV and TRBV chains fused to mTRAC and cloned into the MP71 vector. (B) Transduction efficiency of dominant TRAV/TRBV chain combinations. (C) Functional and EBV specific combinations of TRAV / TRBV chains. LTR: long terminal repeat, Ψ: retroviral psi packaging element; UT: untransduced.

Tab. 8. Functional and EBV-specific combinations of TRAV/TRBV chains isolated using TOPO cloning and NGS.

TCR	Donor	EBV antigen/ HLA combination	TRAV/TRBV chain combination	Transduction efficiency of CD8/mTRBC (%)	Secretion of IFNγ (pg/ml)					
					dLMP1	LMP2A	EBNA1	EBNA3C	MIN	MAX
TCR 6	1	dLMP1+LMP2A+ EBNA1 HLA-A*02:01	TRAV21*01 TRBV10-2*02	18.2	9.31	**1218.59**	5.07	-	0	2475.59
TCR 50	1	dLMP1+LMP2A+ EBNA1 HLA-B*57:01	TRAV8-2*01 TRBV9*01	18.2	**978.03**	6.47	6.47	-	0	3821.43
TCR 64	1	EBNA3C HLA-C*06:02	TRAV27*01 TRBV28*01	6.84	-	-	-	**2113.80**	0	6243.80
TCR 83	2	dLMP1+LMP2A+ EBNA1 HLA-C*15:02	TRAV1-2*01 TRBV3-1*01	11.0	**11251.98**	262.69	77.63	-	0	2888.21
SCDR3	3	LMP2A HLA-A*02:01	TRAV12-3*01 TRBV11-2*01	6.69	-	**2307.27**	-	-	0	6030.02
TCR h27	3	EBNA3C HLA-B*07:02	TRAV26-2*01 TRBV27*01 (1)	14.4	-	-	-	**414.65**	0	3759.11
TCR 27_01	3	EBNA3C HLA-B*07:02	TRAV26-2*01 TRBV27*01 (2)	8.31	-	-	-	**2476.56**	0	2210.29
TCR 25	4	EBNA3C HLA-B*44:02	TRAV8-2*01 TRBV2*01 (2)	6.55	-	-	-	**857.78**	0	1606.28
TCR 58	4	EBNA3C HLA-B*44:02	TRAV12-1*01 TRBV2*01 (1)	8.96	-	-	-	**1171.09**	0	6478.34

5.7. Functional analysis of EBV-specific TCR-engineered T cells

The functional TRAV/TRBV chain combinations were used to generate EBV-specific TCR cassettes. Within the cassette, the chains were combined as follows; TRBV chain, P2A self-cleaving peptides and TRAV chain (Figure 13A). This rearrangement ensures the most efficient expression of TRA and TRB chains on the T cell surface (Leisegang et al. 2008).

The TCR cassettes were cloned into the MP71 vector and used to transduce CD8-positive T cells. Donors of the CD8-positive T cells did not carry HLAs recognized by the tested TCRs and/or were EBV negative. This selection of donors helps to avoid unspecific T cell stimulation and response.

To analyze functionality of EBV-specific TCRs, the TCR-engineered T cells were cocultured with EBV-positive cell lines that were divided into three groups. The first group, consist of K562 cell lines, which were additionally endowed with desired HLAs and EBV antigens. The second group, consist of cancer derived cell lines (L591 and RPMI6666) which naturally expressed EBV antigens. However, in most cases, they were additionally endowed with desired HLAs. The third group consist of LCLs which naturally expressed desired HLAs and EBV antigens.

After overnight coculture, the T cell responses were analyzed by measuring IFNγ secretion using ELISA.

Figure 13B indicates a positive response of the TCR6-engineered T cells to the K562+A*02:01+LMP2A cells (3568.29 pg/ml), two cancer derived cell lines (RPMI6666 (620.73 pg/ml), L591+A*02:01 (2179.0 pg/ml)) and two LCLs (BSM (961.20 pg/ml), EK (2263.79 pg/ml)). The TCR6-engineered T cells did not recognize the K562+A*02:01 cells (157.50 pg/ml) used as control. Figure 13C indicates a positive response of the TCR50-engineered T cells to the K562+B*57:01+dLMP1 cells (1351.79 pg/ml), one cancer derived cell line L591+B*57:01 (1106.03 pg/ml) and two LCLs (WIN (656.92 pg/ml), DEM (298.55 pg/ml)), while K562+B*57:01 control cells (14.22 pg/ml) and RPMI6666+B*57:01 (117.14 pg/ml) were not recognized. Figure 13D indicates a positive response of the TCR64-engineered T cells to the K562+C*06:02+EBNA3C cells (4754.54 pg/ml), two cancer derived cell lines (RPMI6666+C*06:02 (278.42 pg/ml), L591+C*06:02 (2458.42 pg/ml)) and two LCLs (WIN (1094.31 pg/ml), KAS011 (511.60 pg/ml)). The TCR64-engineered T cells did not recognize the K562+C*06:02 control cells (0.0 pg/ml). The untransduced T cells did not indicate a significant response to any of the tested EBV-positive cell

lines compared to the TCR-engineered T cells. All functional EBV-specific TCRs from all donors were depicted in the Table 9.

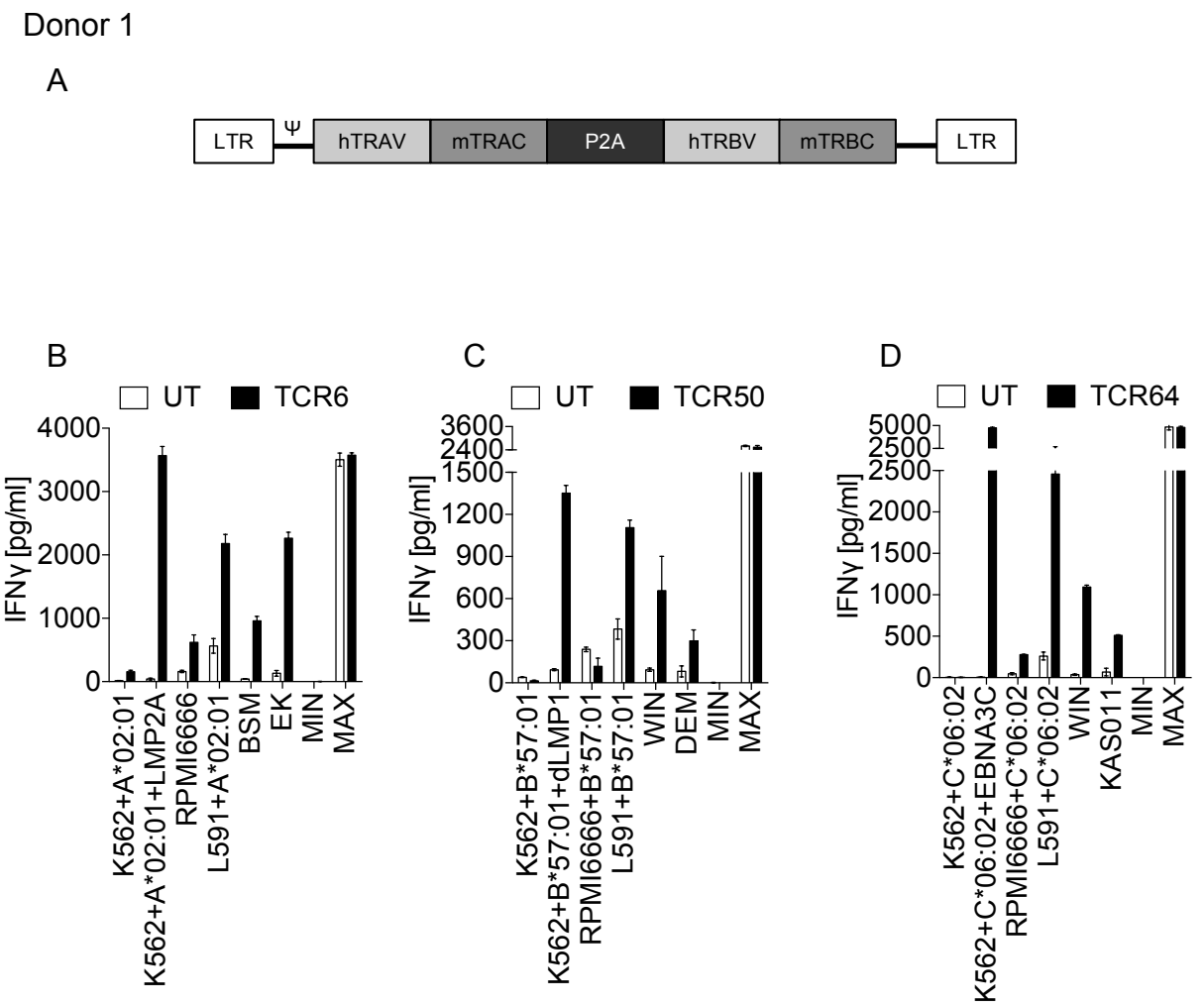


Fig. 13. The EBV-specific TCR-engineered T cells recognized EBV-positive cell lines. EBV-specific TCRs were transduced into CD8-positive T cells and cocultured with EBV-positive cell lines (K562; cancer derived cell lines: RPMI666 and L591; LCLs: BSM, EK, WIN, KAS011, DEM). Effector to target cell ratio was 1:1 ($2x10^4$ ($CD8^+$/mCb^+):$2x10^4$). Effector cell number was calculated according to the $CD8^+$/mCb^+ transduction efficiency. After overnight coculture (>16 h), IFNγ secretion was measured by ELISA. Untransduced (UT) T cells were used as a negative control. Assays were repeated using another donors with similar results. The data show means ± s.d. (A) TCR-cassette cloned into MP71 vector. (B-D) Functional assay of EBV-specific TCR-engineered T cells with EBV-positive cell lines.

Tab. 9. Recognition of target cell lines by EBV-specific TCRs.

TCR	Donor	EBV antigen/ HLA combination	Target cell lines	IFNγ secretion (pg/ml) by TCR-engineered T cells	IFNγ secretion (pg/ml) by UT-T cells	EBV^+ cell line recognition
TCR 6	1	LMP2A	K562+A*02:01	157.50	13.78	-
		HLA-A*02:01	K562+A*02:01+LMP2A	3568.29	41.58	+
			RPMI6666	620.73	159.97	+
			L591+A*02:01	2179.0	563.76	+
			BSM	961.20	42.13	+
			EK	2263.79	132.42	+
TCR 50	1	dLMP1	K562+B*57:01	14.22	39.71	-
		HLA-B*57:01	K562+B*57:01+dLMP1	1351.79	93.39	+
			RPMI6666+B*57:01	117.14	238.49	-
			L591+B*57:01	1106.03	383.83	+
			WIN	656.92	93.71	+
			DEM	298.55	81.78	+
TCR 64	1	EBNA3C	K562+C*06:02	0.0	4.71	-
		HLA-C*06:02	K562+C*06:02+EBNA3C	4754.54	5.38	+
			RPMI6666+C*06:02	278.42	47.23	+
			L591+C*06:02	2458.42	260.91	+
			WIN	1094.31	36.40	+
			KAS011	511.60	67.66	+
TCR 83	2	dLMP1	K562+C*15:02	3.73	2.80	-
		HLA-C*15:02	K562+C*15:02+dLMP1	2668.12	10.73	+
			L591+C*15:02	582.0	275.58	+

SCDR3	3	LMP2A HLA-A*02:01	K562+A*02:01 K562+A*02:01+LMP2A RPMI6666 L591+A*02:01 BSM EK	56.73 3570.72 398.78 1595.59 342.6 1200.77	13.78 41.58 159.97 563.76 42.13 132.43	- + + + + +
TCR h27	3	EBNA3C HLA-B*07:02	K562+B*07:02 K562+B*07:02+EBNA3C RPMI6666 L591+B*07:02 SA HO104	9.81 383.36 332.67 1471.08 400.37 256.26	5.17 5.60 74.50 264.04 36.50 14.84	- + + + + +
TCR 27_01	3	EBNA3C HLA-B*07:02	K562+B*07:02 K562+B*07:02+EBNA3C RPMI6666 L591+B*07:02 SA HO104	16.12 2850.78 1018.86 2495.63 586.93 1896.95	5.17 5.60 74.50 264.04 36.50 14.84	- + + + + +
TCR 25	4	EBNA3C HLA-B*44:02	K562+B*44:02 K562+B*44:02+EBNA3C EK	0.0 765.19 858.35	0.0 1.18 99.61	- + +
TCR 58	4	EBNA3C HLA-B*44:02	K562+B*44:02 K562+B*44:02+EBNA3C EK	0.0 2757.01 859.59	0.0 0.0 225.14	- + +

5.8. Identification of epitopes for EBV-specific TCR-engineered T cells

5.8.1. Identification of TCR binding sites on EBV antigens (epitope mapping)

The identified EBV-specific TCRs were isolated from T cells of EBV-positive donors after stimulation with full-length EBV antigens. Thus, epitopes recognized by these TCRs are unknown. To identify EBV-specific epitopes for given TCRs, the dLMP1, LMP2A and EBNA3C antigens were C-terminal truncated and cloned into the pcDNA3.1 vector as shown in figures 14A-16A. This approach reduces the range of the epitope search to a small part of the antigen. Next, K562 cells from the single-HLA cell library were electroporated with the corresponding full-length EBV antigens and their truncated versions. The electroporated K562-HLA cell lines were cocultured with corresponding TCR-engineered T cells. After overnight coculture, T cell responses were analyzed by measuring an IFNγ secretion using ELISA.

Figure 14B indicates a positive response of the TCR6-engineered T cells only to the full-length LMP2A (3270.36 pg/ml). It means that the TCR6-specific epitope is located between nt 1001-1494 of the LMP2A antigen. Figure 15B indicates a positive response of the TCR50-engineered T cells to the full-length dLMP1 (1638.32 pg/ml) and dLMP1/2 (753.77 pg/ml). It means that the TCR50-specific epitope is located between nt 315-624 of the dLMP1 antigen. Figure 16B indicates a positive response of the TCR64-engineered T cells to the full-length EBNA3C (107.7 pg/ml), EBNA3C/3 (372.54 pg/ml) and EBNA3C/2 (268.62 pg/ml). Its means that the TCR64-specific epitope is located between nt 567-1569 of the EBNA3C antigen. More details of epitope mapping for all TCRs are depicted in the Tables 10-12.

TCR 6

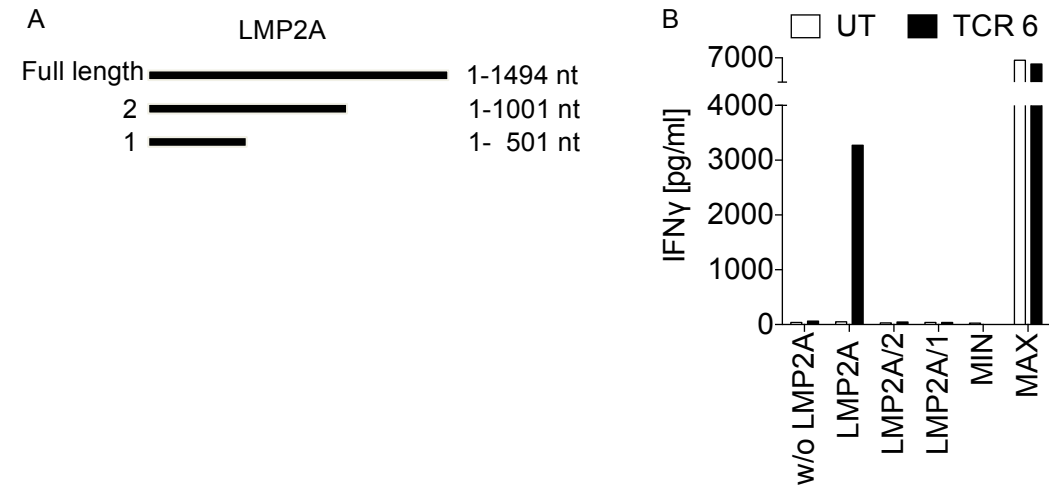


Fig. 14. TCR6-engineered T cells recognized full-length of LMP2A antigen. (A) Truncated versions of LMP2A antigen were cloned into pcDNA3.1 vector. (B) TCR6-engineered T cells were cocultured with full-length and truncated versions of LMP2A antigen. Effector to target cell ratio was 2:1 ($5x10^4$:$2.5x10^4$). After overnight coculture (>16 h), IFNγ secretion was measured by ELISA. Untransduced (UT) T cells were used as a negative control.

Tab. 10. Epitope localization in the LMP2A antigen for given LMP2A-specific TCRs.

TCR	Secretion of IFNγ (pg/ml)					
	w/o LMP2A	LMP2A	LMP2A/2	LMP2A/1	MIN	MAX
TCR 6	60.89	**3270.36**	41.41	34.92	0	6205.2
SCDR3	This TCR did not require epitope mapping. The epitope was found in a peptide loading experiment.					

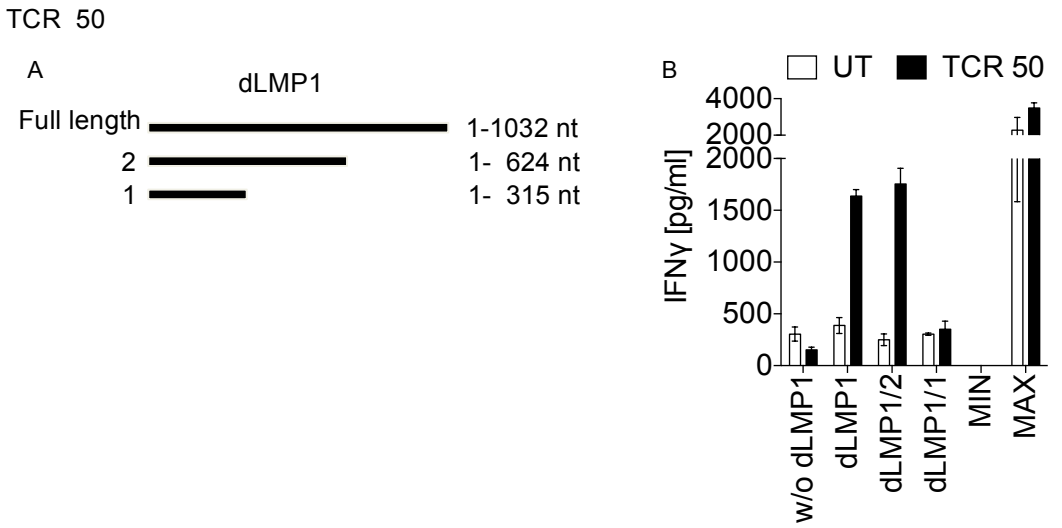


Fig. 15. TCR50-engineered T cells recognized full-length and truncated version 2 of dLMP1 antigen. (A) Truncated versions of dLMP1 antigen were cloned into pcDNA3.1 vector. (B) TCR50-engineered T cells were cocultured with full-length and truncated versions of dLMP1 antigen. Effector to target cell ratio was 2:1 ($5x10^4$:$2.5x10^4$). After overnight coculture (>16 h), IFNγ secretion was measured by ELISA. Untransduced (UT) T cells were used as a negative control.

Tab. 11. Epitope localization in the dLMP1 antigen for given dLMP1-specific TCRs.

TCR	Secretion of IFNγ (pg/ml)					
	w/o dLMP1	dLMP1	dLMP1/2	dLMP1/1	MIN	MAX
TCR 50	152.18	**1638.32**	**1753.77**	351.84	0	3487.98
TCR 83	21.19	**2847.06**	**3485.3**	26.64	0	1479.75

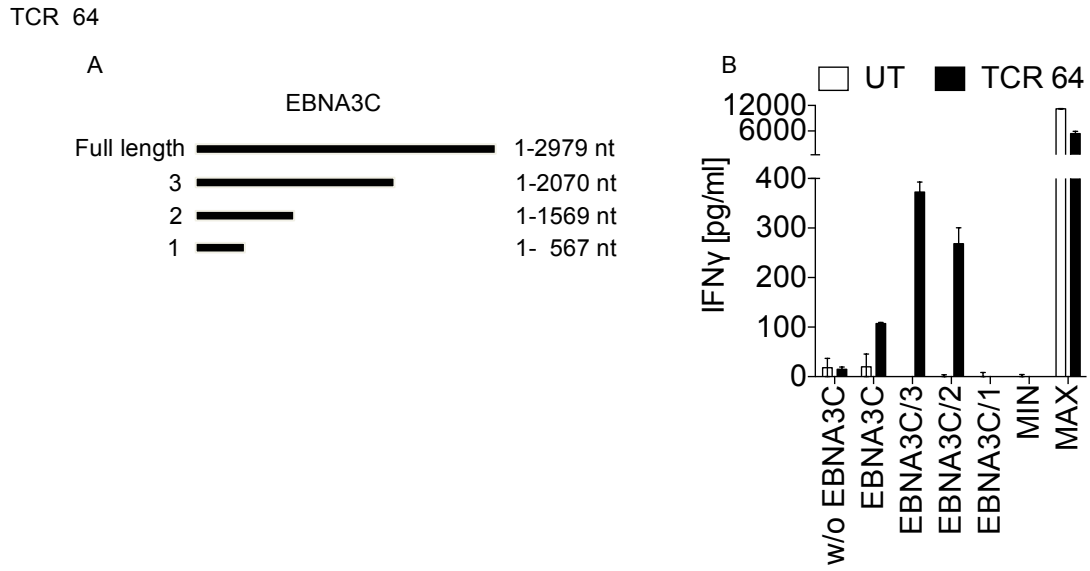


Fig. 16. TCR64-engineered T cells recognized full-length and truncated version 3 and 2 of EBNA3C antigen. (A) Truncated versions of EBNA3C antigen were cloned into pcDNA3.1 vector. (B) TCR64-engineered T cells were cocultured with full-length and truncated versions of EBNA3C antigen. Effector to target cell ratio was 2:1 ($5x10^4$:$2.5x10^4$). After overnight coculture (>16 h), IFNγ secretion was measured by ELISA. Untransduced (UT) T cells were used as a negative control.

Tab. 12. Epitope localization in the EBNA3C antigen for given EBNA3C-specific TCRs. Bold numbers indicate positive responses.

TCR	Secretion of IFNγ (pg/ml)						
	EBNA3C w/o	EBNA3C	EBNA3C /3	EBNA3C /2	EBNA3C /1	MIN	MAX
TCR 64	14.91	**107.7**	**372.54**	**268.62**	0	0	5417.34
TCR h27	-	**2290.32**	248.97	232.42	294.07	0	4978.89

TCR 27_01	-	**2836.07**	104.30	104.30	104.30	0	4797.86
TCR 25	-	**487.89**	**1529.72**	**1528.93**	**1746.39**	0	1609.04
TCR 58	-	**1490.34**	**3300.21**	**3287.55**	**4058.27**	0	5551.91

5.8.2. Prediction of immunogenic epitope sequences for EBV-specific TCRs

Located regions of immunogenic EBV epitopes were used to determine correct epitope sequences for given TCRs using NetMHC 4.0 epitope prediction server.

Figures 17A-19A depict lists of the predicted epitopes for TCR6, TCR50 and TCR64. The tables contain length of the epitopes, TCR affinity for the peptide/HLA class I in nanomolar (nM) and binding level.

To identify immunogenic EBV-specific epitopes for given TCRs, the appropriate K562 cells from single-HLA cell library were loaded with the corresponding peptides at the concentration of 10^{-6} μM. Loaded K562 cell lines were cocultured with corresponding TCR-engineered T cells. After overnight coculture, the T cell responses were analyzed by measuring the IFNγ secretion using ELISA.

Figure 17B indicates a positive response of the TCR6-engineered T cells to two out of 14 epitopes. First response was to MCLGGLLTMV (3716.12 pg/ml) and second respond was to CLGGLLTMV (3182.45 pg/ml). Figure 18B indicates a positive response of the TCR50-engineered T cells to four out of 13 epitopes. Positive responses were identified for IALYLQQNWW (1608.08 pg/ml), IALYLQQNW (1633.89 pg/ml), IIALYLQQNW (1680.71 pg/ml), and ALYLQQNWW (623.47 pg/ml). Figure 19B indicates a positive response of the TCR64-engineered T cells to one out of 27 epitopes. The response was to FRKAQIQGL (713.46 pg/ml). EBV-specific epitopes of other TCRs are depicted in Table 13.

A

Epitope	Length (-mer)	Affinity (nM)	Bind level
FMCLGGLLTM	10	13.92	SB
MLLLIVAGI	9	23.94	SB
NLFCMLLLI	9	28.54	SB
LLIVAGILFI	10	30.70	SB
NLFCMLLLIV	10	35.77	SB
MCLGGLLTMV	**10**	37.39	**SB**
CLGGLLTMV	**9**	75.60	**WB**
LIVAGILFI	9	78.80	WB
FIPNLFCML	9	88.29	WB
IVAGILFIL	9	94.09	WB
MLLLIVAGIL	10	102.10	WB
CMLLLIVAGI	10	107.92	WB
PNLFCMLLLI	10	151.79	WB
FIPNLFCMLL	10	154.14	WB

B

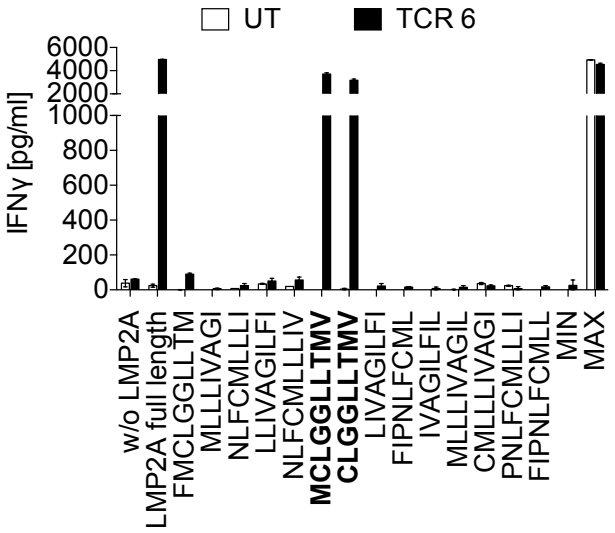


Fig. 17. TCR6-engineered T cells recognize two LMP2A-predicted peptides. (A) List of predicted peptides from LMP2A regions recognized by TCR6. (B) TCR6-engineered T cells were cocultured with the A*02:01-K562 cell line loaded with corresponding peptides at the concentration of $1x10^{-6}$ μM. Effector to target cell ratio was 1:1 ($2x10^4$:$2x10^4$). After overnight coculture (>16 h), IFNγ secretion was measured by ELISA. Untransduced (UT) T cells were used as a negative control. Bind levels: strong binder (SB) and weak binder (WB).

A

Epitope	Length (-mer)	Affinity (nM)	Bind level
WTLLVDLLW	9	12.25	SB
IALYLQQNWW	10	14.84	SB
IALYLQQNW	9	16.32	SB
WWTLLVDLLW	10	32.51	SB
IIALYLQQNW	10	57.84	SB
WTLLVDLLWL	10	111.95	SB
LAILIWMYY	9	231.02	SB
LLFLAILIW	9	234.00	SB
LLLFLAILIW	10	749.75	WB
LAILIWMYYH	10	789.96	WB
ALYLQQNWW	9	853.78	WB
FLAILIWMYY	10	1515.23	WB
NSNEGRHHL	9	2236.90	WB

B

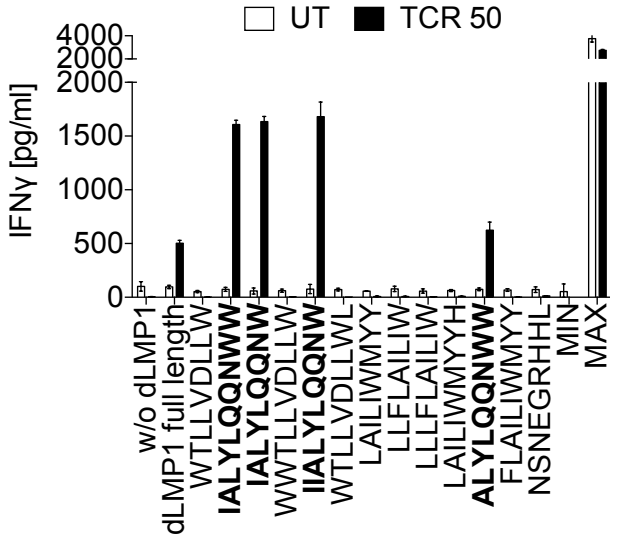


Fig. 18. TCR50-engineered T cells recognize four out of 13 predicted peptides. (A) List of predicted peptides from dLMP1 regions recognized by TCR50. (B) TCR50-engineered T cells were cocultured with the B*57:01-K562 cell line loaded with corresponding peptides at the concentration of 10^{-6} μM. Effector to target cell ratio was 1:1 ($2x10^4$:$2x10^4$). After overnight coculture (>16 h), IFNγ secretion was measured by ELISA. Untransduced (UT) T cells were used as a negative control. Bind levels: strong binder (SB) and weak binder (WB).

A

Epitope	Length (-mer)	Affinity (nM)	Bind level
RRYRRIYDL	9	42.19	SB
FRKAQIQGL	**9**	70.21	**SB**
AREAEVRFL	9	150.90	SB
LRGKWQRRY	9	154.87	SB
ERYAREAEV	9	194.73	SB
SRRRRGACV	9	561.86	SB
NLLDFVRFM	9	644.16	SB
RRIYDLIEL	9	695.45	SB
RRRRGACVV	9	828.70	SB
VRFLRGKWQ	9	966.12	SB
RRRGACVVY	9	1220.16	WB
QRRYRRIYD	9	1447.20	WB
VRFMGVMSS	9	1479.77	WB
YAREAEVRFL	10	1602.13	WB
NRVGADSIM	9	1849.33	WB
LHHIWQNLL	9	1893.43	WB
RRGIKEHVI	9	2063.06	WB
YRRIYDLIE	9	2320.12	WB
RRYRRIYDLI	10	2493.23	WB
ARRGIKEHV	9	2832.43	WB
QRRYRRIYDL	10	2849.15	WB
WQRRYRRIY	9	3037.39	WB
FLRGKWQRRY	10	3388.28	WB
RRGACVVYD	9	3495.79	WB
VYDDDVIEV	9	4973.38	WB
YAREAEVRF	9	5274.33	WB
GCQNAARTL	9	5591.13	WB

B

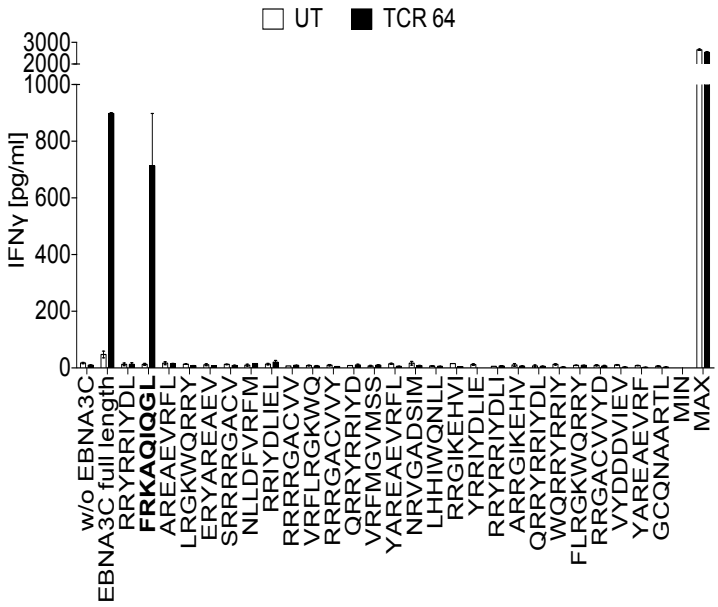


Fig. 19. TCR64-engineered T cells recognize one out of 27 predicted peptides. (A) List of predicted peptides from EBNA3C regions recognized by TCR64. (B) TCR64-engineered T cells were cocultured with the C*06:02-K562 cell line loaded with corresponding peptides at the concentration of 10^{-6} μM. Effector to target cell ratio was 1:1 ($2x10^4$:$2x10^4$). After overnight coculture (>16 h), IFNγ

secretion was measured by ELISA. Untransduced (UT) T cells were used as a negative control. Bind levels: strong binder (SB) and weak binder (WB).

Tab. 13. Epitopes recognized by EBV-specific TCRs.

TCR	TCR-specific epitope	Length (-mer)	Affinity (nM)	NetMHC epitopes ranking	Bind level	½ -max IFNγ release
TCR 6	MCLGGLLTMV	10	37.39	6	SB	$6x10^{-9}$
	CLGGLLTMV	9	75.60	7	WB	$6x10^{-9}$
TCR 50	IALYLQQNWW	10	14.84	2	SB	$2x10^{-7}$
	IALYLQQNW	9	16.32	3	SB	$3x10^{-8}$
	IIALYLQQNW	10	57.84	5	SB	$1x10^{-8}$
	ALYLQQNWW	9	853.78	11	WB	$7x10^{-6}$
TCR 64	FRKAQIQGL	9	70.21	2	SB	$7x10^{-6}$
TCR 83	QQNWWTLLV	9	974.07	2	WB	$2x10^{-9}$
SCDR3	CLGGLLTMV	9	75.60	-	WB	$4x10^{-8}$
TCR h27	SPQPRAPIRPIP	13	96.46	6	SB	$7x10^{-8}$
	QPRAPIRPIP	10	102.53	8	SB	$4x10^{-8}$
	QPRAPIRPIPT	11	1018.57	26	WB	$5x10^{-8}$
TCR 27_01	SPQPRAPIRPIP	13	96.46	6	SB	$6x10^{-8}$
	QPRAPIRPIP	10	102.53	8	SB	$4x10^{-8}$
	QPRAPIRPIPT	11	1018.57	26	WB	$6x10^{-8}$
TCR 25	AEGGVGWRHW	10	12.74	1	SB	$6x10^{-7}$
TCR 58	AEGGVGWRHW	10	12.74	1	SB	$3x10^{-7}$

5.8.3. Analysis of TCR sensitivity to peptides (peptide titration)

EBV-specific TCR sensitivity to the corresponding peptide/HLA class I combinations were performed by challenging the TCRs with titrated amounts of peptides. To do this, appropriate K562 cells from single-HLA cell library were loaded with the corresponding titrated amounts of peptides ($1x10^{-6}$-$1x10^{-13}$ μM). Loaded K562 cell lines were cocultured with corresponding TCR-engineered T cells. After overnight coculture, the T cell responses were analyzed by measuring the IFNγ secretion using ELISA. The TCR sensitivity to given peptide/HLA combination was estimated by measuring the half-maximum IFNγ release. Figure 20A indicates sensitivity of the TCR6 to CLGGLLTMV and MCLGGLLTMV, with the half-maximum IFNγ release at $6x10^{-9}$ μM for both. Figure 20B indicates sensitivity of the TCR50 to four peptides. Two of the peptides were recognized by the TCR50 at low peptide concentration. These were IALYLQQNW and IIALYLQQNW with the half-maximum IFNγ release at

$3x10^{-8}$ and $1x10^{-8}$ μM, respectively. Last two were recognized by the TCR50 at higher peptide concentration. These were IALYLQQNWW and ALYLQQNWW with the half-maximum IFNγ release at $2x10^{-7}$ and $7x10^{-6}$ μM, respectively. Figure 20C indicates sensitivity of the TCR64 to the FRKAQIQGL, with the half-maximum IFNγ release at $7x10^{-6}$ μM. Untransduced T cells did not recognize peptide at any concentration. The half-maximum IFNγ releases of other TCRs are depicted in Table 13. Epitopes that were recognized at the lowest peptide concentration were considered the most specific for given TCRs.

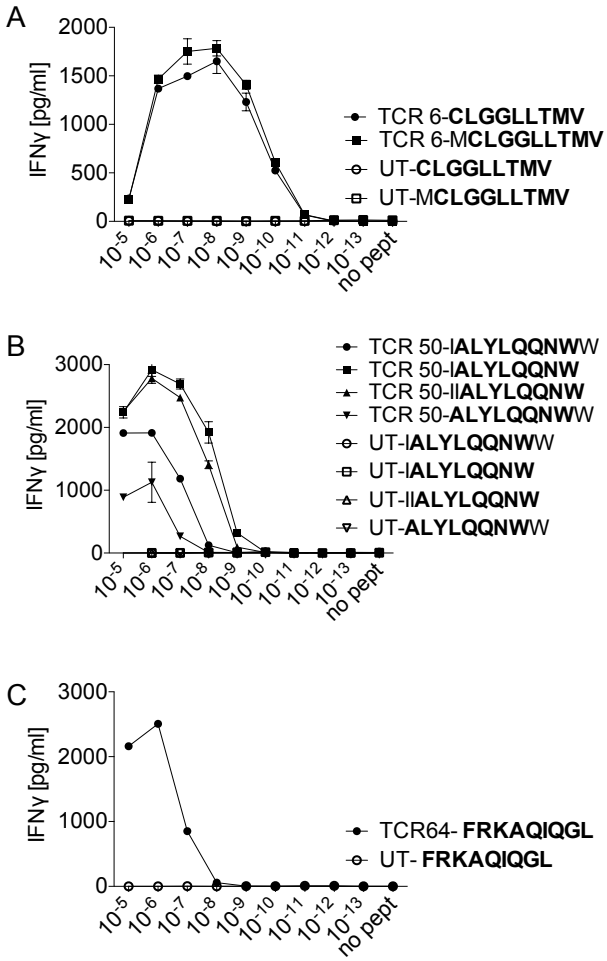


Fig. 20. EBV-specific TCR sensitivity is different for each peptide/HLA complexes.

TCR-engineered T cells were cocultured with peptide loaded single-HLA cell lines. Before coculture, the single-HLA cell lines were loaded with titrated amounts of peptides ($1x10^{-6}$-$1x10^{-13}$ μM). Effector to target cell ratio was 1:1 ($1x10^{4}$:$1x10^{4}$). After overnight coculture (>16 h), the IFNγ secretion was measured by ELISA. The TCRs sensitivity to the given peptide/HLA complexes were estimated by measuring the half-maximum IFNγ release. Untransduced (UT) T cells were used as a negative control.

6. Discussion

TCR gene therapy is a promising immunotherapy in the fight against cancer. Clinical trials using this therapy showed positive results in treatment of melanoma and synovial cell sarcoma patients, targeting antigens such as MART-1 and NY-ESO-1, respectively (Johnson et al. 2009), and (Robbins et al. 2011).

However, the complexity of identifying cancer antigens and isolating cancer-specific TCRs limits this therapy of cancer treatment. Moreover, the current focus on TCRs that recognize antigens presented only on the most common HLA types eliminates many patients from clinical trials. To overcome these problems, in this PhD study, the TCR detection and isolation platform developed in our laboratory was used to generate an EBV-specific TCR library, facilitating TCRs implementation in the clinic.

In total, nine EBV-specific TCRs were isolated and characterized (Table 14). Two LMP1-specific TCRs restricted to the HLA-B*57:01 (TCR50) and HLA-C*15:02 (TCR83). Two LMP2A-specific TCRs restricted to the HLA-A*02:01 (TCR6 and SCDR3) and five EBNA3C-specific TCRs restricted to the HLA-B*07:02 (TCRh27 and TCR27_01), HLA-B*44:02 (TCR25 and TCR58), and HLA-C*06:02 (TCR64).

Of all the TCRs, eight were newly discovered. The exception is the SCDR3 TCR that TRAV/TRBV chains were previously published (Su, Molloy, and LIDDY 2015). However, the SCDR3 TRAV chain differs from the published TRAV chain by one point mutation (c.447S>A) located in the CDR3 region.

The C*15:02-restricted QQNWWTLLV epitope of LMP1 antigen recognized by TCR83 was identified for the first time. In the future, this epitope can therefore be used to stimulate and expand LMP1-specific CTLs or to identify other TCRs using class I pMHC tetramers. Epitopes specific for TCR25, TCR27_01, TCRh27, TCR50, and TCR58 were previously described using revers immunology. In case of TCR6 and SCDR3 they recognize the same CLGGLLTMV epitope as the two published TCRs (Su, Molloy, and LIDDY 2015), and (Stauss, Xue, and Topp 2016). However, the TCR6 has a different TRAV/TRBV chain combination than the published TCRs. Interestingly, the C*06:02-restricted FRKAQIQGL epitope of EBNA3C, recognized by TCR64, was previously shown to be also presented by other HLAs, such as B*08:01, B*14:02, B*27:02, B*27:04, B*27:05 and B*39:0137 (Immune Epitope Database (IEDB)). Thus, one epitope can be used, for example, to isolate TCRs restricted to many HLAs by the pMHC class I tetramers.

Table 14. Summary of EBV-specific TCRs

TCR	EBV antigen/ HLA combination	Recognized epitope	½ max. IFNg	TRAV	TRBV	Recognition of tumor cells
27_01	EBNA3C HLA-B*07:02	QPRAPIRPIP*	$4x10^{-8}$	26-2*01	27*01	+
h27	EBNA3C HLA-B*07:02	QPRAPIRPIP*	$4x10^{-8}$	26-2*01	27*01	+
25	EBNA3C HLA-B*44:02	AEGGVGWRHW	$6x10^{-7}$	8-2*01	2*01	+
58	EBNA3C HLA-B*44:02	AEGGVGWRHW	$3x10^{-7}$	12-1*01	2*01	+
64	EBNA3C HLA-C*06:02	FRKAQIQGL	$7x10^{-6}$	27*01	28*01	+
50	LMP1 HLA-B*57:01	IALYLQQNW*	$3x10^{-8}$	8-2*01	9*01	+
83	LMP1 HLA-C*15:02	QQNWWTLLV	$2x10^{-9}$	1-2*01	3-1*01	+
06	LMP2A HLA-A*02:01	CLGGLLTMV*	$6x10^{-9}$	21*01	10-2*02	+
SCDR3	LMP2A HLA-A*02:01	CLGGLLTMV	$4x10^{-8}$	12-3*01	11-2*01	+

* Most probably true epitope

Functional TCRs can be found by combining dominant TRAV and TRBV chains.

EBV-specific TCRs were generated from dominant TRAV and TRBV chains, which frequency of occurrence exceeded 10% of total V segment reads. Potentially, combinations of TRAV and TRBV chains that were below a set threshold could generate functional EBV-specific TCRs. However, of the nine discovered TCRs, six were generated from a combination of the first two dominant TRAV and TRBV chains. The other three were generated from TRAV and TRBV chains, which frequency of occurrence, at least one of them, was in third position. This statistic suggests that most functional TCRs can be found from a combination of the first three dominant TRAV and TRBV chains. Nonetheless, to avoid losing valuable TCRs, the TRAV and TRBV chains below the set threshold should be examined in the future.

Identification of true epitopes for TCR6, TCRh27, TCR27_01 and TCR50 requires further investigation.

Peptides presented on HLA class I molecules are usually eight to 10 amino acids long. The length of the peptides is restricted by tyrosine residues formed on the edges of the HLA class I grooves, which create hydrogen bounds with the N- and C-terminus of the peptide (Rammensee, Falk, and Rötzschke 1993). However, many studies reported that longer peptides can also bind to HLA class I molecules. The longer peptides can adopt to the HLA peptide-binding groove by bulging in the center or by protrusions (extension of the peptides) at the N- and C-terminus (Stryhn et al. 2000), (Miles et al. 2005), (Ebert et al. 2009), and (Chan et al. 2018). Extensive research of A. Stryhnet et al. (2000) indicated that longer peptides can bind to the HLA class I molecules only when protrusion occurs either at N- or C-terminus but never both at the same time. Additionally, they showed that extension or truncation of the peptide even by one amino acid can abolished T cells stimulation.

In this PhD study, four out of nine EBV-specific TCRs re-expressed in T cells responded to more than one predicted peptide. In each case, these peptides shared the same core motif and differed in length at the N- and/or C-terminus.

An additional methionine (M) at the N-terminus of the CLGGLLTMV epitope did not affect the TCR6 sensitivity to the pHLA, which were the same for both epitopes.

TCRh27 and TCR27_01 recognized three peptides at a similar sensitivity level. Interestingly, two epitopes were expanded at the N-terminus (SPQPRAPIRPIP) or C-terminus (QPRAPIRPIPT) and were longer than 10 amino acids in comparison with the shortest epitope (QPRAPIRPIP). Analysis of the peptide anchor residues using the NetMHC 4.0 Server (usually, these are the second and ninth amino acid of the epitope that specifically bind to the given HLA molecule) revealed that in both cases the second amino acid of the epitopes is proline (P), which is the most preferable anchor residues for HLA-B*07:02. Thus, the true epitope for TCRh27 and TCR27_01 may start with either serine (S) or glutamine (Q), suggesting that these TCRs recognized more than one epitope. Another explanation may be that the longer epitopes become protuberant or bulged in the center to match the specific groove of the HLA-B*07:02 molecule.

TCR50 recognized four epitopes that did not exceed 10 amino acids. However, they differed at the N- and C-terminus. The highest sensitivity of TCR50 was observed only for two out of four examined epitopes presented on HLA-B*57:01 molecules

(IALYLQQNW; IIALYLQQNW). An epitope with an additional tryptophan (W) at the C-terminus (IALYLQQNWW) reduced TCR50 sensitivity by one log down. An epitope without any Isoleucine (I) at the N-terminus (ALYLQQNWW) reduced TCR50 sensitivity by two logs. The most preferred peptide anchor residue for the HLA-B*57:01 molecule is alanine (A) at the second position and W at the ninth position. Only one epitope (IALYLQQNW) meet these requirements. However, the IIALYLQQNW epitope similarly stimulates TCR50-engineered T cells, suggesting that this epitope may become protuberant at the N-terminus or bulged in the center.
Based only on the research from this PhD thesis, it is impossible to identify a true epitope for any of the above-mentioned TCRs. In order to indicate them further research, such as cryptographic analysis, should be done in the future.

EBV-specific TCRs recognized epitopes that were not selected by the NetMHCpan4.0 prediction program as the most immunogenic.

Epitope prediction programs, which algorithms mimic the intracellular pathways of epitope processing and presentation were developed to generate and indicate epitopes with the strongest affinity to the HLA molecules and thus, the most immunogenic (Martini et al. 2020). However, studies demonstrated that these programs also generate immunogenic epitopes, which are not processed *in vivo* and therefore unable to induce T cell response. Here, examples are the only predicted TEL-AML1 epitope (VAYGRQVYL) and the strongest binding $hTERT_{540–548}$ epitope (ILAKFLHWL). Both were able to induce a specific T cell response to the peptides but failed to recognize APCs. Further analysis confirmed that these epitopes were not processed and do not occur on the surface of APCs (Popović et al. 2011), and (Purbhoo et al. 2007).
To overcome this issue, the TCR detection and isolation method, used in this study, was designed to identify antigen-specific TCRs that recognize epitopes in an unbiased manner, that is, without preselected target epitopes. Here, the TCRs recognize epitopes presented by antigen-presenting DCs, which during intracellular processing selected the most suitable epitopes of a given antigen for the corresponding HLA type. This approach ensures not only epitope processability, but also selection of the most suitable epitopes for given TCRs. Indeed, further analysis indicated that seven out of nine EBV-specific TCRs recognized epitopes, which were not ranked at the highest positions in the NetMHCpan4.0 prediction program. The

exceptions are TCR25 and TCR58. Moreover, epitopes recognized by SCDR3, TCR6, TCRh27, TCR27_01, TCR50 and TCR83 that induced high T cell response were classified as weak binders. Thus, epitope prediction programs can provide useful tools to facilitate and narrow the search for immunogenic epitopes, but each predicted epitope should be tested experimentally to find the one that triggers the most positive and strong T cell response.

TCR detection and isolation platform is not appropriate for identifying TAA-specific TCRs.

Optimal TCR affinity to the cancer peptide/HLA complex is the key to the success of ATT. However, T cells with a high affinity for self-antigens (or TAAs) are deleted during thymus selection to avoid autoimmunity and tissue destruction. Thus, the T cells released to the blood periphery possess only a low affinity for TAAs, which was found to be insufficient for the elimination of tumors (Bos et al. 2012), and (Janicki et al. 2008) .

The TCR detection and isolation platform uses CD8-positive T cells from the PBMCs as a source of TCRs that have the low affinity for TAAs. In consequence, this method is insufficient for isolating TCRs with the high affinity for TAAs. However, the virus antigens constitute a foreign source of peptides, which means that T cells were not verified against them during thymus selection. Thus, the TCR isolation method is a perfect tool to isolate TCRs with high affinity to virus antigens.

EBNA1 epitopes can be naturally processed and presented on HLA class I molecules.

EBNA 1 antigen is expressed in all EBV-associated cancers and is the only antigen that is express in Burkitt's lymphoma, making it an attractive target for ATT. But so far, no EBNA1-specific TCR was identified in this PhD study. This is caused by the amino acid sequence of the EBNA1, which contains a Glycine-Alanine repeat domains preventing intracellular processing and presentation on the HLA class I molecules. However, extensive research performed by Rickinson et al. showed that EBNA1 epitopes can induce a specific response of CD8-positive T cells, isolated from patients with EBV-associated diseases. Moreover, they proved that the full-length EBNA1 antigen can be processed and presented on HLA class I molecules

but only when the antigen is delivered exogenously, suggesting an alternative TAP-independent pathway of antigen processing and presentation (Blake et al. 1997).
The endogenous processing and presentation of epitope derived from the full-length EBNA1 antigen was demonstrated by Voo et al. In this case, the EBNA1-specific CD8-positive T cells recognized a HEK293 cell line expressing EBNA1/HLA-B8, 1359mel cancer cell line and LCL 111. Further analysis showed that the recognized HLA-B8–restricted EBNA1 epitope was derived from short-lived defective ribosomal products (DRiPs), where serine proteases degraded EBNA1 DRiPs before further processed by proteasomes (Voo et al. 2004).
Data from both studies suggest that EBNA1 epitopes can be processed and presented on HLA class I molecules of target cells and can induce the CD8-positive T cell response. Therefore, further efforts to identify and isolate EBNA1-specific TCRs should be taken in the future.

Stimulation of T cells with a mDC sample, primed with more than one antigen, increases chances of isolating EBV-specific TCRs.

Identification and isolation of cancer-specific TCRs is very laborious and time consuming. In addition, many screenings of CD8-positive T cells specific for EBV antigen, such as LMP1, failed due to the lack of these T cells in the tested PBMC samples. Therefore, to increase chances of isolating EBV-specific TCRs and to utilize the full potential of the entire CD8-positive T cell population from the PBMC sample, the T cells were stimulated with a mDC sample primed with more than one EBV antigen. This allowed the isolation of TCRs specific for different EBV antigen/HLA combinations in one experiment as shown for TCR06 (LMP2A/A*02:01) and TCR50 (LMP1/B*57:01).

Generation of TCRs specific to more than one EBV antigen can improve elimination of the EBV-associated malignancies.

EBV-associated cancers vary in the expression of different EBV antigen patterns, ranging from BL, where only EBNA 1 is expressed and ending with PTLD, where all EBV latent antigens are expressed. Further studies showed that the expression of EBV antigens can also vary within the same cancer type. In this case, a study of 35 samples from EBV-positive HL patients showed that only 45.7% of the cases expressed both LMP1 and LMP2A antigens, whereas 54.3% of the cases expressed

only LMP1 (Vistarop et al. 2020). Therefore, to improve the recognition and elimination of cancer cells, it is required to identify TCRs specific to more than one EBV antigen. The EBV-specific TCR library consisting of well-defined TCRs restricted to various EBV peptide/HLA complexes may in future not only provide TCRs for patients with rare HLA types, but also enables rapid generation of therapeutic T cells specific to any EBV antigen.

7. Abbreviations

ALL	acute lymphocytic leukemia
allo-HLA	allogeneic HLA molecules
AML	acute myeloid leukemia
Amp	ampicillin
APC	antigen-presenting cell
ATT	adoptive T cell therapy
BCR	B cell receptor
Bim	Bcl-2-interacting mediator of cell death
BL	Burkitt lymphoma
C	constant
CAR	chimeric antigen receptor
CD	cluster of differentiation
CDKI	cyclin dependent kinase inhibitors
cDNA	complementary DNA
CDRs	complementarity-determining regions
CFP	cyan Fluorescent Protein
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
CMV	cytomegalovirus
Cp	C promoter
CT	cancer-testis
CTL	cytotoxic T cell line
D	diversity
DC	dendritic cell
DLBCL	diffuse large B cell lymphoma
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EBERs	EBV-encoded small RNAs
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus

EBVaGC	EBV-associated gastric carcinoma
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FCS	forward scatter
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GVHD	graft-versus-host disease
HHV	human herpesvirus
HL	Hodgkin's lymphoma
HLA	human leukocyte antigen
HPV	human papillomavirus
HRS	Hodgkin and Reed-Sternberg
HSCT	hematopoietic stem cell transplantation
HSV	herpes simplex virus
IFN	interferon
IL	interleukin
ivt RNA	*in vitro*-transcribed RNA
J	joining
KSHV	Kaposi’s sarcoma-associated herpesvirus
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LP	leader protein
LPD	lymphoproliferative disease
LTR	long terminal repeat
LV	lentivirus
mTRA/TRB	murinized TRA/TRB chain
mAb	monoclonal antibody
MAGE	melanoma-associated antigen 1
MART-1	melanoma differentiation antigen
MCPyV	Merkel cell polyomavirus
MHC	major histocompatibility complex
NGS	next generation sequencing

NK cell	natural killer cell
NPC	nasopharyngeal carcinoma
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGE2	prostaglandin E2
pHLA	peptide/HLA complex
Poly (I:C)	polyinosinic:polycytidylic acid
PTLD	post-transplant lymphoproliferative disorder
R848	resiquimod
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RV	retroviruses
scFv	single-chain variable fragment
SOT	solid organ transplant
SSC	side scatter
TAA	tumor-associated antigen
TCR	T cell receptor
TIL	tumor infiltrating lymphocyte
TME	tumor microenvironment
TNF	tumor necrosis factor
TRA	alpha (α) chain
TRB	beta (β) chain
TRD	delta (δ) chains
TRG	gamma (γ) chain
TSA	tumor-specific antigen
V	variable
VCA	viral capsid antigens
VSTs	virus-specific T cell
VZV	varicella-zoster virus
WBCs	white blood cells
WHO	world health organization
Wp	W promoter

WT1	Wilms tumor 1
ψ	retroviral psi packaging element

8. References

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