Improving EBV-Specific T Cell Therapy: Lineage Tracing and EBV Stem Cell Memory T Cells

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Darya Palianina

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auf Antrag von

Erstbetreuerin: Prof. Dr. Nina Khanna,

Zweitbetreuer: Prof. Dr. Dirk Bumann,

Externer Experte: Prof. Dr. Werner Held

Basel, 15.11.2022

Prof. Dr. Marcel Mayor

The Dean of Faculty

“I solemnly swear that I am up to no good.” [1]

- *Epstein-Barr Virus (probably)*

[1] From J.K. Rowling, “Harry Potter and the Prisoner of Azkaban”

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ABBREVIATIONS

AAV – adeno-associated virus

AB - antibody

ACT – adoptive cell therapy / adoptive cell transfer

Ad - adenovirus

AITL – Angioimmunoblastic T-Cell Lymphoma

ANOVA – analysis of variances

AML – acute myeloid leukemia

APC – antigen-presenting cell

BALF2 – BamHI-A leftward frame 2 DNA-binding protein

BALF3 – BamHI-A leftward frame 3 protein

BALF4 – BamHI-A leftward frame 4 envelope glycoprotein

BALF4 – BamHI-A leftward frame 4 envelope glycoprotein B

BALF5 – BamHI-A leftward frame 1 DNA polymerase

BARF1 – BamHI-A rightward frame 1 protein

BART – BamHI fragment A rightward transcript

BBRF1 – BamHI-B rightward frame 1 protein

BC - barcode

BCL-2 – B-cell lymphoma-2 protein

BCRF1 – BamHI-C rightward frame 1 protein

BDLF3 – BamHI-D leftward frame 3 glycoprotein gp150

BFRF1 – BamHI-F rightward frame 1 protein

BFRF3 – BamHI-F rightward frame 3 smallest capsid protein

BHLF1 – BamHI-H leftward frame 1 transcript

BHRF1 – BamHI fragment H rightward reading frame 1

BILF1 – BamHI-I leftward frame 1 G Protein-Coupled Receptor

BILF2 – BamHI-I leftward frame 1 membrane protein

BKRF2 - BamHI-K rightward frame 2 glycoprotein L

BL – Burkitt’s lymphoma

BLLF1 – BamHI-L leftward frame 1 glycoprotein 350/220

BLLF2 – BamHI-L leftward frame 2 protein

BMLF1 – BamHI M fragment leftward open reading frame 1 protein

BMRF1 – BamHI M fragment rightward open reading frame 1 protein

BNLF2 – BamHI-N leftward frame 2 protein

BNRF1 – BamHI-N rightward frame 1 major tegument protein

BRLF1 – BamHI R fragment leftward open reading frame 1 protein

BVRF2 – BamHI-V rightward frame 2 DA packaging protein

BXLF2 – BamHI-X leftward frame 2 glycoprotein gp85

BZLF1 – BamHI Z fragment leftward open reading frame 1 protein

BZLF2 – BamHI-Z leftward open reading frame 2 glycoprotein

CAR – chimeric antigen receptor

CBF1 – Cp binding factor 1

CCR5 - C-C chemokine receptor type 5

CD – cluster of differentiation (as in CD3, CD4, etc.)

CMV – cytomegalovirus

CNS – central nervous system

CR – complete remission

CRISPR – clustered regularly interspaced short palindromic repeats

CRS – cytokine release syndrome

CTL-L – long-term-expanded (or expanded with lymphoblastoid cell lines) cytotoxic T lymphocytes

CTL-R – rapidly expanded cytotoxic T lymphocytes

CTLA-4 – cytotoxic T-lymphocyte-associated Protein 4

CTLm – cytotoxic T lymphocyte culture medium

CTLs – cytotoxic T lymphocytes

DC – dendritic cell

DLBCL - diffuse large B cell lymphoma

DNA – deoxyribonucleic acid

DPBS – phosphate-buffered saline

dsDNA – double-stranded deoxyribonucleic acid

E – early

EBER - Epstein-Barr virus-encoded small RNAs

EBNA-LP EpsteIn-Barr virus nuclear antigen leader protein

EBNA– EpsteIn-Barr virus nuclear antigens

EBV – Epstein-Barr Virus

EDTA – ethylenediaminetetraacetic acid

EGFP – enhanced green fluorescent protein

ELISpot – enzyme-linked immunosorbent spot

EOMES – Eomesodermin

FACS – fluorescence-assisted cell sorting

GFP – green fluorescent protein

GM-CSF – granulocyte macrophage-colony stimulating factor

gp350 - Epstein-Barr virus glycoprotein 350

GrB – granzyme B

GSK3β – glycogen synthase kinase-3β

GVHD – graft-versus-host disease

GZMA – granzyme A

GZMB – granzyme B

GZMH – granzyme H

GZMK – granzyme K

HCT or HSCT – hematopoietic stem cell transplantation

HDR – homology-directed DNA repair

HER2 – human epidermal growth factor receptor 2

HHV-4 – Human herpesvirus 4

HL – Hodgkin lymphoma

HLA – human leukocyte antigen

HLA-DRA – Major Histocompatibility Complex, Class II, DR Alpha isotype

HLH – Hemophagocytic Lymphohistiocytosis

HRS cells – Hodgkin/Reed-Sternberg cells

HSC – hematopoietic stem cells

ICAM-1 – Intercellular Adhesion Molecule 1

ICC – intracellular cytokines

IE – immediate early

IFNg - interferon g

IgG – immunoglobulin G

IHC – immunohistochemistry

IL – interleukin

IM – infectious mononucleosis

ITAM – immunoreceptor tyrosine-based activation motif

ITR – inverted terminal repeats

K+ – here: elevated Potassium concentration

L – late
LAG-3 – lymphocyte-activating gene 3
LCL – lymphoblastoid cell line
LCLs - lymphoblastoid cell lines
LCM-10 – lymphoblastoid cell culture medium
LHA – left homology am
LMP – latent membrane protein
LPD – lymphoproliferative disease
mAB – monoclonal antibody
MHC – major histocompatibility complex
miR – microRNA precursor
miRNA – micro-ribonucleic acid
mRNA – messenger ribonucleic acid
NGS – next-generation sequencing
NHEJ – non-homologous end joining
NHL – non-Hodgkin lymphoma
NK – natural killer cell
NKG7 – Natural Killer Cell Granule Protein 7
NKT – Natural killer T cell
ND – no disease
NPC – nasopharyngeal carcinoma
NR – no response
NSG – NOD scid gamma (mice)
ORF – open reading frame
PBMCs – peripheral blood mononuclear cells
PBS – Dulbecco's phosphate-buffered saline
PID – primary immune deficiency
PD – progressive disease
PD-1 – Programmed cell death protein 1
PEL – primary effusion lymphoma
PR – partial response
PRF1 – perforin 1
PTLD – post-transplant lymphoproliferative disorderd
RHA – right homology am

RNA – ribonucleic acid

RNP – ribonucleoprotein

scRNA-seq – single cell RNA sequencing

SOT – solid organ transplantation

SV40 polyA – simian vacuolating virus 40 polyadenylation signal

TAA – tumor-associated antigen

TarGET – polyclonal antigen-specific T cell-targeted genome editing

TCM – central memory T cell

TCR – T cells receptor

TEM – effector memory T cell

TEMRA – terminally differentiated effector T cell

Th – helper T cell

TIGIT – T cell immunoreceptor with Ig and ITIM domains

TIM-3 – T cell immunoglobulin domain and mucin domain 3

TME – tumor microenvironment

TN – naïve T cell

TNFα - tumor necrosis factor α

TRAC – T cell receptor α constant

T_{reg} – regulatory T cell

TSCM – stem cell memory T cell

TTM – transitional memory T cell

TWS-119 – the reversible inhibitor of Wnt/β-catenin signaling pathway, glycogen synthase kinase-3β inhibitor

UMAP - Uniform Manifold Approximation and Projection

VCA – viral capsid antigen

VST – virus-specific T cell

WT – wild type

SUMMARY

Viral infections remain an important cause of morbidity and mortality, especially after transplantation, and treatment options are often limited. Adoptive therapies with virus-specific T cells (VST) have shown to be promising in restoring virus-specific immunity and thereby preventing and treating viral infections over the past 25 years. Donor-derived Epstein Barr virus (EBV)-specific cytotoxic T-cell lines (CTLs) have demonstrated prolonged overall survival in patients with EBV-driven post-transplant lymphoproliferative disease (PTLD), lymphomas and diseases, but approximately 30% of patients show no response indicating a need for further improvements (the overview of the clinical trials with EBV-CTLs is summarized in Appendix I).

Naturally, after antigen stimulation and activation, T cells undergo a series of cell proliferation and differentiation stages, from naïve to stem cell memory (T_{SCM}), central memory (T_{CM}), transitional memory (T_{TM}) effector memory (T_{EM}), and terminally differentiated, short-lived effector T cells (T_{EMRA}). During differentiation, the effector function of T cells gradually increases, while their self-renewal capacity correspondingly declines. As one of the major challenges of adoptively transferred T cells is their poor ability to persist *in vivo* upon the infusion., early differentiated i.e., stem cell memory T cells (T_{SCM}) became relevant for the adoptive therapies due to their high proliferation, engraftment, and persistence potential in different human diseases and have shown promising results in ACT against cancer. Protocols that are used for EBV-specific ACT to date mainly exploit late-differentiated T_{EM} or at best T_{CM}. Data on EBV-specific T_{SCM} in adoptive therapy is very limited and no protocol for clinical application is available.

In **chapter II**, I present a **clinically-scalable protocol for T_{SCM}-enriched expansion of Epstein-Barr virus (EBV)-specific T cells**. In collaboration with Prof. Dr. Christian Münz and the group of Dr. Obinna Chijioke from the University of Zurich we compared its anti-tumor efficiency with conventionally expanded, EBV-transformed lymphoblastoid cell line-stimulated T cells in the *in vivo* model of post-transplant lymphoproliferative disorder (PTLD). Rapidly expanded T_{SCM}-enriched EBV-specific T cells **efficiently controlled the PTLD, showed a better tumor infiltration rate, robust *in vivo* proliferation and persistence potential with functional CD4+ and CD8+ cells and a broader reconstitution of EBV specificity**. The method and these

data together should help to establish the next generation of unmodified antigen-specific cell therapies beyond EBV diseases.

Another way to improve the cell therapies is to study their dynamic after infusion, thus being able to characterize the most promising clones.
Determining the fate of T cells following patient infusion hinges on the ability to track them *in vivo*. While this is possible by genetic labeling of parent cells, the applicability of this approach has been limited by the non-specificity of the edited T cells. Recently, it was shown that CAR T cells can persist in patients as many as 10 years after infusion. While CAR T cells are readily identifiable, non-engineered therapeutic T cells are difficult to distinguish from naïve T cells. In chapter II, I describe **a novel method for CRISPR-targeted genome integration of a barcoded gene into Epstein-Barr virus-antigen-stimulated T cells** that we devised in collaboration with the group of Prof. Sai T. Reddy from ETH Zurich. We demonstrated its use for exclusively identifying expanded virus-specific cell lineages. Our **method facilitated the enrichment of antigen-specific T cells**, which then mediated **improved cytotoxicity against EBV-transformed target cells**. Single-cell and deep sequencing for lineage tracing revealed the **expansion profile of specific T cell clones and their corresponding gene expression signature**. This method has the potential to enhance the traceability and the monitoring capabilities during immunotherapeutic T cell regimens.

INTRODUCTION

Epstein-Barr virus

Taxonomy

Epstein Barr virus (EBV), or human herpesvirus 4 (HHV-4) belongs to *Herpesviridae* family, *Gammaherpesvirinae* subfamily, *Lymphocryptovirus* genus [1].

Genome

EBV has a linear double-stranded DNA encoding over 85 latent and lytic genes. Most of these genes are translated into proteins and some encode small and micro-RNA [2, 3]. EBV type 1 (type A) and EBV type 2 (type B) are the two major EBV genotypes which differ by the sequences of the proteins encoding nuclear antigens (EBNA-3A, -3B and -3C) [4]. EBV persist in a cell as a circular episome or by integrating into the host genome. EBV episome mimic the behavior of the hosts DNA: it is packaged with histones, replicates once during S phase of the cell cycle [5] and is equally distributed into daughter cells during mitosis [6].

Structure

It is the first human virus identified as oncogenic. As a typical herpesvirus, it includes the toroid-shaped core that contains viral dsDNA; the core is surrounded by the capsid consisting of 162 capsomers; the virus is protected by the envelope (the outer lipid membrane) coated by many glycoproteins called spikes that help the virus to attach to a potential host; the space between the envelope and capsid is called tegument filled with proteins needed for replication (Figure 1) [7].

Epidemiology

Naturally EBV infects only humans. Epidemiological studies reveal that EBV is highly prevalent and infects over 90% of the world's population. Primary infection normally occurs in the childhood and is often asymptomatic. Normally remains harmlessly in the body life-long [8, 9]. In most populations, EBV-1 is observed more frequently as EBV-

1 immortalizes B cells more efficiently than EBV-2 *in vitro* [10]. However, EBV-2 is also highly prevalent in New Guinea an equatorial Africa, especially in the patients with Burkitt's Lymphoma [11]. In addition, both EBV types are often found in immunocompromised patients [12].

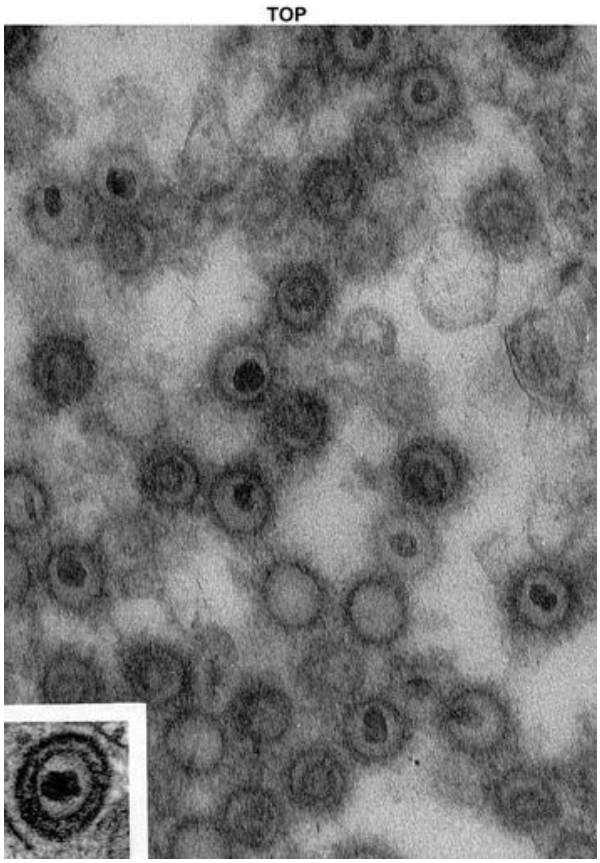

Figure 1. Ethe first electron-microscopic image of EBV virions. Adapted from [13].

Primary infection

Being transmitted via saliva EBV infects the oropharyngeal epithelium after which B cells get infected via interaction of the viral envelope protein gp350 and CD21 expressed on B cells [14]. There are two views on the type of B cells that get primarily infected Figure 2, a). One of them is that EBV targets naïve B cells and then drives them into memory state by mimicking physiological B cell memory induction through trafficking into germinal center in lymphoid tissues where B cells normally undergo somatic immunoglobulin-gene hypermutation to acquire memory. However, it was shown that acutely infected B cells localize to extrafollicular areas instead of germinal

centers and do not undergo hypermutation *In vitro*, both naïve and memory B cells were equally susceptible to the infection [15-17].

In the childhood, EBV often passes without diagnosis, however, in a minority of individuals, especially in adulthood, it causes infectious mononucleosis (IM). In acute infection, EBV releases proteins causing cell proliferation which creates an excess of white mononuclear cells giving its name to the disease. As a result of a primary infection, virus is getting released from the infected B cells which are also getting destroyed in the process, after which EBV can be again recovered from saliva [18].

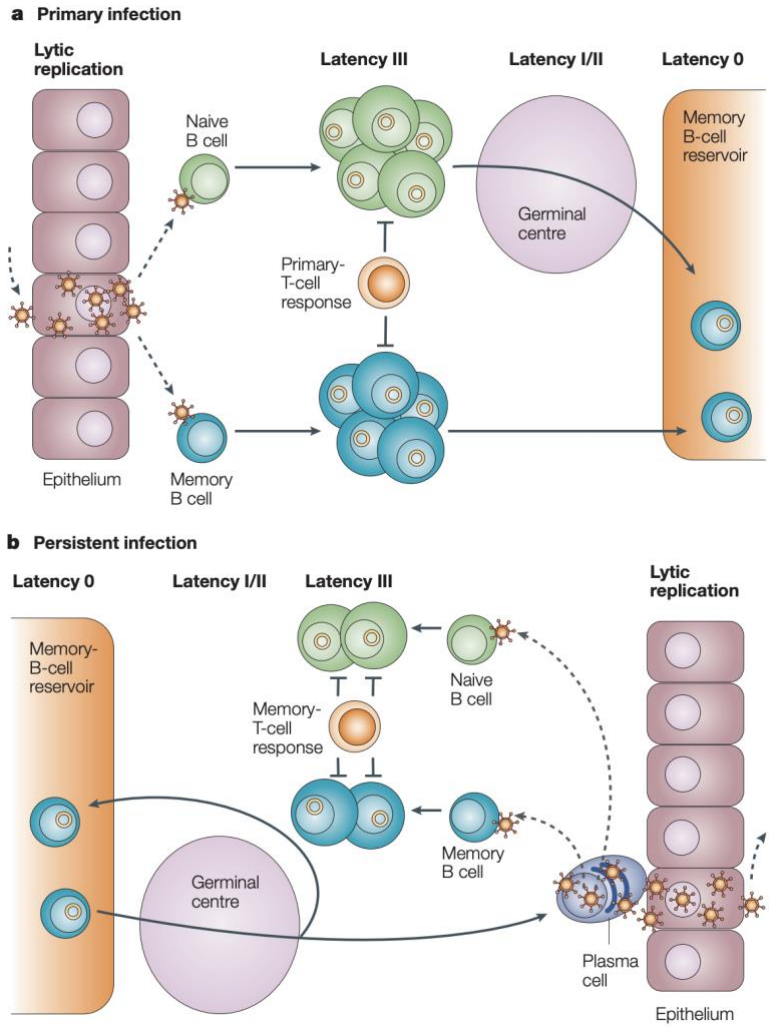

Figure 2. Schematic of EBV primary (a) and persistent (b) infection routs, adapted from [15].

Latency

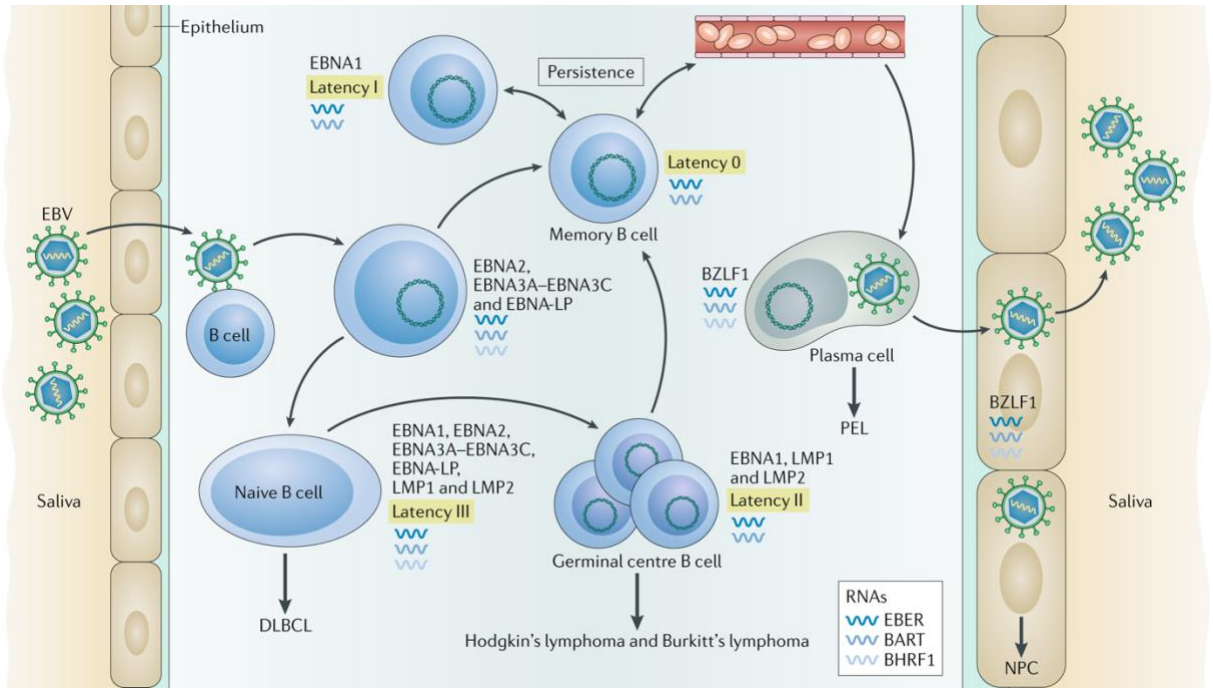

Figure 3. Models of EBV latent infection. Different latency programs and lymphomas they may lead to. DLBCL stands for diffuse large B cell lymphoma, PEL - Primary effusion lymphoma, NPC – nasopharyngeal carcinoma. Adapted from [19].

The infection causes cytotoxic T cell (CTL) response which eventually controls infected B cell proliferation. Afterwards, the virus spreads in the lymphoid tissues as a growth-transforming B cell infection (latency III) (Figure 2). These cells can also be controlled by latent-specific CTLs. A fraction of B cells escapes the recognition remaining infected in a quiescent state when viral antigens are largely downregulated (latency 0). These resting memory B cells persist and serve as viral reservoir.

Occasionally, infected resting B cells can be recruited to germinal centers where they can change the latency programs or differentiate to plasma cells and traffic to mucosal or oropharynx sites where they can re-enter lytic cycle releasing the virions and continuing the infection; however, this process of re-infection is interrupted by well-established by this time immunological control mechanisms [7, 20].

In type I latency which is common Burkitt's lymphoma and stomach cancer, EBNA-1 expression dominates [21, 22]. Type II latency is characterized by expression of EBNA-1, LMP-1 and LMP-2. It can be observed during Hodgkin lymphoma, nasopharyngeal carcinoma, and in the state of heavy hyperinflammation in the form of hemophagocytic lymphohistiocytosis [1]. In type III latency, all nine latent proteins are expressed. It is found in EBV-associated post-transplant lymphoproliferative disease (PTLD) [23]. EBV antigens expressed during different latency programs are summarized in Figure 3.

Mechanisms of immunological control of EBV

- Innate immune responses

The mechanism of the EBV control by Natural Killer (NK, CD3-CD56+) cells remains unclear, however there is a correlation between NK cell expansion and peripheral viral loads. NK cells were also found to appear in blood before the CD8+ T cell response in blood and effectively lyse cells with the replicative virus in humanized mice [24]. The depletion of NK cells led to elevation of serum pro-inflammatory cytokines and increased CD8+ cell expansion [25]. NK subsets with $CD56^{dim}CD16-$ expression are found to proliferate more during IM and better recognize cell with the replicative virus. The frequency decrease of this subset with age might explain the higher occurrence of infectious mononucleosis in adults [26].
Other innate immune cells such as monocytes dendritic cells (DCs) are shown to mediate early EBV infection control indirectly so far only *in vitro* [27-29].

- Adaptive immune responses

Antibody responses
Antibody response against lytic EBV antigens is formed during acute infection. IgM against viral capsid antigen (VCA) appears early, is detectable in the first 2-3 months and then disappears whereas anti-VCA IgG remain life-long. Antibodies to the main envelope glycoprotein gp350 were reaching peaks up to a year after IM detection [30]. Anti-EBNA-2 IgG appear after the disease peak subsequently declining whereas anti-EBNA-1 IgG appear only after 3-6 months and remain stable afterwards [31, 32].

CD8+ T cell responses
CD8+ T cell response is the most extensive immune reaction during IM. Dramatic amounts of CD8+ cells specific to immediate early or early lytic epitopes are produced while responses against delayed early and late proteins are significantly less frequent [33, 34] (Figure 4). This is due to viral immune evasion strategies that impair antigen presentation strength of infected cells as the cycle progresses [35, 36]. Responses to latent EBV antigens are less strong in the acute infection and focus mainly on EBNA3

proteins however depending on HLA alleles, presentation of different latent antigens may be equally frequent [33, 37].

Once the IM is resolved and the latency is established, CD8+ T cells return to normal frequencies. Then the frequency of cells specific to lytic antigens may comprise up to 2% of the CD8+ pool whereas up to 0.5% of CD8+ cells might be specific to latent antigens. Some lytic responses disappear and some new latent responses develop, otherwise immunodominance / clonotypic composition present during IM largely remains life-long [38-42].

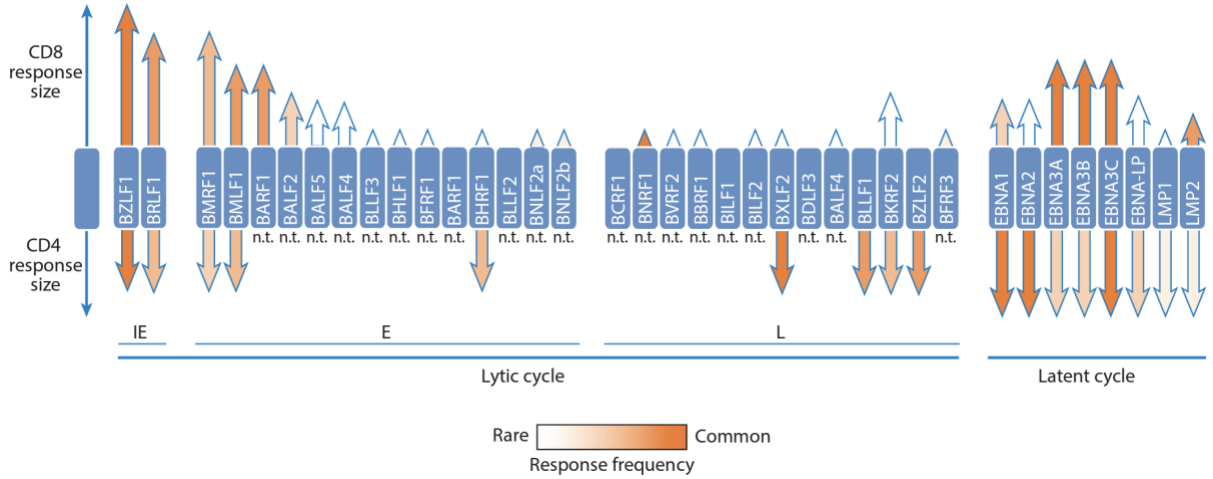

Figure 4. Lytic and latent epitope choices by CD8+ and CD4+ T cells. IE – immediate early, E – early, L – late. Summarized results from comparative studies, adapted from [20].

CD4+ T cell responses

CD4+ reactivity can also be detected during IM although it results in substantially less frequency increase than for CD8+ cells [43, 44]. In healthy carries or subjects with resolved infection, the CD4+ EBV-specific T cell frequency comprises up to 0.1% [43]. The CD4+ responses against single latent epitopes are more abundant than those against single lytic ones (Figure 3), however they seem to be broader than for CD8+ cells with less immunodominance base [20]. Thus, CD4+ latent responses are present during all cycle stages and in equally strong frequencies [45-47].

B cell immortalization by EBV

In vitro, the absence of the CTL response, latently EBV infected B lymphocytes, can become immortalized, or tumorigenic. These EBV-transformed lymphoblastoid cell

lines (EBV-LCLs) are usually polyclonal but with time individual become dominant [48]. EBV-LCLs carry type III latency [49] and thus can serve as a model of PTLD [50]. B cells *in vivo* can also undergo this transformation which becomes Burkitt's lymphoma (type I latency) [51].

EBV products

- EBV nuclear antigens (EBNAs)

There are six EBNA proteins: EBNA-1, -2, -3A (EBNA-4), -3B (EBNA-5), -3C (EBNA-5) and -LP (EBNA-6) [52]. All EBNAs beside EBNA-3B are important for B cell immortalization [53-55].

EBNA-2 and EBNA-LP are the first EBV genes detected upon the infection [56], they interact in order to drive B cells into G1 phase by biding and inactivating tumor suppressor gene products [57]. EBNA-2 is a transcriptional coactivator the primary function of which is expression upregulation of viral and cellular genes, e.g., CD23 (B cell marker of activation), c-myc (cellular proto-oncogene) and viral EBNA-C promoter [58-60]. EBNA-2 acts through binding transcriptional factors, for example those of the Notch signaling pathway which contribute to lymphoma development.

EBNA-1 is a DNA-binding nucleophosphoprotein required for EBV genome replication and maintenance. It segregates viral episomes during mitosis and is crucial for maintaining latent phase of EBV infection [7].

EBNA-3A-C are transcriptional regulators [55] interacting with Cp binding factor 1 (CBF1) which is involved in Notch signaling and its overexpression has been observed in human malignancies [61, 62].

- LMP-1

LMP-1 (latent membrane protein 1) is most actively involved in oncogenesis. It acts as a constitutively active receptor CD40 and thus mimics a growth signal [63]. LMP-1 interacts with several essential signaling pathways, which allows it to induce cell growth, transformation and control apoptosis [64-68]. For example, LMP-1 elevates levels of BCL-2 and A20 thus inhibiting p53-mediated apoptosis [69].

- LMP-2

LMP-2 proteins (LMP-2A and LMP-2B) contain an ITAM domain in its tail (see below) [70] and drive and maintain EBV latency. LMP-2A is expressed in Hodjkin's disease and nasopharyngeal carcinoma thus it plays a role in oncogenesis however its contribution is yet unknown [55].

- EBV-encodded small RNAs and miRNAs

EBV-Encoded RNAs 1 and 2 (EBERs 1 and 2) are non-coding RNAs that are abundantly expressed in all latency types in most EBV tumors [55]. They are involved in oncogenesis [71, 72] although studies showed that EBV with EBER knock-outs can still transform B cells [73].
EBV can encode approximately 23 precursors and 44 mature miRNAs [72]. miRNAs play a contributing role in oncogenesis: mutant EBV strains lacking miRNA cluster located within Bam HI fragment H rightward open reading frame (BHRF-1) gene encoding a Bcl-2 homolog had a substantially reduced transforming capacity [74]. EBV BamHI-A rightward transcript (BART) miRNAs are overexpressed in many EBV tumors and play diverse roles [75-77] (reviewed in [72]).

Immune evasion and adaptation

DNA viruses are less capable of mutations to adapt to a host than RNA viruses because DNA is a more stable molecule [78]. However, large genomes of dsDNA viruses allowed to encode a machinery to subvert host defense mechanisms, and the ability to switch to latency is the most fundamental part of it [79]. Latent EBV antigens ca lead to tumorigenesis by interfering into cell signaling pathways [80].
Herpesviruses produce proteins that prevent the appearance of viral peptide:MHC class I complexes on the infected cells by blocking either the peptide traffic into endoplasmic reticulum or the peptide:MHC class I complexes traffic to the cell surface or by lysing MHC class I [81, 82]. One of the functions of NK cells is to recognize and lyse cells with downregulated MHC-class I presentation, however there are also viral mechanisms to produce inhibitory receptors to block NK lysis [83].
Mechanisms specific to immune evasion by EBV include but are not limited to (summarized in Figure 5):

- Production of BARF-1, a lytic protein partially homologous to Intracellular Adhesion Molecule 1: as a result, lymphocyte adhesion to infected cells is blocked [84];
- Downregulation of IFNγ and TNF receptors by immediate-early protein BZLF1 [85, 86];
- Inhibition of Th-1 response. This includes reduction of IFNγ production by producing a viral homolog of IL-10 (BCRF-1) [87];
- HLA class II/peptide blockade. Envelope glycoprotein gp42 in association with HLA class II blocks its interaction with T cell receptors (TCR) [88], early protein BGLF5 degrades MHC class II mRNAs [89], and BZLF1 interferes with the invariant chain post-transcriptionally [90];
- The viral LMP2A contains an immunoreceptor tyrosine-based activation motif (ITAM) in its tail [70]. ITAMs were evolutionarily acquired from the host's B cell receptor complex and are required for BCR signaling. ITAM allows EBV trigger B cell proliferation [91];
- Degradation of the proteasome. During the lytic phase, many viral proteins are expressed in order to support viral replication which provides a source for peptide presentation. However, in the latency state, Epstein-Barr virus nuclear antigen 1, EBNA-1, interacts with the proteasome preventing its degradation into peptides and thus preventing the CTL recognition of infected cells [18, 92]. However, studies reveal that EBNA-1 can be processed by cross-presentation and then recognized by both CD4+ and CD8+ specific T cells [93-95];
- Down-modulation of LMP-2A expression by miR-BART22. This might permit the escape of EBV-infected cells from host immune surveillance, which may facilitate NPC carcinogenesis [75];
- Mutations in dominant peptides. For example, in small isolated populations in South-East Asia around 60% of people carry HLA-A11 allele. It was found that EBV in these populations often carries a mutation in a dominant peptide that is normally presented by this allele, and as a result, this peptide can no longer bind to it [96].

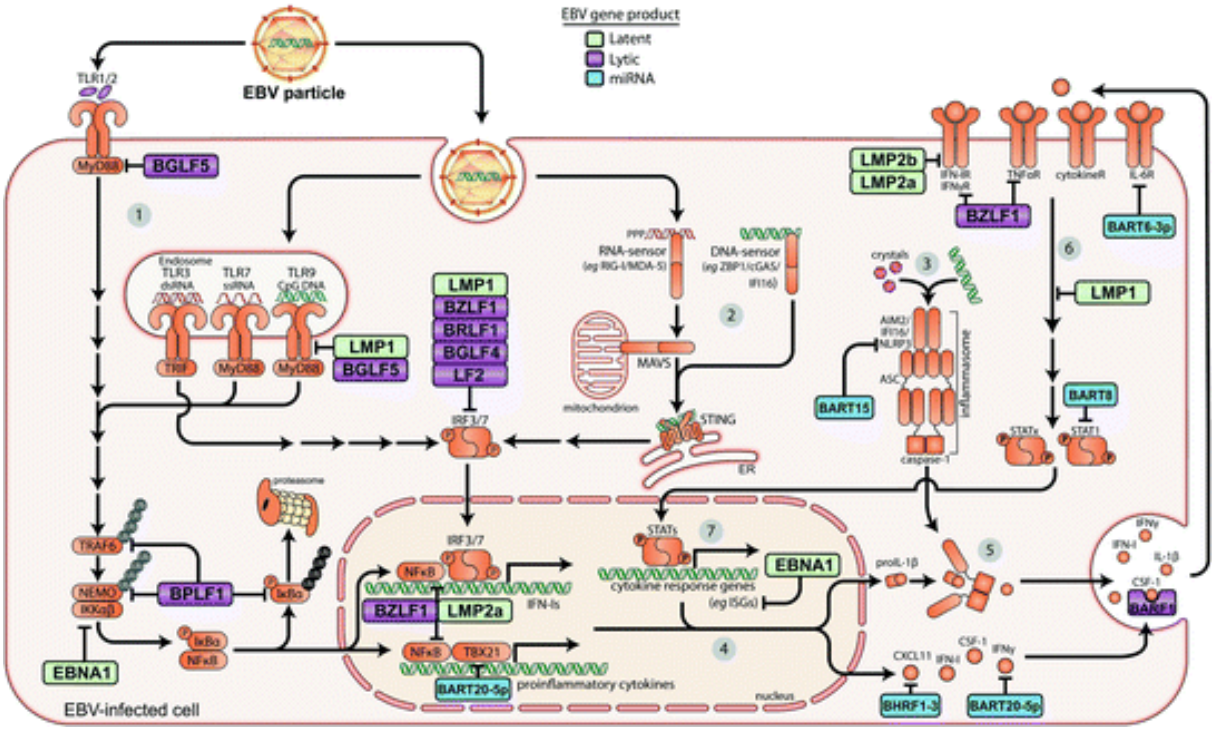

Figure 5. Overview of innate signaling pathways subjected to EBV modulation. Adapted from [97].

EBV-associated malignancies

Beside acute infection, infectious mononucleosis, EBV causes a variety of diseases (Figure 6). They can arise as a result of impaired immunity, e.g., in patients with primary immunodeficiencies or during immunosuppression after hematopoietic stem cell or solid organ transplantation. However, EBV can also spontaneously develop lymphomas in an immunocompetent host [98]. In fact, 200'000 EBV-associated malignancies develop annually worldwide, causing 150'000 deaths and comprising 1.5% of all cancer cases [99, 100]. In addition, it has been associated with pathophysiology of autoimmune diseases such as systemic lupus erythematosus and multiple sclerosis [20, 101].

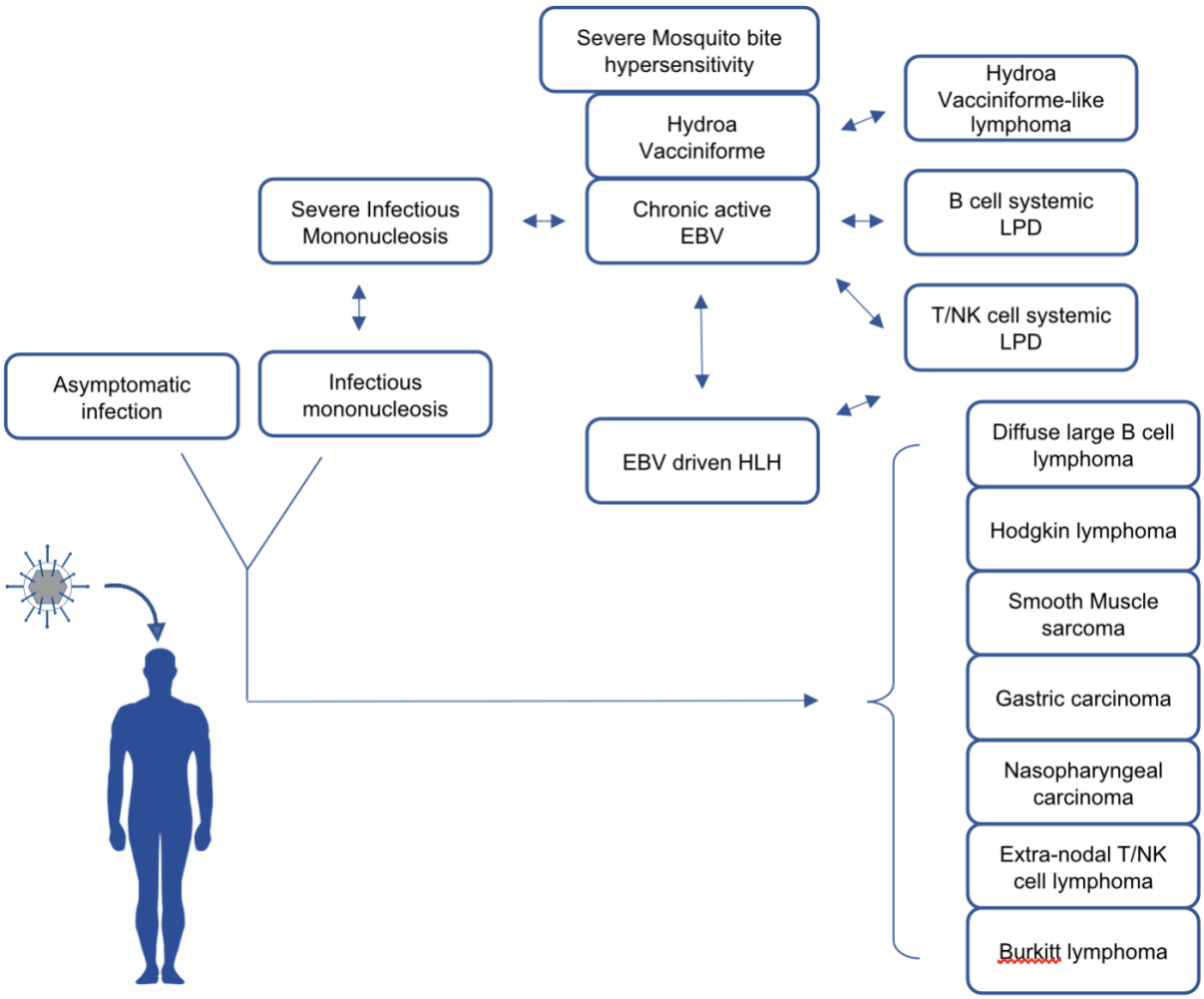

Figure 6. The variety of EBV complications. Adapted with changes from [98].

Burkitt's Lymphoma

EBV was originally discovered in Burkitt's lymphoma, thus it is the first virus-associated tumor described [102, 103]. BL is characterized by clonal proliferation of lymphocytes. results from a translocation between chromosome 8 and chromosome 14, 2 or 22. As a result, *c-myc* proto-oncogene gets misplaced and thus deregulated [104]. It is the most aggressively growing human tumor (the fastest tumor cell doubling time). BL is an AIDS-defining illness, it occurs in HIV-infected subjects is 10-100-fold more frequently than sporadically. BL represents 50% of pediatric cases [105]. In EBV-involved cases latency I is observed (characterized by expression by low-immunogenic EBNA-1) [106], however, the mechanism of EBV role in this disease is not well understood [7].

Endemic BL. This form is EBV-involved in 98% of cases. It occurs mostly in children, and the incidence is usually associated with immune-suppressive co-factors such as malaria (endemic BL is observed south of the Sahara Desert and Papua New Guinea) [107].

Sporadic BL. This form is observed outside of the endemic BL area. 30-40% of cases are EBV-associated. The median of incidence fall on children and adolescents, however, 60% of cases occur in adults over 40 years old [105].

Hodgkin Lymphoma

The hallmark of HL is Hodgkin/Reed-Sternberg (HRS) cells which presumably arise from germinal center pre-apoptotic B cells but have lost the B cell identity [108]. EBER, EBA-1, LMP-1 and LMP-2A are expressed in these cells in EBV+ HL (latency II). HL is usually found in children below 10 years old (75% of cases are EBV-associated) and elderly people over 75 years of age (95% are EBV-associated). Higher occurrence of HL in these ages may be a result of the primary EBV infection in children and of a decrease of immunological EBV control, respectively [1]. Genetic susceptibility may also play a role. For example, HLA-A02 is associated with a reduced risk and HLA-A01 – with an increased risk of EBV+ HL development [109].

Diffuse large B-Cell Lymphomas (DLBCL)

These lymphomas have a diverse morphology and localization depending on a subtype. Nearly all EBV-positive cases are aggressive and have a poor prognosis [110].

EBV-positive DLBCL in Elderly. EBV is present in up to 8-10% diagnosed patients in Asia although the disease is uncommon in Western populations [111]. LMP-1 and EBER can be observed in the infiltrate of polymorphic large cells with an inflammatory background [112].

DLBCL Associated With Chronic Inflammation. Occurs mostly in older persons with the majority of cases being EBV-associated (expressing usually LMP-1) [113]. It is an

aggressive lymphoma described as a massive infiltrate typically in the pleural cavity around the lung although the localization may vary [114].

Primary Effusion Lymphoma. Diagnosed in HIV-positive persons, patients with severe immunodeficiencies and EBV infection [115]. Although the lymphoma lacks B-cell markers and is abundant in T cell ones, EBER is detected in 70% of cases. The lymphoma is characterized by an effusion in one of the body cavities without lymph node enlargement. A median survival is six months. [116]

Lymphomatoid Granulomatosis. A rare disease characterized by the presence of an infiltrate composed of polymorphic cells including small T cells and HRS-like cells that are EBER-positive and LMP-positive or negative [1, 117].

Primary DLBCL of the Central Nervous System. Occurs in CNS and bone marrow and recurs in CNS. Typically happens late in the HIV infection and is almost exclusively associated with EBV [118]. Prognosis is 2-12 months [119].

Plasmablastic Lymphoma (PBL). A rare and highly aggressive disease that usually develops on oral ad nasal mucosal membranes of HIV-positive patients but can affect also immunosuppressed patients after organ transplantation, myeloma patients and even in older patients without immune deficiency. Nearly 100% of cases are EBV-positive [120-123].

EBV-associated NK/T-Cell Lymphomas

Angioimmunoblastic T-Cell Lymphoma (AITL). A diffuse lymphoma with retained abnormal follicles in the lymph node. The neoplastic cells are positive for CD3+ and CD4+, scattered large immunoblasts are often CD20+ and EBER+ [124]. The disease develops between 57-68 years of age, often associated with autoimmunity [125]. Characterized by fever, lymph node enlargement, hepato- and splenomegaly. Clinical outcome varies from spontaneous remission to rapid fatal disease progression [126].

Extranodal NK/T-Cell Lymphoma, Nasal Type. A rare EBV-associated lymphoma highly prevalent in Asia, Mexico and South America. The mean age at diagnosis is 50

years and the 5-year survival is 40-65%. The disease is characterized by malignant transformation of NK cells and develops most frequently in the upper airways, especially the nasal cavity, frequently followed by inflammatory symptoms. Characterized by an aggressive and angiodestructive inflammatory-proliferative process [115, 127-129].

Aggressive NK-Cell Leukemia / Lymphoma. Characterized by neoplastic NK cell proliferation in peripheral blood and bone marrow, massive hepato- and splenomegaly. The disease is highly aggressive – prognosis for most patients is 2 months after diagnosis. Median age of diagnosed patients is 40 years [129, 130].

EBV-positive Lymphoproliferative Diseases of Childhood

Hydroa Vacciniforme-Like Lymphoma. This disease is most prevalent in children and youth in Asia, Mexico, Latin America. Characterized by proliferation of lymphocytes in the epidermis, dermis and subcutaneous tissue and presence of EBER. A 2-year survival is 43%, however treatment with IFNα can improve the outcome [131-133].

Systemic EBV-Positive T-Cell Lymphoproliferative Disorders of Childhood. Occurs in immunocompetent children, adolescents and young adults (median age 20 years) with a higher frequency in Asians and native Americans and develops as a result of primary or chronic active EBV infection. The cells are CD3+, CD8+ if the EBV infection was acute, or CD3+ CD4+ if chronic active, and EBER+. The lymphoma progresses rapidly and is associated with high morbidity and mortality [134-136].

Nasopharyngeal Carcinoma

Undifferentiated nasopharyngeal carcinoma (NPC) is EBV-associated, affecting individuals in mid-40s with a high prevalence in Hong Kong, Taiwan, Chinese province of Canton, among the Inuits. In NPC, EBV infects the epithelial cells of the posterior nasopharynx. NPC in Inuits develops with EBV-2, in the other groups – by EBV-1. EBV undergoes latency II program in this disease [7, 137].
NPC cells are well recognized by EBV-specific CTLs, yet they are not eliminated. The mechanism of immune escape is currently under investigation. One of the hints is

increased expression of viral IL-10 homologue in NPC [138]. The current theory proposes a combination of immune evasion mechanisms that alter infected cell signaling, switch off tumor control genes and allow apoptosis escape [139].

Gastric Carcinoma

EBV presence varies in different gastric carcinomas. While it is detected in over 90% of lymphoepithelioma-like gastric carcinomas, it is observed in 5-25% of gastric adenocarcinomas. The pathogenic role of EBV in these tumors remains unclear [7].
In gastric adenocarcinomas, EBV develops a novel latency program where it expresses BARF-1 (a homologue of human colony-stimulating factor 1 receptor and ICAM-1) but not LMP-1 [140].

Posttransplant Lymphoproliferative Disorders (PTLDs)

This disease is highly heterogeneous, EBV-associated in most of the forms and develops as a result of immune suppression after organ transplantation [141]. Depending on the organ being transplanted, recipient's age, EBV serostatus of the donor and the recipient and immunosuppression therapy used, the frequency of PTLD development can be 0.5-30% especially commonly occurring in solid organ transplants [7]. Most commonly, PTLDs represent B-cell neoplasms. A variety of different forms have been described which includes plasmacytic hyperplasia, polymorphic lymphoproliferative disorder, malignant non-Hodgkin's lymphoma, and multiple myeloma [142, 143]. Proliferating polyclonal infected B cells are highly susceptible to genetic alterations which subsequently leads to malignant growth [142].

Treatment

Reduction of immunosuppression. In the post-transplant patients, the initial therapeutic action for PTLD / EBV lymphomas is the immunosuppression reduction (IR), however this often leads to the transplant rejection [144].

Monoclonal Antibodies. After IR, Rituximab, the anti-CD20 monoclonal antibody (mAb), has been routinely used in the treatment of CD20-positive lymphomas. It has

been effective against lymphoproliferative disorders; however the response may vary depending on the PTLD stage (76% early onset vs. 47% late onset) [145]. That's why sometimes this treatment is followed by chemotherapy which also includes anti-CD20 mAb) [144].
As EBV lymphomas use IL-6 as a growth factor, anti-IL-6 mAb has also been useful to treat these diseases with a 67% response rate reported [146].

Antivirals. Broad-spectrum antiherpesvirus agents such as ganciclovir, famcyclovir, acyclovir, valaciclovir (a prodrug of acyclovir), foscarnet, and cidofovir are implemented against EBV-associated diseases [7].
Acyclovir and ganciclovir are affecting vial thymidine kinase which is only expressed in the lytic phase; therefore, these drugs are not affective against EBV cancers as those are undergoing latency states. To circumvent this, arginine butyrate can be administered in addition to ganciclovir in order to selectively activate EBV thymidine kinase genes. This approach has been used to treat patients after solid organ transplantations undergoing EBV-associated lymphoproliferation [147]. Foscarnet (demonstrated to be affective against PTLD) and cidofovir are directed against viral DNA polymerase [148-150].

Adoptive Immunotherapy. However, the first two approaches do not help to restore suppressed host's anti-EBV immune response. Moreover, anti CD20 mAb is not helpful against CD20-negative EBV cancers.
An alternative approach is adoptive transfer of donor-derived, HLA-matched EBV-specific T cells which may help to restore EBV-specific immunity [151]. It also allows to develop polyspecific and polyclonal EBV-specific T cells thus lowering the risk of acquired immune evasion by EBV. This method has been safe and effective both as prophylaxis and treatment of a diverse range of viral complications not restricted only to EBV [152-154]. As the process of T cells for adoptive transfer is costly and laborious, the banks of pre-developed antigen-specific CTLs are used to simplify the procedure [153].

Vaccines. EBV vaccines could be useful as a protection in immunocompromised patients ot as a booster for patients with EBV tumors. The investigation of this subject has been ongoing since decades. The main targets of the vaccines are EBV envelope

glycoproteins such as gp350 and EBV latent genes. Currently, clinical trials of the EBV RNA vaccine (Moderna) and gp350–ferritin nanoparticle are ongoing [155].

Adoptive cell therapy

Concept

Restoration of function is presumably better accomplished by therapeutic cells generated for a specific therapeutic duty than by any chemical drug. The burden of chronic diseases, including cancers, opportunistic infections and immune-mediated disorders [156], and the advent of systems medicine [157], have prompted the idea of developing Advanced Therapy Medicinal Products (ATMPs) as an alternative to existing treatments by chemical compounds. ATMPs are aimed at harnessing cells to treat debilitating conditions and are divided into subtypes such as: 1) manipulated somatic cell therapies; 2) gene therapies; and 3) engineered tissues [158]. By now, ATMPs, and especially cell therapies, have progressed from theory to novel treatment strategies some which are becoming standard of care [159].

Adoptive cell therapy preparation typically starts from leukapheresis (a procedure of separating donor's or patient's own leukocytes from other blood cells) [160] or sometimes from regular blood collection and isolation of peripheral blood mononuclear cells (PBMCs) [153]; leukocytes are then manipulated appropriately – genetically modified and / or stimulated with antigens, and infused either directly or after *in vitro* expansion (Figure 7) [161].

Typically, cell therapies involve adoptive transfer of therapeutic T cells, in rarer cases – NK cells or hematopoietic stem cells.

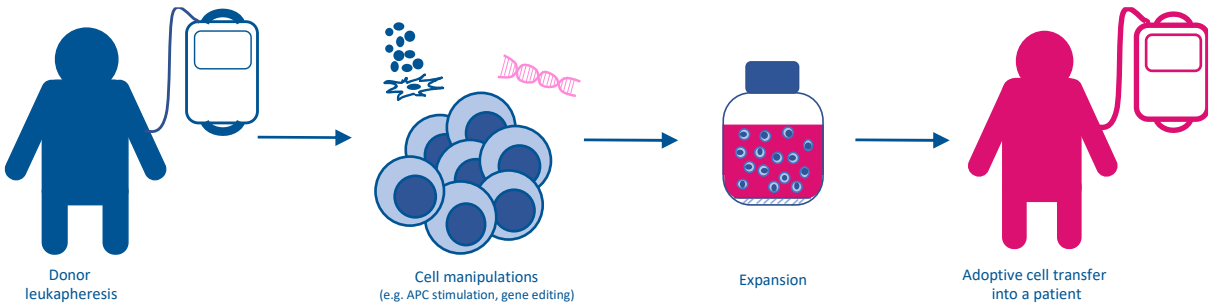

Figure 7. Adoptive cell therapy concept. Donor (or patient's own) leukocytes are isolated, manipulated (e.g., genetically modified and / or stimulated), expanded in vitro (sometimes this step can be omitted), and infused into a patient.

Both autologous and allogeneic T cells are produced for ACT [162-164]. Allogeneic ACT allows the development of “universal”, or “off-the-shelf” ACT banks which would allow the fastest delivery of therapeutic cells to patients [165].
Some of the common ACT approaches are described in more detail below.

Genome engineering methods in cell and gene therapy

Traditional genetic engineering for therapeutic cells utilizes transgenes encapsidated into viral vectors (most often, adenoviral, adeno-associated and retroviral) which transfer the transgene into desired cells or tissues. Normally, a viral vector includes the protein capsid and/or envelope that defines the vector’s tissue or cell tropism; the transgene of interest; and the “regulatory cassette” that controls stable or transient transgene expression as an episome or as a chromosomal integrant [166].
Adenoviruses (Ad) were the first vectors to use for gene therapy, and are still the most popular choice comprising half of ongoing clinical trials and gaining an increased popularity for vaccine development. These vectors possess certain safety risks as they are extremely immunogenic and cause strong cytokine storms in humans [167].
Adeno-associated viruses (AAV), on the contrary, are not immunogenic, do not bare a replicative potential, and normally do not integrate the non-transgene related parts into a genome. Nonetheless, as they are increasingly being used in combination with CRISPR/Cas-9 (targeted gene integration technology discussed below), it was shown that AAV can actually integrate themselves at the sites of double stranded DNA breaks during CRISPR-mediated transgene integration [168]. Currently commercially available AAV gene therapies are Glybera, for lipoprotein lipase deficiency; Luxturna, for retinoid isomerohydrolase RPE65 delivery to fix Leber’s congenital amaurosis (causing progressive blindness); and Zolgensma, for the treatment of spinal muscular atrophy. Zolgensma is the most expensive drug in the world to date [166].

An early gene therapy clinical trial used *gammaretroviral vectors* for transgene insertion, however it let to development of leukemia in 4 out of 9 treated patients [169]. Despite that *Lentiviral vectors* from the same family but with a reduced genotoxicity are widely used nowadays for genetically modified cell therapies as they efficiently and stably integrate into the host’s genome with a preference for transcriptionally active sites [170]. The difference between the two vectors is that gammaretroviral vectors not

only insert into transcriptionally active sites but tend to prefer the transcriptional start sites and oncogenes [171]. Integrase-deficient lentiviral vectors are now being introduced to deliver CRISPR/Cas9-trangene cassettes [172].

The lack of understanding of viral biology led to the lethal cases in the early cell-and-gene therapy trials. Viral vectors still bare strong safety concerns such as risk of viral replication (retroviruses and Ad), risk of oncogene integration that can lead to cell immortalization (retroviruses) and strong immunogenicity (Ad). Another challenge is high cost of virus-based cell-and-gene therapy manufacturing and as a result – treatment [166]. For this reason, researchers have been looking for a more affordable alternative which could potentially be *transposable elements (transposons)* which are easier to produce and have a safer integration profile [173], however its coupling to precise gene integration via CRISPR/Cas9 is still in development and thus the safety risks of random integration are still present [174].

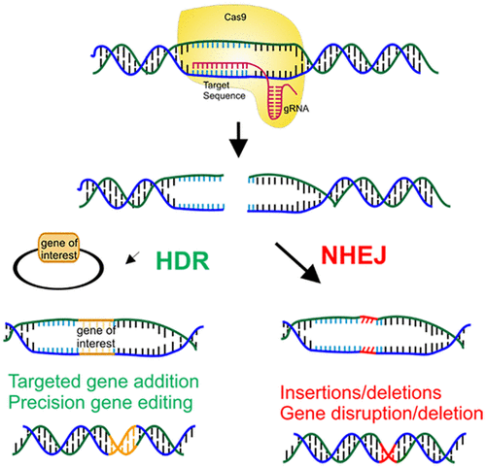

Figure 8. A schematic of HDR and NHEJ DNA repair mechanisms. Adopted from [175].

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats*) /Cas9* is a so-called bacterial anti-viral immune system [176] which was successfully adapted by scientists in order to either knock-out genes of interest or integrate genetic information in a precise location in the genome by generating double-stranded DNA breaks which are followed by either of two DNA repair mechanisms: non-homologous end-joining (NHEJ, useful to generate knock-outs) or DNA integration via homologous DNA repair (HDR) (Figure 8) [177, 178].

Chimeric antigen receptor cell therapy

CAR-T cells. Anti-CD19 and anti-CD20 (B-cell cancer-directed) chimeric antigen receptor (CAR) T cells (Figure 9) have become widely used in clinics since the first CAR T cell therapy for pediatric acute lymphoblastic leukemia (ALL) was approved by FDA in 2017 [179]. The development of improved CAR T cells has been ongoing ever since because despite dramatic results with up to 93% complete remission (CR) rate in ALL patients, the therapy was not as effective for other conditions, e.g., chronic lymphoblastic leukemia (CLL) and large B cell lymphoma– CR was maintained in 15-30% and 40-50% of treated patients, respectively [180]. Nonetheless, already now CAR-T cell therapy outstanding clinical results led to a paradigm shift in cancer treatment, and are now recommended as a standard of care for a second-line treatment of relapsed or refractory large B-cell lymphoma [159].

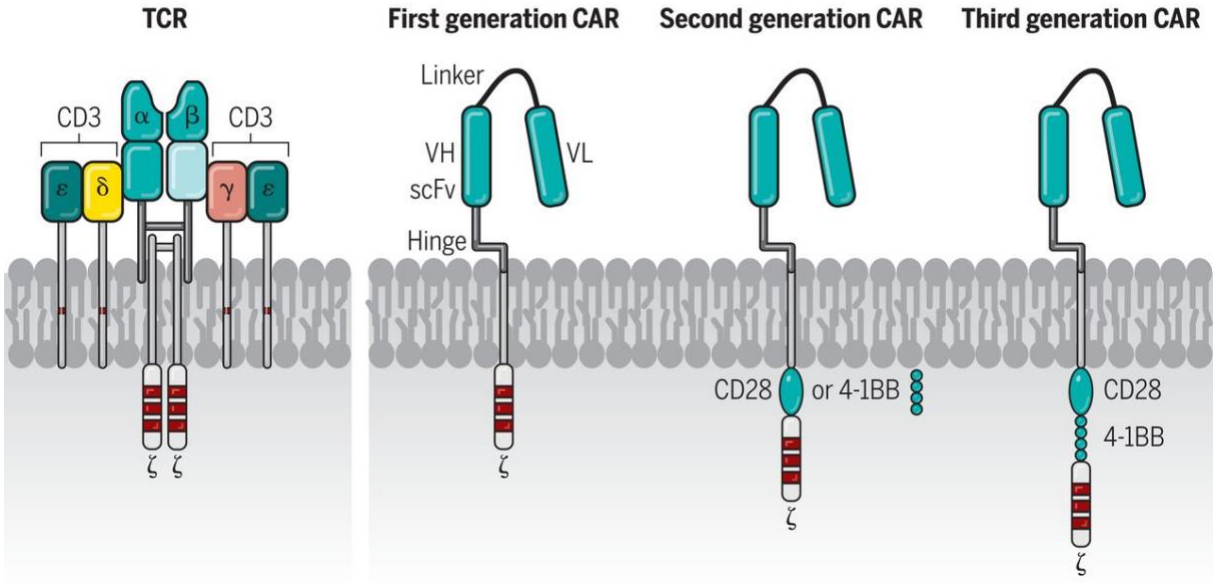

Figure 9. Design of TCR- (left) and CAR-T (right) cells. Transgenic TCR represents in essence a cloned TCR sequence with a defined specificity. CAR T cells are artificial constructs consisting of the extracellular and intracellular domains. Extracellular domain represents a fusion protein of the variable antibody regions – single-chain variable fragment (scFv) which allows the recognition of a target antigen. Intracellular parts are normally derived from those of a T cell receptor and allow CAR signaling transfer. Introduction of one or more co-stimulatory domains into CAR constructs (2nd and 3rd generations) facilitated enhanced anti-tumor activity, T cell survival and expansion [181]. Adopted from [162].

In addition to anti-CD19 CAR-T cells, other targets are being explored to more precisely target diverse cancers, especially CD19/CD20-negative ones. For example, human epidermal growth factor receptor 2 (HER2)-directed CAR-T cells are being

developed as this antigen is overexpressed in a myriad of human tumors [182]. Another promising target is CD123 which is overexpressed on acute myeloid leukemia (AML) blasts and has only a limited expression on normal hematopoietic stem cells. Anti-CD123 CAR T cells showed a relevant clinical anti-AML activity without myelosuppressive effects. CAR-T approaches for specific solid tumors such as neuro- and glioblastoma are gaining more and more attention [183].

CAR-NK and -NKT cells. CAR-NK cells is an alternative approach to CAR-T cells because they allow to overcome some of CAR T limitations such as NK cells have a low risk of cytokine storm and neurotoxicity (covered below in the “Limitations of ACT”), reduced risk of GFHD, multiple mechanisms to activate cytotoxicity, and a higher feasibility of off-the-shelf manufacturing. However, the limitations for massive use of NK cell engineering for ACT is low efficacy of transgene delivery and ex vivo proliferation and maintenance of NK cells [184].
Natural killer T cells (NKT) cells is a conserved subset of innate lymphocytes which has inherent antitumor function and infiltrates tumor more efficiently than T cells which makes them also an attractive target for CAR engineering. Moreover, in contrast to NK cells, they are long-lived thus being able to persist long-term [185, 186].

CAR-T_{reg} cells. Regulatory T cells are crucial for maintaining immune tolerance by modulating (suppressing) immune responses. Thus, engineering of T_{reg}s can be directed for specific purposes, e.g., to mitigate such conditions as GVHD, diabetes and autoimmune diseases, where increased immune tolerance would be of help. The limitation is the rarity of Tregs in peripheral blood [187, 188].

TCR-engineered T cells

A potential advantage of engineered TCR T cells is the possibility of high personalization of the therapy. However, the efficiency of TCR-based ACTs seems to be quite low to date [189]. This is often due to low affinity of selected TCRs to self-cancer antigen due to negative selection in the thymus [190]. Fine-tuning the affinity is critical because engineered high-affinity TCR-T cells also impair T cell function and increase the risk off cross-reaction with structurally similar autoantigens which can promote dangerous pathologies [191].

To tackle this challenge, high-throughput neoantigen discovery pipelines involving next-generation sequencing and single-cell RNA sequencing for TCR identification, novel epitope prediction methods, mass-spectrometry and T cell-based validation assays have been in development [189]. Such pipelines help significantly in advancing the discovery process for the efficient detection of immunogenic neoantigens and matching TCR sequences. Thus, TCR engineering raises a potential for the development of highly personalized therapies [192, 193].
Native TCRs are often more preferred to CARs as TCR signaling is naturally better regulated than that of exhaustive tonic CAR signaling. For example, in this study CAR was introduced precisely into TRAC locus of a TCR which resulted in an improved the anti-tumor efficiency [194].

Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TILs) are T cells derived from tumor microenvironment. They are supposedly tumor-specific T cells and thus are being successfully exploited for melanoma and other cancers [195, 196]. One of the limitations of their use is low numbers of starting TILs obtained from the biopsies. In order to expand sufficient amounts of TILs *in vitro*, long-term culture and high dosages of IL-2 are required [197]. Moreover, TIL administration is usually followed with further high-dose IL-2 infusions in order to promote their proliferation and maintenance *in vivo*, thus already exhausted state of initially isolated TILs is further worsened by IL-2 [198] which effects their function and persistence [199]. To overcome this, check-point blockade therapy can be administered together with TIL infusion or check-point knockout can be engineered in TILs [200, 201]. Furthermore, using additional supplements during in vitro expansion that decreases T cells differentiation helps to increase TIL longevity and functionality after infusion [202].

Antigen-specific T cells with pathogen-specific native TCRs

Patients undergoing immunosuppression post-transplantation are at risk for developing potentially life-threatening conditions caused by opportunistic pathogens, including viruses and fungi. The most common opportunistic viruses are EBV, CMV and Adenovirus, BK virus, Varicella Zoster virus and JC virus (polyoma virus 2) [203].

Treatment of these infections with monoclonal, polyclonal single-virus and polyclonal multi-virus-specific T cell has been proven safe and effective. Despite this, mortality from EBV complications remains among the highest of all among dsDNA viruses (Figure 10) (the overview of the clinical trials with EBV-CTLs is summarized in Appendix I).

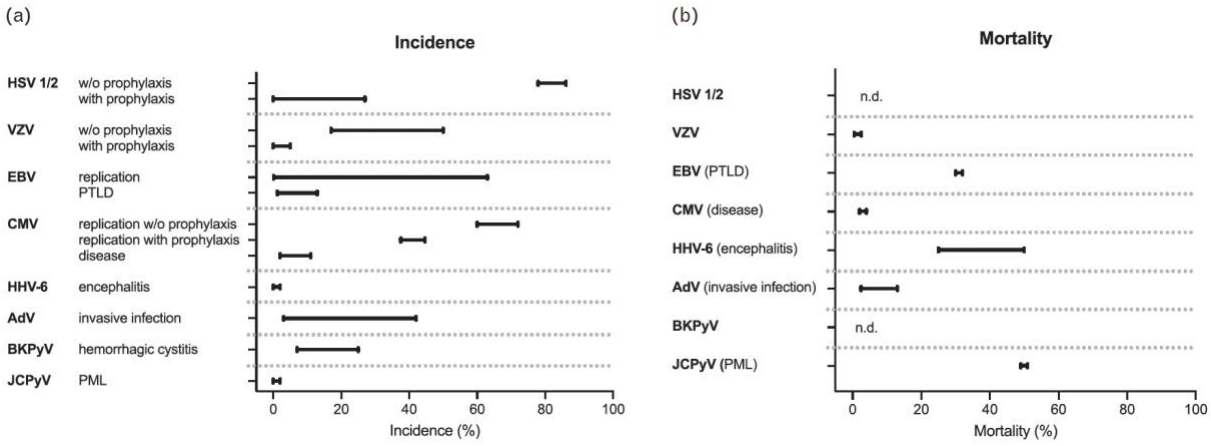

Figure 10. Incidence (a) and mortality (b) of different dsDNA viruses in HSCT recipients [152].

Methods of virus-specific T cell isolation

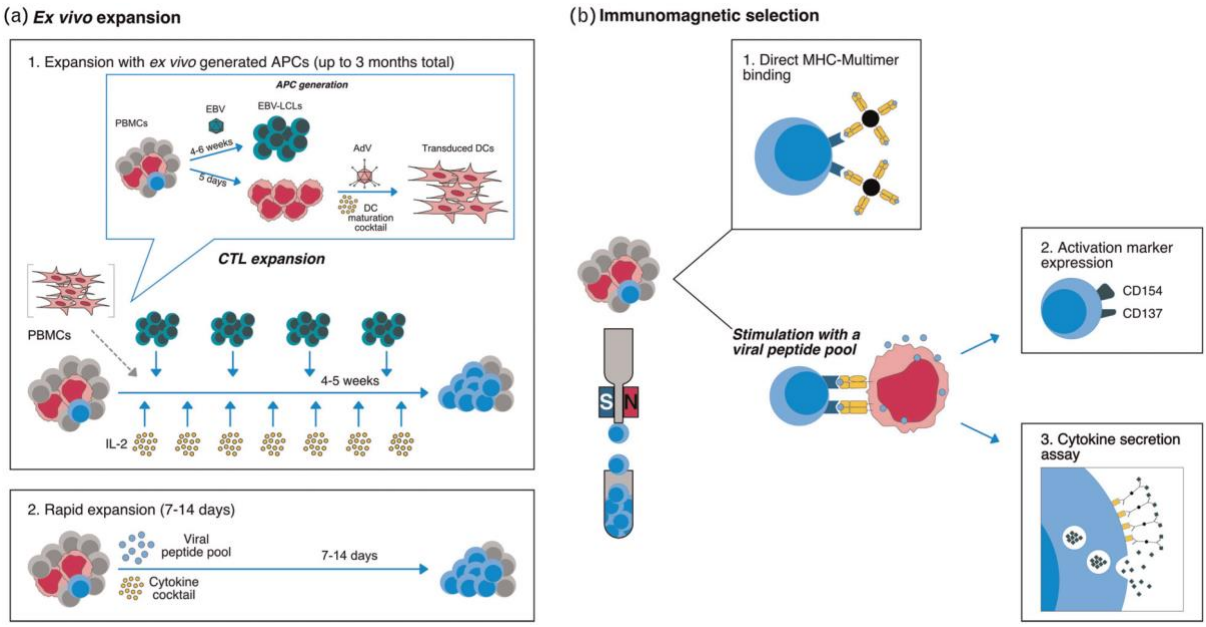

Figure 11. Methods of virus-specific T cell isolation. VSTs can be expanded *ex vivo* (a) or immunomagnetically selected (b). (a) Traditional *ex vivo* expansion involves prior generation of antigen-presenting cells (APCs), which are subsequently used for stimulation of cytotoxic T cells. EBV-transduced lymphoblastoid B-cell lines or dendritic cells (DCs) transduced with AdV and carrying a viral antigen of interest can be used as such APCs. Alternatively, rapid expansion can be used: PBMCs are stimulated with viral peptides presented to T cells on monocytes. (b) Immunomagnetic selection is based on binding of molecules via conjugated magnetic beads. Beads can be conjugated with viral peptide-

loaded MHC class I-multimers, allowing VST selection via TCR (1). For activation marker-dependent selection (2) and the cytokine capture assay (3), PBMCs are first stimulated with viral peptides, and activated T cells are selected with beads labeled with antibodies against T-cell activation markers or against cytokines secreted after stimulation. VST are shown in blue, monocytes in pink, and other PBMCs in gray. PBMC, peripheral blood mononuclear cells. Adopted from our recently published review [152].

Methods for different virus-specific T cell (VST) isolation are depicted in Figure 11. Traditional *ex vivo* expansion involves prior generation of appropriate antigen-presenting cells (EBV-transformed lymphoblastoid cell lines or DCs transduced to express specific viral antigens, *etc.*). These cells are then used for recurrent T cell stimulation during prolonged culture in the presence of IL-2. Both multiple restimulation rounds and culture with IL-2 cytokine drive cells to exhaustion as mentioned above and late stages of differentiation which may affect cellular persistence and long-term functionality *in vivo* [204, 205]. On the other hand, rapid expansion with direct stimulation of PBMCs by viral peptide mixes and subsequent culture in a closed bioreactor offers a fast and simple approach. Alternatively, cells can be isolated by immunomagnetic selection: either by direct staining binding to EBV-antigen loaded MHC-class I-multimers or by EBV peptide pool stimulation prior to selection through activation markers or cytokine capture [152]. The drawback of this method, however, is low number of isolated cells.

Limitations of ACT

Some key challenges of ACT products include:

1. cytokine release syndrome (CRS, cytokine storm) and [206]neurotoxicity due to a massive cytolytic activity after therapeutic cell infusion [207, 208]. CRS is usually triggered by increase of TNFα, IFNγ, IL-1b, IL-2, IL-6, IL-8, and IL-10 although the central role is attributed to patient's monocyte-derived IL-6 and IL-1 [209] blockade of which is used for CRS prevention and treatment [210].

   Neurotoxicity is not understood in detail but it is thought to develop as a result of severe inflammation in endothelium of the central nervous system (CNS) which is also associated with cytokine release. The treatment options are administration of corticosteroids (inhibit pro-inflammatory cytokine production) as well as blockade of IL-1, IL-6 and granulocyte macrophage-colony stimulating factor (GM-CSF) [211].

2. Tumor antigen escape. Potential tumor antigen escape is a serious limitation especially for CAR cell therapies and TCR-C therapies where usually a single antigen is targeted [206, 212]. Development of polyclonal and polyspecific therapies might increase the probability of tumor recognition [213].
3. On-target, off-tumor cytotoxicity is a result of targeting cells by ACT that express the same antigen as the tumor [214]. For example, CD33-CAR T cells expressed severe on-target, off-tumor toxicity due to CD33 expression on healthy hematopoietic stem cells and on hepatocytes [215]. In order to overcome the risk of such a side effect, neoantigens (antigens expressed only by a tumor) should be investigated [216].
4. Mutations in the ACT products that can be caused not only by viral integration into protooncogene but also through the off-target gene integration which can occur even with CRISPR/Cas9 technology [217] – all this can lead to ACT-associated cancer development. To mitigate this risk, more precise Cas9 nucleases can be developed [218], safe harbor loci can be targeted (loci that are located far from any vital genes and oncogenes and thus even off-target transgene integration would not lead to the cell dysregulation) [219], or suicide genes can be introduced which allows depletion of engineered cells when they show an increased risk after patient infusion [220].
5. The major challenge of ACT is the poor ability of therapeutic effector T cells to persist long-term in order to control tumor outgrowth and prevent relapses [221, 222]. This subject is addressed in detail below.

Key characteristics of an ACT product

Main qualities determining a successful outcome of ACT are functionality and persistence [222] which correlates with better outcomes [223].

Memory composition. After antigen stimulation and activation, T cells undergo a series of cell proliferation and differentiation stages, from naïve to terminally differentiated, short-lived effector T cells (T_{EMRA}) (Figure 12). During differentiation, the effector function of T cells gradually increases, while their self-renewal capacity correspondingly declines [224]. As one of the major challenges of adoptively transferred T cells is their poor ability to persist *in vivo* upon the infusion., early

differentiated i.e., central memory (T_{CM}) and particularly stem cell memory T cells (T_{SCM}) became relevant for the adoptive therapies due to their high proliferation, engraftment, and persistence potential in different human diseases and have shown promising results in ACT against cancer [225]. This topic is addressed in more detail in chapter II.

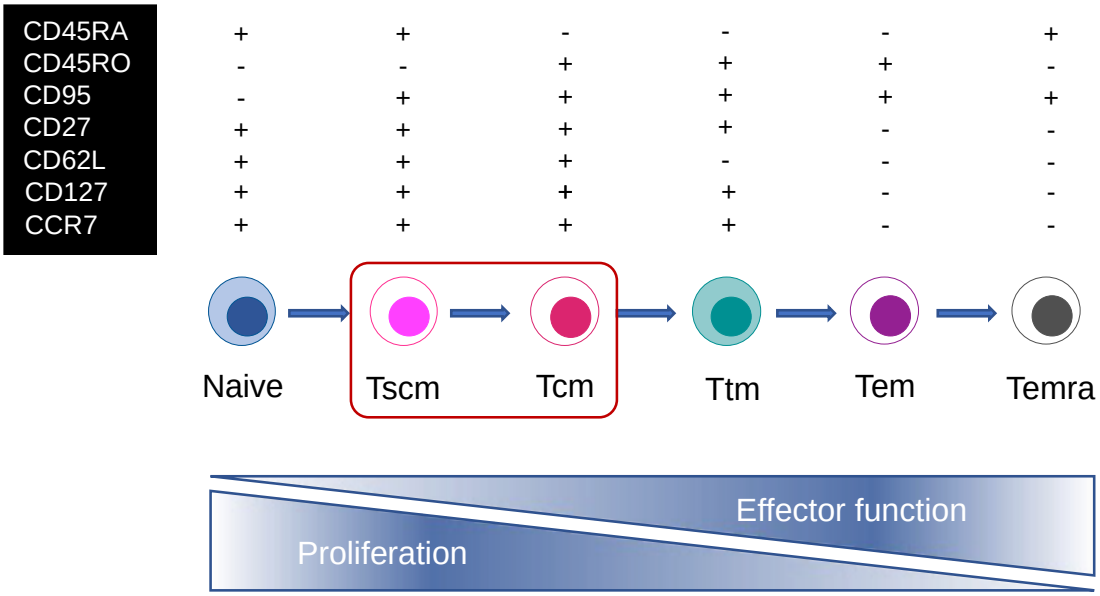

Figure 12. Differentiation stages of T cells after antigenic stimulation and some of the markers defining them. Tscm – stem cell memory, Tcm – central memory, Tem – effector memory, Temra – terminally differentiated T cells. Summarized from [224, 226]

Different approaches to maintain early differentiation stages in ex vivo ACT preparations can be applied. It has been shown that culture with IL-7, IL-15 or IL-21 cytokines can result in decreased T cell differentiation [227-229]. Furthermore, signaling pathways can be modulated by supplementation with special inhibitory/ inducing agents. For example, normally, Glycogen synthase kinase-3 beta (GSK3β) blocks β-catenin pathway. Inhibiting GSK3β results in the pathway induction which suppresses T cell differentiation without blocking their expansion [230]. Thus, T_{SCM} T cells can be enriched. Alternatively, an elegant method was discovered by the Restifo group. They studied the melanoma tumor microenvironment (TME) and discovered that TILs accumulated there possess stem cell characteristics. This was due to increased potassium concentration in the TME as a result of tumor cell necrosis. This induced metabolic starvation of the surrounding TILs which blocked the synthetic processes and thus suppressed the differentiation (and cytotoxicity). Culture in the

elevated potassium conditions ex vivo also resulted in stemness preservation of T cells [202].

Exhaustion. Exhaustion is a progressively declining T cell function owing to chronic TCR stimulation in the setting of persistent antigen exposure. Exhausted T cells are characterized by reduced cytokine production and expression of inhibitory receptors (exhaustion markers) such as CTLA-4, LAG-3, PD-1, TIGIT, TIM-3, *etc.* [231]. Tumor-infiltrating lymphocytes are often expressing these markers due to the chronic antigen exposure and are unable of killing the tumors because of their exhausted state [232]. In ACT setting, exhausted cells do not only show reduced functionality but also do not persist and are associated with inferior outcomes in patients [233]. As discussed above, chronic antigen stimulation and culture with IL-2 cytokine drive cells to exhaustion and late stages of differentiation [204, 205], thus these should be avoided in the ACT preparation.

CD4/CD8 balance. A key aspect of an ACT product is the balance between CD4+ and CD8+ populations [46, 234]. In the early T cell therapies, researchers focused on CD8+ CTLs for ACT because of their cytotoxic role [234]. However, there is a substantial amount of data indicating a key role of CD4+ T cells in cancer immunity. Studies in murine models have shown that effective CD8+ T cell responses against MHC class-II-negative tumors required the helper function of CD4+ T cells [235]. CD4+ helper T cells can suppress inhibitory receptors in CD8+ T cells and are essential for the formation of functional CD8+ memory T cells [236, 237]. Moreover, Th1-CD4+ T cells can display cytolytic activity and provide effective cancer immunity upon the adoptive transfer [238, 239]. Patients with a higher CD4/CD8 ratios of adoptively transferred virus-specific T cells show better clinical outcomes against PTLD [240]. EBV Virion antigen-specific CD4+ T cells proved to be cytolytic and able to prevent the outgrowth of primary B cells infected with EBV in vitro. Thus, virion antigen-specific CD4+ T cells might aid in controlling EBV infection *in vivo* [46, 241]. CD4+ EBV-specific T cells were also shown to equally well control EBV PTLD in mice compared to CD8+ T cells [242].

Clonal diversity. It was shown in multiple studies that broader repertoire may provide choices that allow for the selection of higher avidity clones [243]. Higher clonal diversity of HIV-specific CD8+ cells in HIV-infected patients correlated with slower disease

progression [244]. Furthermore, patients receiving polyclonal EBV-CTL have improved responses against EBV PTLD [245]. High polyclonality and polyspecificity of infused T cells might circumvent tumor and viral immune evasion mechanisms [246, 247].

Lineage tracing of infused therapeutic cells

Single cell antigen specificity and clonality and phenotype characterization can be assessed prior to transfer through methods such as flow cytometry, ELISPOT and TCR RNA- or transcriptome-sequencing. These assessments become especially important during treatment. Beyond monitoring needs, the ability to identify the most therapeutically-relevant clones and phenotypes is of significant interest, particularly for long term efficacy. Recently, it was shown that CAR T cells can persist in patients as many as 10 years after infusion [248]. Lineage tracing of infused therapeutic cells should facilitate the investigation of therapeutically relevant clones: their identification, clonal and phenotypic characterization. His topic is addressed I Chapter II.

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AIMS OF THE THESIS

In this work, I present two different strategies to improve EBV-specific T cell therapy.

Chapter I – investigating the potential of T_{SCM}-enriched EBV-CTLs

In this project, I hypothesized that EBV-specific early differentiated T cells can be enriched from donor-derived PBMCs and restore T cell immunity to EBV and treat it more efficiently than conventionally *ex vivo* expanded EBV CTLs, due to their robust proliferation and persistence.
I aimed to establish a simple and robust, clinically applicable protocol for rapid expansion of EBV-CTLs (cytotoxic T cell lines) that enriches for the T_{SCM} population and to investigate the potential of EBV control by EBV-specific T_{SCM} virus-specific T cells *in vitro* and *in vivo* compared to conventionally *ex vivo* expanded EBV CTLs.

Chapter II – developing a method of precise transgene integration into PBMC-derived EBV-CTLs

Here, I hypothesized that CRISPR/Cas9 so far used to insert transgene only in bulk T cells can be directed to edit antigen-stimulated and activated virus-specific T cells. I aimed at devising a method of polyclonal antigen-specific T cell-targeted genome editing for adoptive therapy applications utilizing T cells with native TCRs.

CHAPTER I. Investigating the potential of T_{SCM}-enriched EBV-CTLs

The following manuscript is a preprint for submission to *Blood*.

Stem-cell memory EBV-specific T cells control post-transplant lymphoproliferative disease and persist *in vivo*

Darya Palianina[1], Juliane Mietz[2], Claudia Stühler[1], Brice Arnold[1], Glenn Bantug[1], Christoph Hess[1], Christian Münz[2], Obinna Chijioke[2], Nina Khanna[1]

[1] Department of Biomedicine, University and University Hospital of Basel, Switzerland
[2] Institute of Experimental Immunology, University of Zurich, Switzerland

ABSTRACT

Adoptive T cell therapy (ACT) intends to therapeutically transfer defined T cell immunity to patients and offer great potential in the fight against different human diseases including difficult-to-treat viral infections. Viral infection predominately those developing after transplantation are associated with poor prognosis. Patients who fail standard therapies have limited therapeutic options, representing a significant unmet medical need. Viral proteins are recognized by T cells providing opportunities for virus-specific T-cell therapy (VST). Indeed, infusion of VST e.g., for Epstein-Barr virus (EBV) increases overall survival, but ~30% of patients show no response indicating a need for further improvements. Recently, very early differentiated i.e., stem cell memory T cells (T_{SCM}) became relevant due to their high self-renewal, engraftment, and persistence potential in different human diseases and have shown promising results in ACT against cancer.

Here, we developed a clinically-scalable protocol for T_{SCM}-enriched expansion of Epstein-Barr virus (EBV)-specific T cells and compared its anti-tumor efficiency with conventionally expanded, EBV-transformed lymphoblastoid cell line-stimulated T cells in the *in vivo* model of post-transplant lymphoproliferative disorder (PTLD). Rapidly expanded T_{SCM}-enriched EBV-specific T cells efficiently controlled the PTLD, showed

a better tumor infiltration rate, robust *in vivo* proliferation and persistence potential with functional CD4+ and CD8+ cells and a broader reconstitution of EBV specificity. The method and these data together should help to establish the next generation of unmodified antigen-specific cell therapies beyond EBV diseases.

INTRODUCTION

Cell therapies have become one of the most promising strategies in treatment of hemato-oncological diseases [1, 2] and offer a great potential in the fight against difficult-to-treat viral infections and autoimmune diseases [3, 4]. One of the most important properties for the efficacy of cellular therapies is their ability to be activated in response to antigens and persist *in vivo* for sustained defence [5]. After antigen stimulation and activation, T cells undergo a series of cell proliferation and differentiation stages, from naïve to stem cell memory (T_{SCM}), central memory (T_{CM}), transitional memory (T_{TM}) effector memory (T_{EM}), and terminally differentiated, short-lived effector T cells (T_{EMRA}) [6]. During differentiation, the effector function of T cells gradually increases, while their self-renewal capacity correspondingly declines [7]. Thus, early differentiated T cells may be promising for cell therapy because of their longevity and robust proliferative potential.

Indeed, multiple studies have shown that adoptively transferred stem cell memory chimeric antigen receptor (CAR) T cells, engineered T-cell receptor (TCR)-T cells and tumour-infiltrating lymphocytes (TILs) can self-renew *in vivo* and exhibit improved anti-tumour capabilities [8]. The dominant clones of genetically modified lymphocytes preferentially originate from early differentiated phenotypes and have the ability to persist for up to 12 years in patients after infusion [9]. Moreover, early memory phenotype of infused therapeutic CAR T cells has been associated with complete and durable responses [10], early proliferation and long-term [11]. Regarding infection control, long-lasting antigen-specific T_{SCM} were identified after yellow fever and bacillus Calmette–Guerin (BCG) vaccination [12] [13]. In addition, it has been shown that CD8+ T_{SCM} support T-cell responses during chronic LCMV infection [14] and were associated with improved prognosis in chronic HIV-1 infection [15].

Viral infections remain an important cause of morbidity and mortality, especially after transplantation, and treatment options are often limited. Adoptive therapies with virus-

specific T cells (VST) have shown to be promising in restoring virus-specific immunity and thereby preventing and treating viral infections over the past 25 years [16, 17].
Donor-derived Epstein Barr virus (EBV)-specific cytotoxic T-cell lines (CTLs) have demonstrated prolonged overall survival in patients with EBV-driven post-transplant lymphoproliferative disease (PTLD), lymphomas and diseases, but approximately 30% of patients show no response indicating a need for further improvements [18]. In addition to clinical factors (e.g., disease state, net state of immunosuppression), limited long-term efficacy, including low persistence capacity and increased exhaustion of adoptively transferred T cells, may account for lower response rates. Most clinical applications for EBV lymphomas are based on VST using a long-term expansion protocol relying on continuous re-stimulation with genetically modified or EBV-transformed lymphoblastoid cell lines (LCLs) [19], potentially driving the cells to late differentiation stages and exhaustion [20]. In the last decade, a rapid expansion method using a single stimulation with a viral peptide mixture has been introduced for the generation of multi-virus-specific T cells, but information on memory composition of the T cell products and persistence for EBV lymphomas is lacking [21, 22].
Therefore, in this study, we aimed to establish a simple and robust, clinically applicable protocol for rapid expansion of EBV-CTLs (cytotoxic T cell lines) that enriches for T_{SCM} and to investigate the potential of EBV control by EBV-specific T_{SCM} VSTs *in vitro* and *in vivo* compared to conventionally *ex vivo* expanded EBV CTLs. We hypothesize that EBV-specific early differentiated T cells can restore T cell immunity to EBV and treat it more efficiently than conventionally *ex vivo* expanded EBV CTLs, due to their robust proliferation and persistence.

METHODS

Peptides

The EBV target specificity were assessed using the EBV Consensus peptide pool (Miltenyi Biotec), and single peptides from various EBV antigens (latent: EBNA-LP, EBNA2, EBNA3a, EBNA3b, EBNA3c, LMP1; lytic: BARF1, BMLF1, BMRF1, BRLF1, BZLF1, GP350/GP340). Peptide libraries were purchased from JPT Peptide Technologies or Miltenyi Biotec.

Blood donors, cell culture and generation and expansion of EBV-specific T-cell lines

Blood was obtained after informed consent from healthy donors in accordance with the Declaration of Helsinki. The study was approved by the local ethic committee (Ethikkommission Nordwest- und Zentralschweiz, Project ID PB_2018-00081). Donors were typed for HLA class I and class II alleles. Human peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood of healthy donors and [23] EBV-transformed lymphoblastoid cell lines (LCL) were generated and cultured in LCM-10 media according to previously published protocols[24] (Supplemental Materials). Long-term EBV-CTL expansion with LCL re-stimulations and rapid expansion protocols were performed as previously described [19] [21]. LCL and T cell cultures are described in Supplemental Methods.

In vitro assays

All *in vitro* assays are described in Supplemental methods.

Procedures in vivo

Animal experiments were conducted according to the licence approved by the veterinary office of the canton of Zurich, Switzerland (ZH049/20). NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (#005557)) or NSG-A2 (NOD.Cg-*Mcph1*$^{Tg(HLA-A2.1)1Enge}$ *Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (#009617)) mice were purchased from The Jackson Laboratory and bred and housed under specific pathogen-free conditions at the Laboratory Animal Services Center (LASC) Zurich. Experiments were initiated at 6-12 weeks of age. LCL tumors were injected subcutaneously into the left flank under isoflurane narcosis. $2x10^6$ tumor cells were resuspended in PBS and right before injection mixed in a 1:1 V/V ratio with Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Milian). Three days after tumor injection, $1x10^7$ T cells were adoptively transferred by tail vein injection. T cell expansion was supported by i.p. injection of 10^5 IU recombinant huIL-2 (3x/week, Peprotech), or as stated otherwise. Tumor size was monitored by calipering (3x/week) and bioluminescent imaging (2x/week). General health was monitored by weighing and health parameter scoring 3x/week or daily, according to the animal license. Peripheral blood composition and expansion of adoptively transferred T cells were monitored by weekly tail vein bleeding and flow cytometric analysis (Supplementary Materials) on BD Fortessa. White blood cell counts were determined from full blood with an automatic cell counting machine (DxH 500, Beckman Coulter). For bioluminescent imaging, mice were injected with 5μl/g body

weight of 15mg/ml VivoGlo™ Luciferin (Promega) and imaged 10 minutes after injection in an IVIS machine (PerkinElmer) under isoflurane narcosis. Animals were euthanized when they met pre-defined criteria stated in the animal license, or when the control group met the end-point criteria.

Statistics

Analyses were conducted using Prism software (GraphPad). Data of individual donors are shown as representative experiments or means with SD. Combined data of different donors are given as median with range.

RESULTS

Rapid expansion in the presence of IL-4 / IL-7 and TWS-119 yields the highest proportion of T_{SCM}

In order to enrich antigen-specific T_{SCM} we tested various cytokines including IL-7, IL-15, and IL-21, which promote T cell growth but limit differentiation [21, 25, 26], potassium-rich medium that can promote T cell stemness preservation [27], as well as a glycogen synthase kinase-3β (GSK3β) inhibitor TWS119, which induces Wnt-beta-catenin signaling and promotes generation of T_{SCM} by limiting cell differentiation [28].

We adopted the previously established rapid expansion approach [21] and stimulated PBMC of healthy EBV-seropositive donors with the EBV consensus peptide pool containing 43 peptides of 8-20 aa in length deriving from 13 different lytic and latent EBV-proteins restricted to 14 frequent HLA-class I and II molecules in presence of different cytokines in the G-Rex cell culture device for 10 days (Fig. 1, A).

T_{SCM} population among expanded EBV-CTLs was defined as CD45RA+CD45RO-CD62L+CD27+ using FlowSOM algorithm in FlowJo [29] (Supplemental Fig. 1, A).

Expansion in presence of IL-4 / IL-7 with or without IL-21 yielded the highest proportion of T_{SCM} (Fig. 1, B). Combinations with IL-15 resulted in low T-cell purity and high enrichment of NK and NKT cells (Supplemental Fig. 2, A-C).

Expansion with increased potassium or TWS-119 further enriched T_{SCM}, with the IL-4/IL-7/TWS-119 condition yielding the highest percentage of T_{SCM} (median 29%) (Fig. 1, B). with similar proportions of CD4+ and CD8+ T cells (Supplemental Fig. 1, B).

Next, we investigated the expansion rates of EBV-specific T cells using Elispot assay and MHC class I-EBV-multimer staining (Fig. 1, C, Supplemental Fig. 3, A), which were similar in all tested conditions. We observed comparable total cell expansion folds and CD4/CD8 ratios between the conditions (Supplemental Fig. 3, B-C).

While short-term cytotoxicity against EBV-lymphoblastoid cell lines (LCLs) was generally lower in the higher T_{SCM}-containing conditions, likely due to the slower activation rate of the early differentiated T cells (Fig. 1, D), long-term outgrowth assay showed a similar ability of all CTL groups to control LCLs (Fig. 1, E).

Furthermore, we observed decreased expression of exhaustion markers in the T_{SCM}-enriched CTLs; this was particularly significant under IL-4 / IL-7 / TWS-119 conditions (Fig. 1, F).

Although the IL-4 / IL-7 / IL-21 condition exhibited higher short-term cytotoxicity than the condition without IL-21, it was not better in long-term LCL outgrowth control, and the cells showed higher PD-1 expression. Because this condition did not yield higher T_{SCM} enrichment, we considered IL-21 supplementation to be dispensable.

Collectively, these data demonstrate that a protocol using IL-4 / IL-7 supplemented with TWS119 yields promising EBV-specific CTLs with favorable properties for virus-specific T-cell therapy such as high proportion of T_{SCM}, low exhaustion and efficient long-term *in vitro* cytotoxicity.

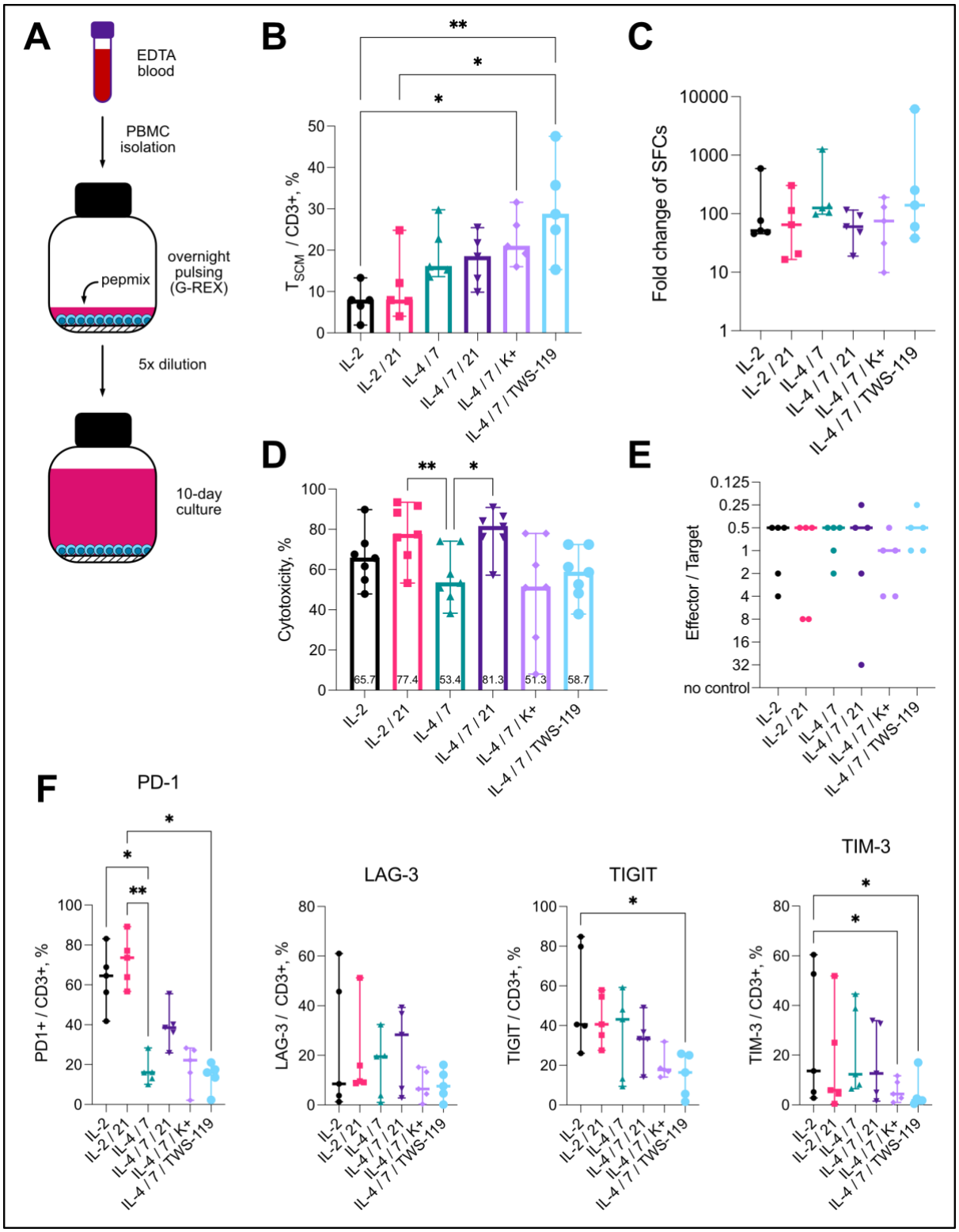

Figure 1. Establishing rapid T_{SCM}-enriched EBV-CTL *ex vivo* expansion protocol. (A) Adopted rapid expansion approach. Isolated PBMCs were stimulated with EBV Consensus pepmix in complete media. After overnight pulsing, pepmix was diluted 5x with complete media following 10-day incubation. (B) T_{SCM} proportions after culturing EBV-CTLs in the presence of different conditions (different cytokine combinations, in elevated potassium concentration (K+) or with the edition of TWS-119) as detected by flow cytometry; n=5, medians with range. (C) Expansion folds (PBMCs vs. after rapid expansion) of

spot-forming cells after culturing in different conditions; IFNγ ELISPOT with EBV pepmix stimulation, n=5, medians with range. (D) Short-term cytotoxicity, medians with range. (E) 4-week EBV-LCL outgrowth control by expanded T cells. Cells were incubated at different effector : target ratios, and the outgrowth-controlling E:T ratios were determined microscopically and confirmed by flow cytometry; n=5; medians of controlling E : T were shown. (F) PD-1, LAG-3, TIGIT and TIM-3, exhaustion marker expression of expanded CTLs; n=5, medians with range. B-F were analyzed by Friedman test, α=0.05, non-significant p-values (ns) not shown, * correspond to $p<0.05$, ** - $p<0.005$, etc..

CD4+ and CD8+ expanded T_{SCM} are EBV-specific and proliferate in response to restimulation

T_{SCM} are a population of very early differentiated T cells that represent only 2-4% of total T cells in peripheral blood [30]. There is limited evidence for their antigen specificity. Our next objective was therefore to investigate the presence of EBV-specific T cells within the stem cell memory compartment of expanded CTLs. We sorted different memory populations of IL-4 / 7 / TWS-119-expanded CTLs with a purity over 95% (Fig. 2, A). Recovered memory populations were stained for proliferation tracing and co-cultured with irradiated autologous EBV-transformed LCLs for one week at an effector : target ratio 1:1. Proliferations of CD4+ as well as CD8+ T cells were observed in all memory populations. In general, the proliferation capacity was higher among CD8+ T cells. The EBV-specificity of proliferating T_{SCM} cells was confirmed by restimulation with EBV pepmix (Fig. 2, B) and by staining with MHC-class I EBV multimers (Fig. 2, C).

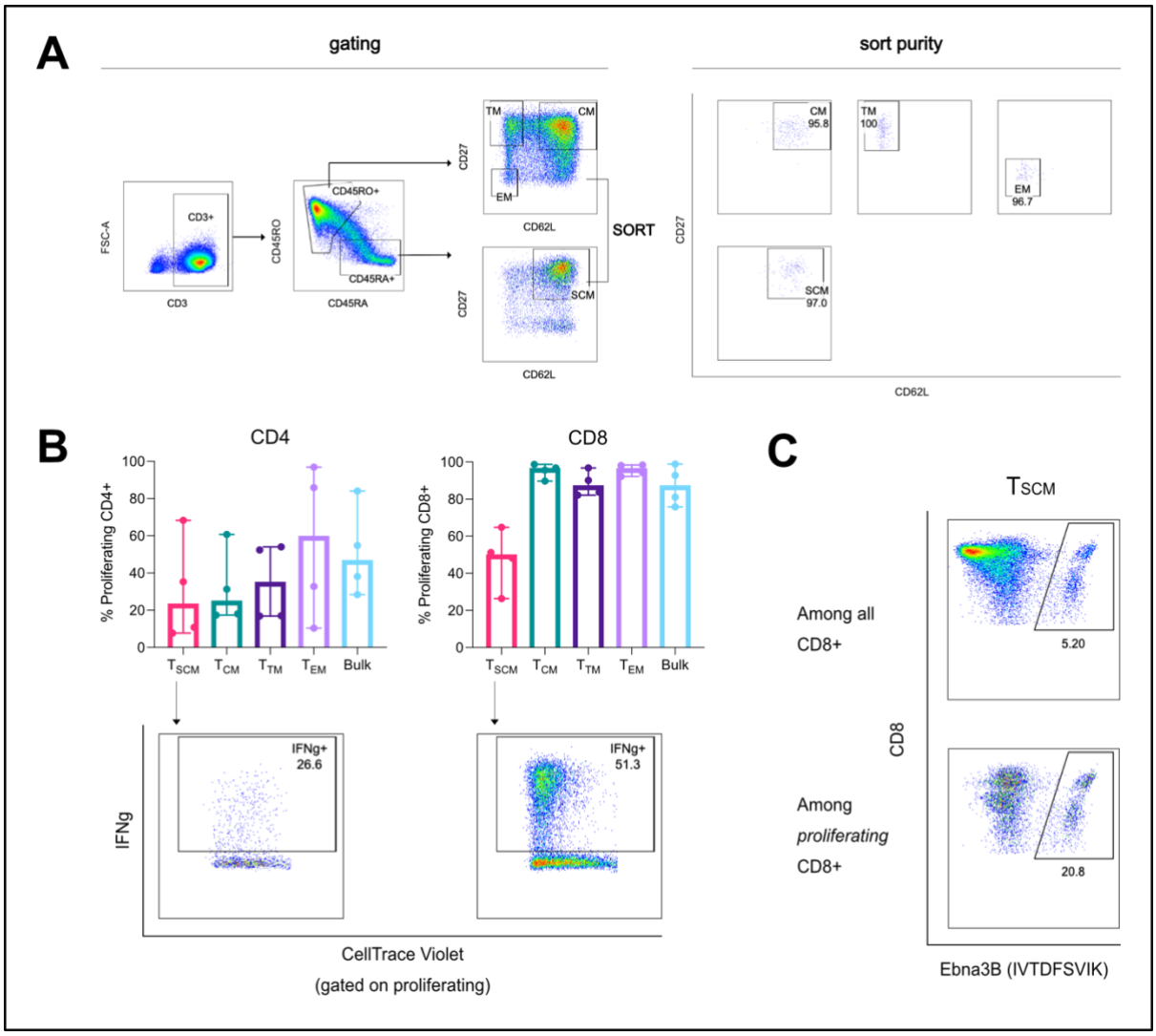

Figure 2. EBV-specific T cells among T_{SCM}. (A) Sorting of different memory population: gating strategy and representative plots of sort purity. Proliferation of sorted and CTV-stained CD4+ and CD8+ populations after 7-day co-culture of the sorted populations with EBV-LCLs (flow cytometry, n=4, medians with range) and specificity of proliferating T_{SCM} cells (IFNγ expression upon re-stimulation with EBV pepmix, representative plot). (C) Proliferation of specific T_{SCM} (stained with a respective MHC class I multimer, representative plot. Proliferating cells in B-C are all cells which proliferated once or more. Proliferating cells in B-C are cells that proliferated at least once. SCM – stem cell memory, CM – central memory, TM – transitional memory, EM – effector memory.

Expanded T_{SCM}-enriched EBV-CTLs exhibit a more favorable phenotype and broader antigen specificity compared to EBV-LCL-expanded CTL

The most widely used and published clinical protocol to date for the manufacturing of EBV CTLs uses EBV-transformed LCLs as antigen-presenting cells (APCs) [18, 19].

As LCLs exhibit EBV type III latency, they express all latent antigens [31] thus being suitable APCs for many EBV-associated pathologies. Unlike rapid expansion (CTL-R), conventional EBV-CTL expansion (CTL-L) involves long-term culture in the presence of IL-2 and multiple restimulations with EBV-LCLs (Fig. 3, A). We compared the two protocols to evaluate the differences regarding specificity and phenotypes.

The total expansion rate of CTLs was higher in CTL-L than in CTL-R (Fig. 3, B) with a higher CD4+ T cell proportion in the CTL-R compared with CTL-L (Fig. 3, C). Although the overall specificity of expanded T cells to EBV consensus peptide pool was similar in both conditions (Fig. 3, D-E), the CTL-R exhibited a broader single-antigen specificity for both latent and lytic peptides (Fig. 3, F). Notably, T cells specific for lytic BARF-1, BMLF-1, BMRF-1, BRLF-1 and GP350 were exclusively present in CTL-R (Fig. 3, F).

As presumed, the memory phenotypes differed considerably: whereas CTL-R consisted largely of earlier differentiation stages (T_{SCM} and T_{CM}), CTL-L resulted mainly in later differentiation stages such as T_{TM} and T_{EM} (Fig. 3, G). Accordingly, exhaustion markers such as PD-1 and TIGIT were significantly more expressed in CTL-L, and a large proportion of them were double positive for both markers (Fig. 3, H).

Taken together, while CTL-L show a higher expansion rate, T_{SCM}-enriched CTL-R cover a broader antigen specificity diversity and exhibit a more favorable memory and exhaustion phenotype.

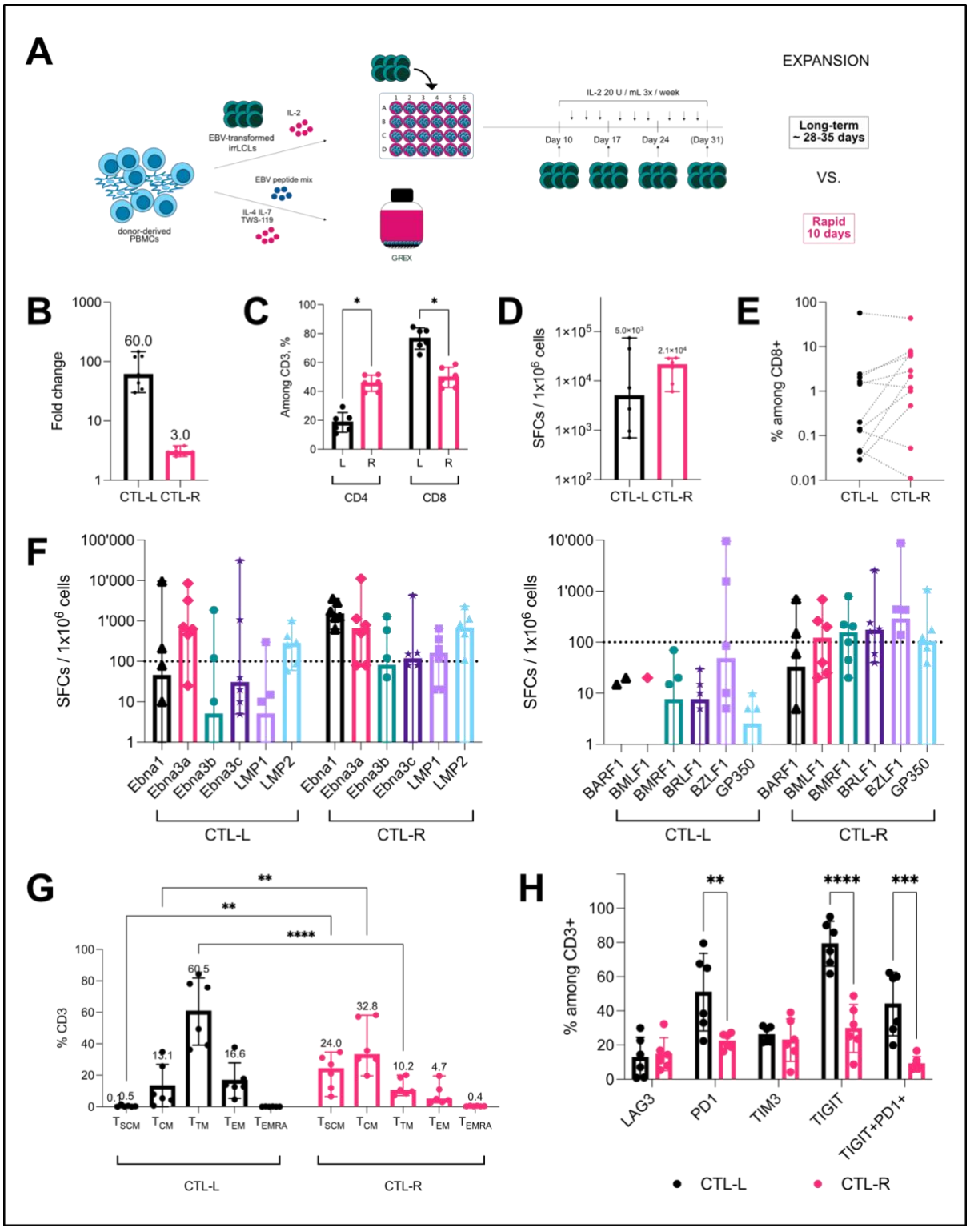

Figure 3. Comparison of rapidly expanded (CTL-R) and long-term (CTL-L) conventionally expanded EBV-CTLs. (A) Schematic of two expansion methods. Traditional long-term expansion protocol of polyspecific EBV-CTLs involves a 10-day incubation of PBMCs with autologous irradiated EBV-LCLs at a 40:1 E:T ratio without cytokines following rounds of weekly LCL re-stimulation (E:T= 4:1) and regular IL-2 re-supplementations. (B) Expansion rates of total cells. n=7, medians with range. (C) CD4+ and CD8+ proportions in expanded cells. 2way ANOVA, n=6, means with standard deviation (SD). (D) Frequencies of EBV-specific T cells in the expanded products, IFNγ ELISpot after re-

stimulation with EBV pepmix, the dotted line indicates a threshold; n=6, medians with range, Wilcoxon matched pairs signed-rank test. (E) Pair-wise comparisons of proportions of different single EBV antigen-specific T cells measured by respective MHC class I-multimer staining, flow cytometry. n=11, Wilcoxon matched pairs signed-rank test. (F) Frequencies of single protein-specific T cells in the expanded products (latent – left graph, lytic – right graph). IFNγ ELISpot after re-stimulation with peptide pools derived from single EBV proteins, the dotted line indicates a threshold. n=6, medians with range, Wilcoxon matched pairs signed-rank test. (G) Memory phenotypes and (H) exhaustion marker expression, flow cytometry. n=6, means with SD, 2way ANOVA. For C-H: α=0.05, non-significant p-values (ns) not shown, * correspond to p<0.05, ** - p<0.005, *etc*.

T_{SCM}-enriched EBV-CTLs control tumor growth, proliferate, persist and release pro-inflammatory cytokine *in vivo*

Based on the promising data *in vitro*, we next investigated the ability of tumor control and persistence of T_{SCM}-enriched EBV-CTL *in vivo*. We adopted a well-established mouse model mimicking EBV-driven post-transplant lymphoproliferative disease (PTLD) by using EBV-LCLs as tumor cells [32]. Autologous CTL-L or CTL-R and luciferase-expressing LCLs were injected at a ratio of 5:1 into NSG mice supplemented with high doses of human IL-2 to support T cells in the NSG system (Fig. 4, A). Tumor growth dynamic tracked by calipering and bioluminescence showed that both CTL expansion protocols successfully controlled tumor growth three weeks after tumor seeding and beyond (Fig. 4, B-C). We observed that 23% of the mice in the CTL-R groups lost weight approximately 4 weeks after tumor seeding compared with the CTL-L groups (supplemental Fig. 5), which was accompanied by a significant increase in white blood cells (WBCs) (Fig. 4, D). Accordingly, the spleen weights and splenocytes counts were significantly higher in the CTL-R groups than in the CTL-L and tumor-only groups after sacrifice on day 28/30 (Fig. 4, E, supplemental Fig. 4, A). A higher proportion of human CD45+ (hCD45+) cells was detected in all organs of the CTL-R groups, most of which were CD3+ indicating a strong *in vivo* expansion of CTL-R T cells (Fig. 4, F, Supplemental Fig. 4, B). In contrast, in the CTL-L groups, the proportion of CD3+ among hCD45+ cells was lower and, consequently, the proportion of CD19+ was higher indicating a poorer tumor cell control compared with CTL-R. T cells in the CTL-L group were predominantly composed of CD8+ cells. In contrast, while CD8+ T cells also expanded more in the CTL-R groups during the first two weeks, CD8+/CD4+ ratios returned to pre-infusion levels towards the end of the experiment (Fig. 4, F, supplemental Fig. 4, C).

In line with the observed results, we found increased IFNγ and TNFα levels in the sera of the CTL-R mice compared to the CTL-L mice corresponding to the greater proliferation of CTL-R T cells (Fig. 4, G).

These data provide evidence that both long-term and rapidly expanded EBV CTLs efficiently control tumor growth, whereas only T_{SCM}-enriched CTLs eradicate tumor cells in organs owing to their better ability to expand CD4+ and CD8+ T cells and persist *in vivo*.

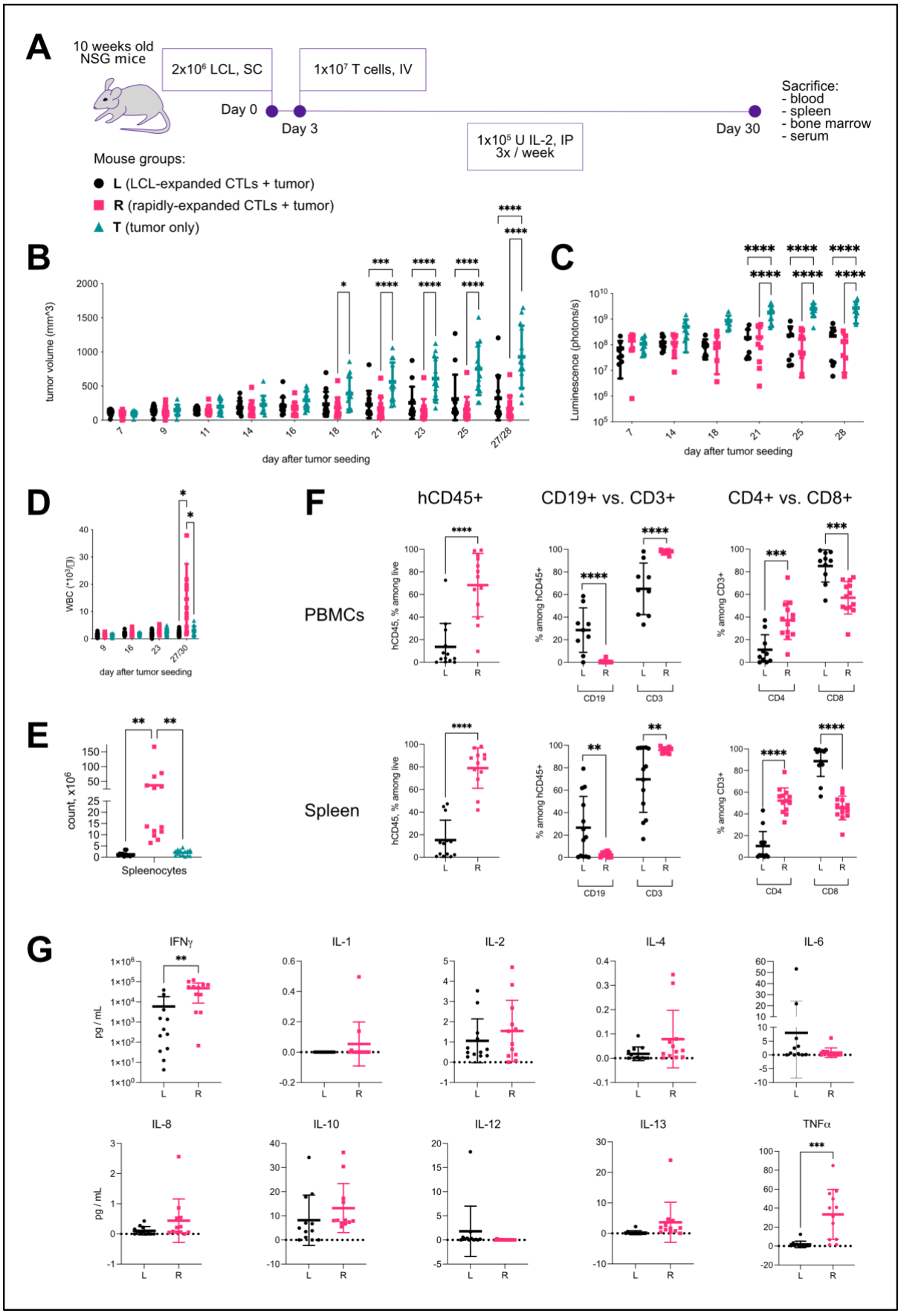

Figure 4. Expansion of CTL-R *in vivo*. (A) Schematic of the in vivo experiments. 2x10^6 tumor cells (luciferase-expressing EBV-LCLs) / mouse were injected into NSG mice subcutaneously, and on day 3

$1x10^7$ autologous long-term or rapidly expanded EBV-CTLs per mouse were infused intravenously. Groups of tumor-only mice were kept as a negative control. All mice were supplemented with $1x10^5$ U / hIL-2 3x / week. Mice were sacrificed after ~4 weeks and organs were collected. Pooled data from three independent experiments (13 mice / group, 4-5 mice / group / per experiment, 1 different PBMC donor / experiment) is shown in further plots unless there was no sample available. Data from CTL-L-injected mice marked in black circles, CTL-R – in pink squares, and tumor-only mice – in green-blue triangles. Tumor growth dynamic measured by calipering ~3x / week (B) and tumor luminescence measured 1-2x / week (C). (D) In vivo white blood cell (WBC) expansion dynamic, flow cytometry of weekly bleedings. (E) Splenocyte counts after sacrifice. (F) Proportions of human CD45+ cells, CD3+ and CD19+ among them, and CD4+ and CD8+ among human CD3+, fow cytometry. (G) Analysis of human cytokines in the murine sera collected after sacrifice, Mesoscale. For B-F, mixed-effects analysis, α=0.05, non-significant p-values (ns) not shown, * correspond to p<0.05, ** - p<0.005, *etc*.

T_{SCM}-enriched EBV CTLs infiltrate the tumor more efficiently and reconstitute broad antigen specificity in vivo

To investigate the specificity of *in vivo* expanded EBV CTLs, we first examined tumor infiltration by immunohistochemistry (IHC) in tumor samples (Fig. 5, A). We found high CD8+ infiltration in both CTL-L and CTL-R groups with a trend toward higher infiltration in the CTL-R group (Fig. 5, B). CD4+ infiltration was not assessed by IHC. There was a negative correlation between tumor volume and infiltration of CD8+ T cells, i.e., tumor size decreased with increasing infiltration of CD8+ T cells (Fig. 5, C).

To describe the profile of tumor-infiltrating lymphocytes (TILs) in more detail, we performed flow cytometric analysis. A significantly higher CD3+ infiltration of CTL-R compared with CTL-L (Fig. 5, D) was observed, and furthermore, we found CD4+ among CTL-R TILs, whereas this population was almost absent in CTL-L groups (Fig. 5 E).

We further compared the memory phenotypes and antigen specificity of TILs between the two treatment groups and also between T cells from different organs (Fig. 5, F, Supplemental Fig. 6, A-C). We observed that CTL-R TILs exhibited a less differentiated phenotype than CTL-L. While almost no T_{SCM} cells were found in either group, we still noted a higher proportion of T_{CM} cells among CTL-R TILs. Interestingly, the TILs in both groups had a more differentiated phenotype than in the organs, and a T_{EM} phenotype dominated in contrast to the organ-resident cells, where the T_{TM} phenotype predominated. With regard to antigen specificity, the CTL-R group had a higher proportion of EBV-specific TILs than CTL-L cells and splenocytes of either group (Fig. 5, G).

We next investigated the contribution of CD4+ and CD8+ CTL-R responses *in vivo*. CD4+ and CD8+ splenocytes were separated after sacrifice and short-term *in vitro* cytotoxicity against EBV-LCLs was performed. These experiments were limited to the CTL-R group because there was not enough material from CTL-L groups, as they did not expand *in vivo*. Both CD4+ and CD8+ CTL-R splenocytes performed equitably (Fig. 5, H, pooled data from three donors). We then analyzed the specificity for 14 different EBV proteins using ELISpot and compared the presence of specific T-cell responses between bulk, CD4+, and CD8+ splenocytes from a CTL-R group with CTL-L and CTL-R data before injection (Fig. 5, I, Supplemental Fig. 7; pooled data from one donor). Bulk CTL-R splenocytes showed higher EBV specificity than both CTLs before injection. Strikingly, CD4+ CTL-R splenocytes showed a more diverse response to EBV antigens than CD8+ splenocytes. This substantiates the robust proliferation potential and ability to reconstitute a wide antigen diversity of the different T cell compartments and the longevity of the EBV-T_{SCM}-enriched CTLs.

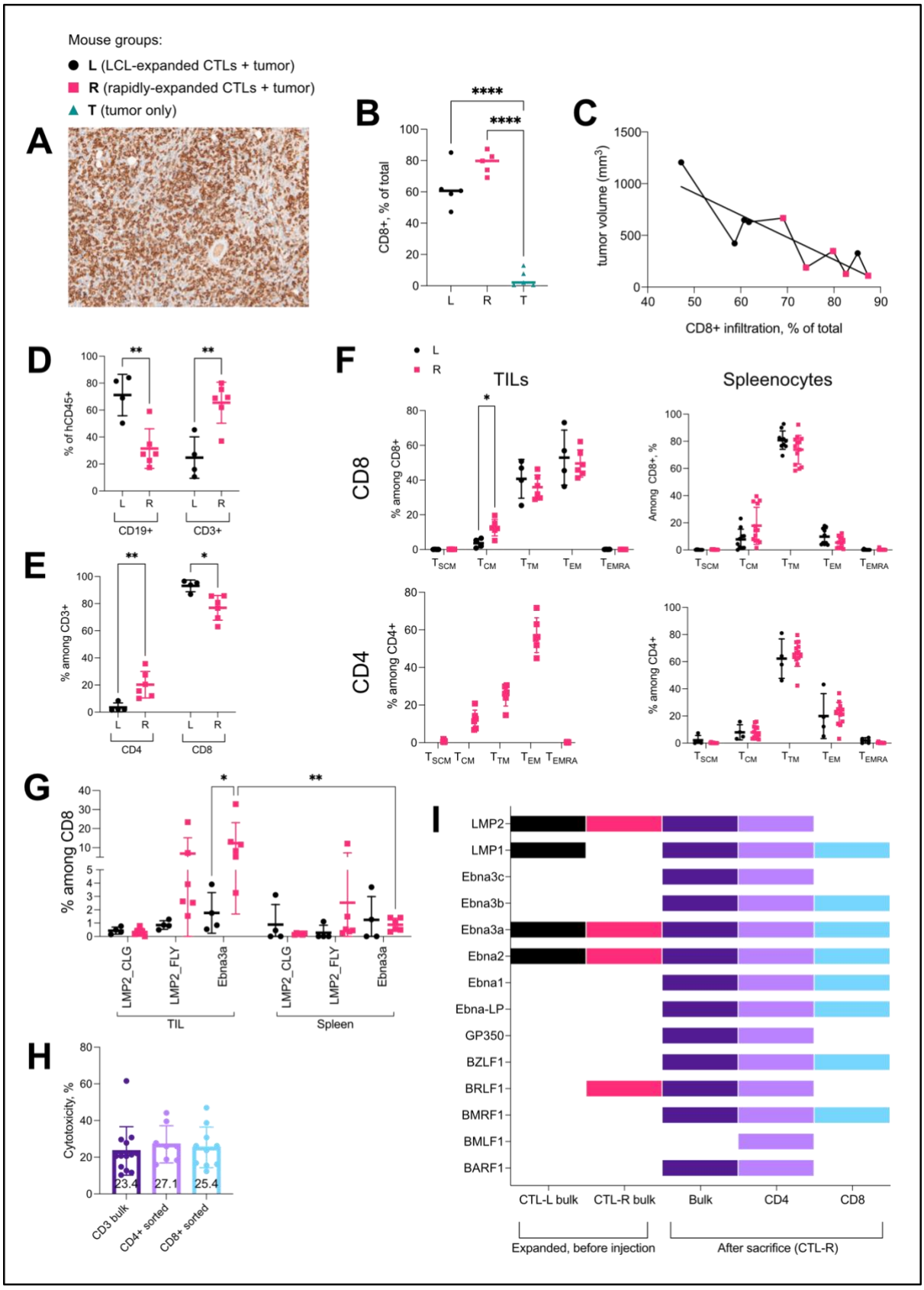

Figure 5. Specificity of expanding EBV-CTLs in vivo. Immunohistochemistry analysis of CD8+ tumor infiltrating lymphocytes (data from one experiment, 5 mice / group): (A) representative picture, CD8+ cells are stained in brown, nuclei – in blue; (B) proportions of tumor infiltrating CD8+ lymphocytes by treatment group; and (C) linear regression line fitted into a correlation plot between CD8+ infiltration and

tumor volume, only data on mice injected with EBV-CTLs plotted, Pearson correlation, n=10, p=0.0014, R^2=0.739. (D) Proportions of CD3+ vs. CD19+ among human CD45+ cells in TILs, and (E) proportions of CD4+ vs. CD8+ among CD3+, measured by flow cytometry. (F) A shift of CD8+ and CD4+ memory phenotypes in TILs vs. spleen (pooled available samples and only samples with >100 recorded events / analyzed population are shown). (G) Proportions of EBV-specific MHC class I-multimer-stained CD8+ T cells. (H) *in vitro* short-term cytotoxicity of bulk, separated CD4+ and CD8+ splenocytes against autologous LCLs (pooled data from all available samples). (I) Presence of specific response of bulk CTL-L and CTL-R cultures before injection vs CTL-R bulk, CD4+ and CD8+ splenocytes after sacrifice to stimulation with single-EBV protein antigen pools measured by ELISpot (based on mean data from all available samples collected from one experiment / one donor). For B, D-G, mixed effects analysis statistics was used.

DISCUSSION

There is strong scientific rationale for evaluating the adoptive transfer of unmodified T cells for clinical medicine based on the function of adaptive T cell immunity in human health. Adoptive T cell therapy (ACT) intends to therapeutically transfer defined T cell immunity to patients. The efficacy of this approach often requires long-term maintenance of the transferred cells, which depends on the presence and persistence of memory T cells. However, persistence of highly differentiated memory T cell subsets after adoptive transfer is still difficult to attain. Recently, very early differentiated T_{SCM} cells with high self-renewal, engraftment, and persistence potential, which can reconstitute all types of effector and memory T cell subsets, became very relevant [33]. Few encouraging and consistent data are available on adoptively transferred T_{SCM}-enriched T cell therapy [9-11].

Viral infections like EBV remain important causes of morbidity and mortality after transplantation. As treatment options are often limited, ACT using virus-specific unmodified T cells has gained attention in recent decades and phase III trials are currently underway (clinicaltrials.gov: NCT03394365, NCT04832607, NCT04832607, NCT04832607). Virus-specific T cells are intended to enhance virus-specific T-cell reconstitution, control viral infection, and persist in the event of recurrence. Current response rates however are around 70% for most viruses [18] indicating an unmet medical need. Thus, VST enriched with T_{SCM} might be superior to highly differentiated T cells due to their longevity, robust proliferation potential, and ability to reconstitute a large antigen diversity, but detailed knowledge about T_{SCM} for VST in the adoptive transfer setting is lacking. Existing protocol of T_{SCM} EBV-CTL generation *ex vivo* involves a complex procedure comprising T cell priming and several steps of cell sorting making it difficult to translate to the clinical setting [34]. Moreover, this protocol completely depletes CD4+ T cells which are important for sustaining adoptive immune responses [35]. We designed an approach which enriches both CD4+ and CD8+ T_{SCM} EBV-CTL from PBMCs with minimal handling.

The most widely used method to expand EBV-specific T cells utilizes continuous restimulations with EBV-LCLs. Although this method has been shown to be safe and effective [36], there are several concerns. These include the length of time required to produce a product suitable for clinical use (4-8 weeks) and repeated ex vivo manipulation which result predominantly in late-stage T_{EM} phenotypes as shown

previously [32, 37]. Moreover, continuous re-stimulations of T cells with target antigens promote CTL exhaustion [20], and as a result, exhausted T cells have a reduced functional capacity [38]. By contrast, the available rapid multi-virus-specific expansion protocol favors an enrichment of central memory phenotype [21], and although the clinical data are so far limited, it was proved to be safe and effective [39]. As CTLs derived from T_{CM} but not T_{EM} are superior in antiviral activity and persistence [40, 41], we used rapid expansion approach as a basis to establish a new T_{SCM}-enriched EBV-CTL generation procedure. We developed a robust expansion protocol that allows enrichment of early differentiated EBV-specific CTLs and can be easily transferred to the clinic with minimal cell manipulations. We compared a broad spectrum of cytokine combinations such as IL-2, IL-2 / IL-21, IL-4 / IL-7, IL-4 / IL-7 / IL-21 as well as several conditions with IL-15. IL-7, IL-15 and IL-21 were previously shown to direct T_{SCM} expansion [42-44]. In our study, IL-4 and IL-7 cytokines together facilitated the highest enrichment of T_{SCM} with or without IL-21 unlike to a previous report on CAR-T cell expansion in which IL-4 / IL-7 / IL-21 combinations were significantly more efficient for T_{SCM} expansion [44]. Additionally, we compared two other reported methods to maintain T cell stemness: culture in elevated potassium concentration or induction of Wnt/β-catenin pathway – and discovered that the latter was resulted in a more efficient T_{SCM} enrichment. Importantly, we could demonstrate that all memory subsets including T_{SCM} of the rapidly expanded EBV-CTLs contained antigen-specific T cells.

Lower overall expansion rate of CTL-R compared with CTL-L Is not a limiting factor as sufficient starting number of PBMCs ffor rapid EBV-CTL expansion can be easily obtained both via leukapheresis and standard blood donation. More importantly, both protocols yielded a comparable overall EBV specificity. Compared to conventionally expanded CTL-L, CTL-R covered a broader antigen diversity targeting additionally lytic antigens such as BARF-1, BMLF-1, BMRF-1 and BRLF-1, which was also confirmed *in vivo*. The limited EBV-specificity of CTL-L might be due to the antigen presentation of EBV-LCLs which exhibit latency III and express predominantly latent EBV antigens [31]. Only recently the relevance of the presence of T cells specific for various lytic EBV antigens, e.g., BMLF1 and BZLF1 in patients with nasopharyngeal carcinoma (NPC) has been demonstrated [45], [46]. Moreover, lytic-antigen specific CTLs might be efficient for prophylaxis of EBV-associated B cell malignancies and NPC, as lytic phase of EBV contributes to oncogenesis [47-49]. Thus, the wider diversity of antigen

specificity is of great importance as it broadens the cell therapy scope and makes them less susceptible to relapse due to antigen escape [50].

A key aspect of an ACT product is the balance between CD4+ and CD8+ populations [51, 52]. It has been reported that patients who received EBV-specific T cells with a higher proportion of CD4+ cells responded better against PTLD [53]. We proved the enrichment of CD4+ and CD8+ EBV-specific T cells in all memory populations including T_{SCM}, which proliferated successfully after stimulation with EBV-LCLs *in vitro*. In the *in vivo* experiments, the higher CD4/CD8 ratio was also maintained in the CTL-R groups, and CD4+ CTL-R infiltrated the tumor whereas CD4+ cells were virtually absent in all organs and TILs of the CTL-L groups. CD4+ and CD8+ T cells isolated from CTL-R splenocytes exhibited comparable anti-tumor cytotoxicity *ex vivo*. In one donor, we demonstrated that both populations recovered a broad antigen-specific profile *in vivo* which was even higher for CD4+ CTL-Rs. This provides a further insight into the potential and importance of EBV-specific CD4+ cells, which may play a role as a reservoir to control various EBV diseases.

Limitations of this study include that long-term data in mice are not yet available for either protocol. We demonstrated persistence for EBV-TSCM-enriched CTL-R for 4 weeks. However, high T-cell engraftment in organs may further be associated with the development of xeno-GVHD [54], signs of which (weight loss) were observed in some mice in the CTL-R groups. Nevertheless, the more efficient CTL-R infiltration into tumors, as well as the *in vitro* cytotoxicity and tested antigen specificity of splenocytes, proved the specificity of the expanded T cells.

Our work demonstrates to our knowledge for the first time that rapid expansion from PBMCs stimulated by an EBV antigen pool, cultured with IL-4 / IL-7 and supplemented with TWS119 yields promising EBV-specific T_{SCM}-enriched CTLs with favorable properties for VST, such as early differentiated memory composition, low exhaustion, better tumor infiltration, efficient CD4+ and CD8+-mediated cytotoxicity, long-term persistence potential, and broader antigen specificity. The method and these data together should help to establish the next generation of unmodified antigen-specific cell therapies beyond EBV indications. The clonal diversity of a CTL-R response remains to be investigated in the upcoming clinical trial.

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Authorship contributions

DP, CS and NK designed the study. DP, CS and BA performed *in vitro* experiments and analysis; GB and CH assisted with the EBV-LCL transformation. DP, JM, OC, CM and NK designed *in vivo* experiments; OC and CM supervised *in vivo* experiments; DP and JM performed *in vivo* experiments and analysis; DP and NK wrote the manuscript.

Disclosure of Conflicts of Interest

The authors declare no conflicts of interest.

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SUPPLEMENTAL MATERIALS

Supplemental materials:

Supplementary methods
Supplementary figures 1-7

SUPPLEMENTAL METHODS

Media used

All media contained PenStrep (Gibco) and were sterile-filtered when supplemented with the human serum (HS).

CTL-M

RPMI 1640 (Sigma Aldrich) with PenStrep (Gibco) and 5% HS.

CTL-M with elevated K^+ concentration

Powdered NaCl-free RPMI 1640 media (Gibco) was reconstituted as previously described [1]. Briefly, NaCl 63.4 mM, NaHCO3 23.8 mM, and *additional* KCl 40 mM had to be added for complete reconstitution. Then PenStrep and 5% HS was added.

LCM-10

RPMI 1640 (Gibco) with PenStrep 100x (Gibco), 5% human serum, 50x Glutamax (Gibco), 100x MEMNEA (Gibco) and 100x Sodium Pyruvate (Gibco).

Concentrations of peptides, cytokines and TWS-119:

Reagent	Supplier	Final concentration
EBV Consensus pepmix	Miltenyi Biotec	0.1 μg/peptide/mL
IL-2	Proleukin	20 U/mL
IL-4	R&D	400 U/mL
IL-7	R&D	10 ng/mL
IL-15	Peprotech	10 ng/mL

IL-21	Peprotech	30 ng/mL
TWS-119	Selleckchem	5μM

T cell expansion from donor-derived PBMCs

All T cells were expanded in CTL-M.

For rapid expansion, PBMCs were cultured in a GRex bioreactor (Wilson&Wolf). $3x10^6$ PBMCs / well of a 24-well GRex plate or $1.5x10^7$ / well of a 6-well GRex plate were cultured. On day 0, cells were pulsed overnight in CTL-M (or CTL-M with high K^+ when applicable) containing EBV Consensus pepmix and supplemented with cytokines (and TWS-119 when applicable). Afterwards the pepmix (and TWS-119 if applicable) was diluted 5x with CTL-M supplemented only with cytokines. Cell culture went on up to day 10-12 without further supplementation.

For long-term EBV-CTL expansion, PBMCs were stimulated with autologous EBV-LCLs at effector : target (E:T) = 40:1 for 10 days ($2x10^6$ PBMCs/well of a 24-well cell culture plate) without cytokine supplementation. Afterwards T cells were re-stimulated weekly at E:T=4:1) and supplemented with 20 U/mL IL-2 3x / week until day 28-35.

EBV-LCL generation and culture

PBMCs were incubated with B95.8 or B95.8-fLuc EBV strain, cultured in LCM-10 media and were treated with 2 μg/ml Cyclosporin A (Sigma Aldrich) and 2 μg/ml CpG ODN 2006 (InvivoGen) weekly until the transformation. Non-irradiated LCLs were always cultured in LCM-10 media (including cytotoxicity and outgrowth assays).

Co-culture with autologous EBV-LCLs

After fluorescence-assisted cell sorting (FACS) (staining described below), sorted cells were recovered for 3 days in CTL-M supplemented with IL-4 and IL-7. Afterwards, autologous LCLs were irradiated and T cells were stained with CellTrace Violet (CTV). Irradiated LCLs were cultured with T cells at a racio 1:1 for one week. Then cells were harvested an analyzed by flow cytometry (see below).

Short-term and long-term in-vitro cytotoxicity

Short-term 6-hour killing assay and long-term 4-week outgrowth assay were adopted as previously published [2]. Briefly, for killing assay, EBV-CTLs were incubated with target EBV-LCLs at an effector (E) to target (T) ratio = 30:1 for 6 h. Afterwards, cells

were stained for viability (Zombie Aqua), apoptosis (CellEvent Blue), CD3+ and CD19+ surface markers (see the panels below). Cytotoxicity was calculated according to the following formula: 100 – ([V_{test} / $V_{control}$] * 100) where V = % viable CD19+ cells (CellEvent$^-$ Zombie Aqua$^-$).

For outgrowth assay (long-term cytotoxicity assay), T cells were incubated with EBV-LCLs at different effector / target ratios in triplicates for 4 weeks. The readout was the lowest E/T ratio controlling the outgrowth of LCLs.

IFN-γ ELISpot, intracellular cytokine staining and V-PLEX

EBV-responsive T cells were identified by stimulation with EBV peptides. Enzyme-linked immunospot assay (ELISpot) [2] and intracellular cytokine (ICC) staining for flow cytometry detection [3] were done as previously published.

Human cytokine presence in murine blood sera was analyzed using V-PLEX human pro-inflammatory panel-1 and detected by Mesoscale system according to manufacturer's instructions.

Immunomagnetic cell sorting

CD4+ and CD8+ T cells were isolated using MACS CD4+ / CD8+ isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

Immunohistochemistry

Tumors were fixed in a 4% paraformaldehyde solution; further sample preparation and immunohistochemistry staining were done commercially by the Pathology Department of the University Hospital of Basel. Slides were acquired on a automated slide scanning brightfield microscope (Vectra) and positive cells were quantified using inForm automated image analysis software (Akoya Biosciences).

Flow cytometry and FACS-based cell sorting

Procedures

If applicable, red blood cells were lysed using ACK lysing buffer until the pellet appeared no longer red. If applicable, whole-cell staining for proliferation tracing and viability staining were performed in PBS according to manufacturers' instructions. Surface staining with antibodies and MHC class I-multimers (if applicable) was performed in FACS buffer (5% FBS, 0.1% NaN3 in PBS). For intracellular staining,

cells were fixed with fixation buffer (Biolegend, 420801) and stained for intracellular markers in the permeabilization buffer (Biolegend, 421002) according to manufacturer's instructions. For combined intracellular/intranuclear staining, cells were fixated and permeabilized using Transcription-Factor Buffer Set (BD, #562574) according to the manufacturer's instructions.

Spectral flow cytometry was performed on Cytek Aurora. Fluorescence-assisted cell sorting was performed with BD FACSMelody. Weekly bleedings were analysed with BD LSRFortessa. Data were analyzed using FlowJo software. FlowSOM algorithm was used to define memory T cell populations: stem cell memory (T_{SCM}) as CD45RA+CD45RO-CD62L+CD27+, central memory (T_{CM}) as CD45RA-CD45RO+CD62L+CD27+, transitional memory T_{TM} as CD45RA-CD45RO+CD62L-CD27+, effector memory T_{EM} as CD45RA-CD45RO+CD62L-CD27-, and terminally differentiated T_{EMRA} as CD45RA+CD45RO-CD62L-CD27-.

Flow cytometry and FACS panels

1. General surface marker analysis (Cytek Aurora)

Dilution	**Marker**	**Dye**	**Clone**	**Supplier**	**Catalog number**
Viability staining					
1:1000	Zombie Aqua			Biolegend	423102
Surface staining					
1:400	CD3	BUV395	UCHT1	BD Biosciences	563546
1:400	CD4	BUV496	SK3	BD Biosciences	612936
1:500	CD8	BUV805	SK1	BD Biosciences	612889
1:50	CD19	PE/Cy7	HIB19	Biolegend	302216
1:30	CD27	FITC	M-T271	Biolegend	356403

1:100	CD45 (anti-mouse) – only for in vivo samples	Alexa Fluor 647	S18009D	Biolegend	160304
1:50	CD45 – only for in vivo samples	Pacific Blue	HI30	Biolegend	304029
1:50	CD45RA	APC	MEM-56	Thermo Fisher	MHCD45RA05
1:100	CD45RO	Alexa Fluor 700	UCHL1	Biolegendd	304218
1:30	CD62L	BV650	DREG-56	Biolegend	304832
1:33	CD127	BV605	A019D5	Biolegend	351334
1:50	CCR7	APC/Cy7	G043H7	Biolegend	353212
1:50	CTLA4	BV785	BNI3	Biolegend	369624
1:50	LAG3	BV711	11C3C65	Biolegend	369320
0:200	PD1	BB700	EH12.1	BD Biosciences	566460
1:100	TIGIT	BV421	A15153G	Biolegend	372710
0:200	TIM3	BV480	7D3	BD Biosciences	746771
1:25	Multimers	PE	-	MBL	See below

2. Staining FACS-based sorting (BD FACSMelody)

Dilution	**Marker**	**Dye**	**Clone**	**Supplier**	**Catalog number**
Surface staining					

1:20	CD3	BV510	UCHT1	Biolegend	300447
1:20	CD27	BV421	M-T271	Biolegend	356418
1:20	CD45RA	PE/ Dazzle 594	HI100	Biolegend	356418
1:20	CD45RO	FITC	UCHL1	Biolegend	304204
1:20	CD62L	PE/Cy7	DREG-56	Biolegend	304822

3. Flow cytometry after 7-day co-culture of sorted memory populations (Cytek Aurora)

Dilution	**Marker**	**Dye**	**Clone**	**Supplier**	**Catalog number**
Whole cell staining (before co-culture start)					
1:1000	CellTrace Violet			Thermo Fisher	C34557
Viability staining					
1:1000	Zombie UV			Biolegend	423108
Surface staining					
1:20	CD3	BV510	UCHT1	Biolegend	300447
1:20	CD27	BV421	M-T271	Biolegend	356418
1:20	CD45RA	PE/ Dazzle 594	HI100	Biolegend	356418
1:20	CD45RO	FITC	UCHL1	Biolegend	304204
1:20	CD62L	PE/Cy7	DREG-56	Biolegend	304822
1:25	Multimers	PE	-	MBL	See below
Intracellular staining					

1:50	Granzyme B	PE/Cy5	QA16A02	Biolegend	372226
1:50	IFNγ	APC	4S.B3	Biolegend	502512

4. Cytotoxicity assay (Cytek Aurora)

Dilution	**Marker**	**Dye**	**Clone**	**Supplier**	**Catalog number**
Apoptosis staining (skipped for outgrowth assay)					
1:1000	CellEvent Caspase-3/7 Green Detection Reagent			Thermo Fisher	C10423
Viability staining					
1:1000	Zombie Aqua			Biolegend	423102
Surface staining					
1:50	CD3	APC	UCHT1	Biolegend	300412
1:50	CD19	PE/Cy7	HIB19	Biolegend	302216

5. Weekly bleedings (BD LSRFortessa)

Dilution	**Marker**	**Dye**	**Clone**	**Supplier**	**Catalog number**
Viability staining					
1:1000	Zombie Aqua			Biolegend	423101
Surface staining					
1:400	CD3	PerCP/Cyanine5.5	UCHT1	Biolegend	300429
1:400	CD4	APC	OKT4	Biolegend	317416
1:500	CD8	BV650	SK1	Biolegend	344730
1:30	CD19	PE/Cy7	HIB19	Biolegend	302216

1:50	CD45	Pacific Blue	HI30	Biolegend	304029
1:50	CD45RA	BV785	HI100	Biolegend	304139
1:30	CD62L	FITC	DREG-56	BD Biosciences	555543
1:33	CD127	APC/Cy7	A019D5	Biolegend	351348
1:50	HLA-DR	BV605	G46-6	BD Biosciences	562845
1:50	PD-1	PE/Dazzle 594	EH12.2H7	Biolegend	329940
Intracellular ad intranuclear staining					
1:50	Granzyme B	Alexa Fluor 700	GB11	BD Biosciences	560213
1:50	TCF-1	PE	7F11A10	Biolegend	655207

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117 MHC class I EBV multimer list

Type	HLA Restriction	Peptide (PE-labeled)	Catalog number
Tetramer	HLA-A*02:01	LMP2 356-364 FLYALALLL	TS-M069-1
Tetramer	HLA-A*11:01	LMP2 SSCSSCPLSK	TS-M111-1
Tetramer	HLA-A*11:01	EBNA3B 416-424 IVTDFSVIK	TS-M029-1
Tetramer	HLA-A*02:01	LMP2 426-434 CLGGLLTMV	TB-M032-1
Tetramer	HLA-A*03:01	EBNA3A 603-611 RLRAEAQVK	TB-M033-1
Dextramer	HLA-A*02:01	BMLF-1 GLCTLVAML	WB2130
Dextramer	HLA-B*3501	EBNA-1	WK2145

		HPVGEADYFEY	
Dextramer	HLA-B*0702	EBNA-3 RPPIFIRRL	WH2166
Dextramer	HLA-B*0801	EBNA-3A FLRGRAYGL	WI2147

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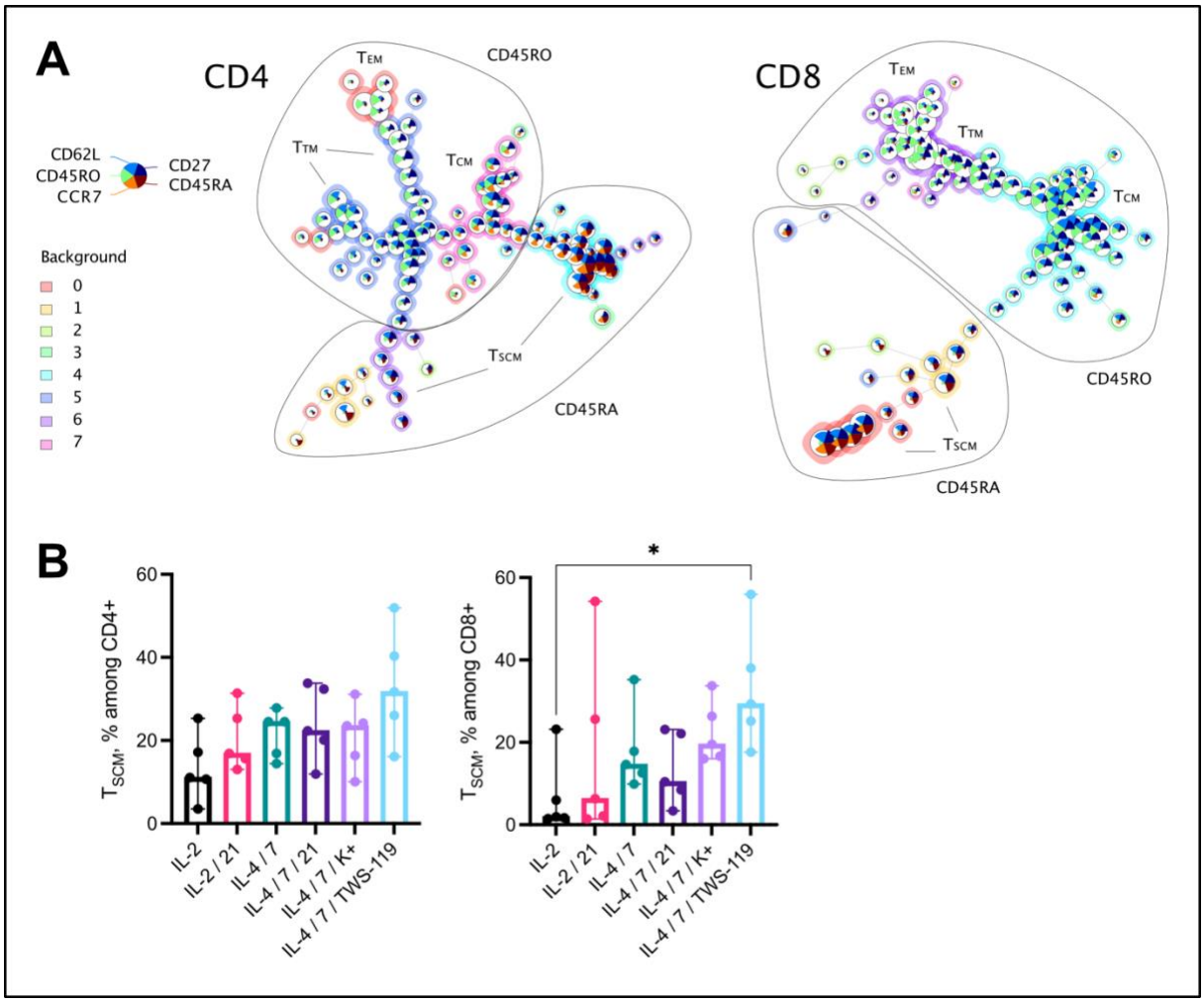
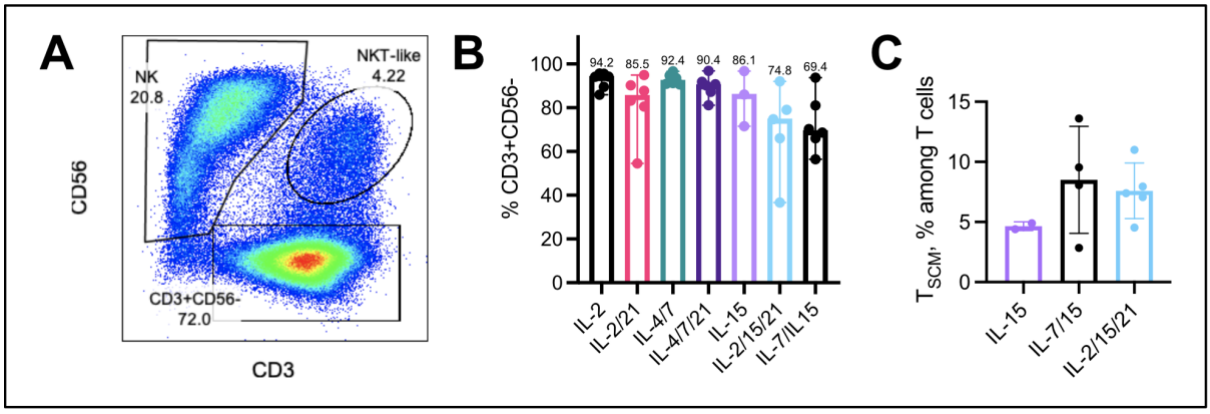
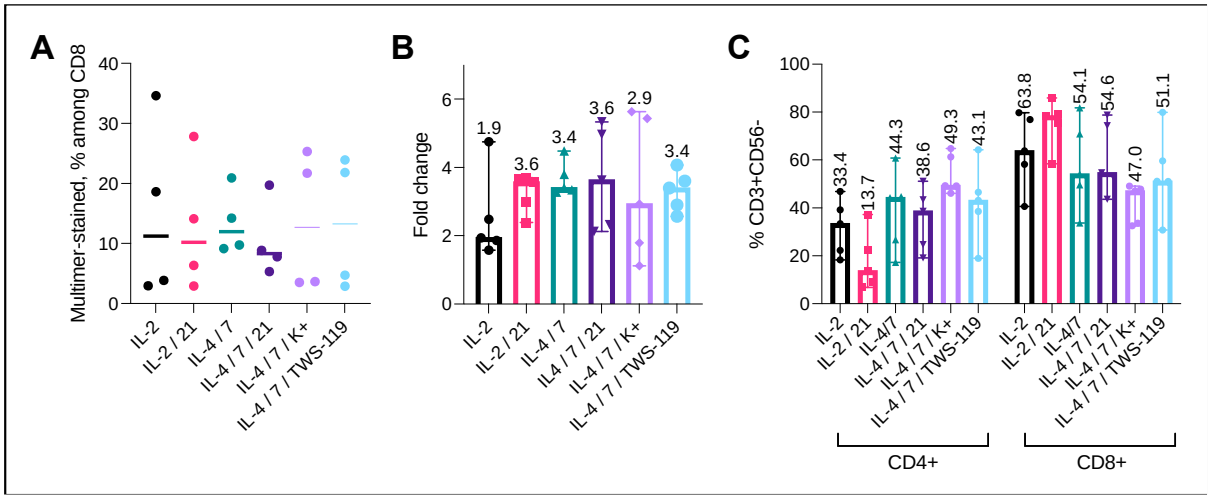
Supplemental figures



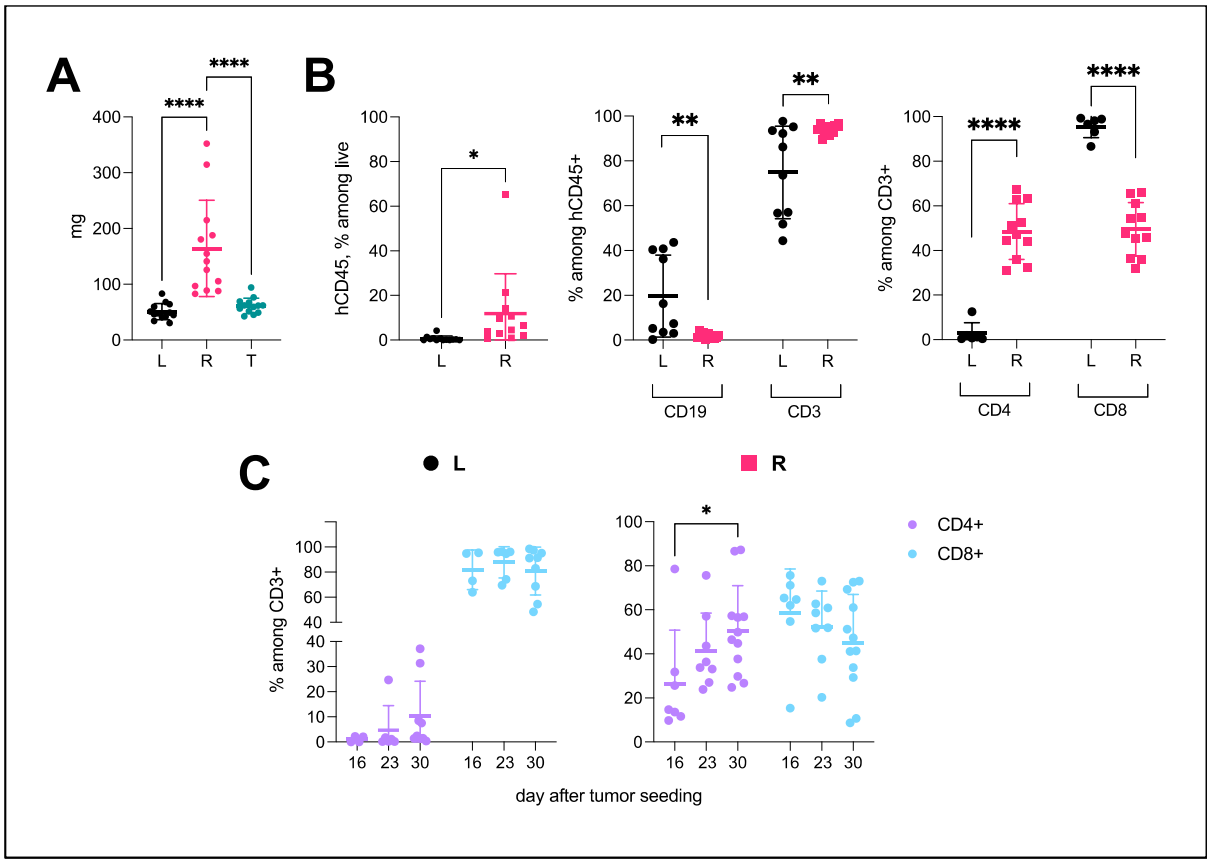
Supplemental Figure 1. Defining markers for T cell memory phenotyping. (A) Memory populations were distinguished among CD4+ and CD8+ cells (separate algorithms for both) by CD27, CD45RA, CD45RO, CD62L and CCR7 markers in the FlowSOM algorithm. Based on the constructed maps, we could identify stem cell memory T_{SCM} ($CD45RA^+CD27^+CD62L^+$), central memory T_{CM} ($CD45RO^+CD27^+CD62L^+$), transitional memory T_{TM} ($CD45RO^+CD27^+CD62L^-$) and effector memory T_{EM} ($CD45RO^+CD27^-CD62L^-$). Short-lived terminally differentiated TEMRA population was almost negligible and, in the analysis, they were defined as ($CD45RA^+CD27^-CD62L^-$). CCR7 was less expressed among the CD8+ cells than CD62L and thus was excluded for simplicity. (B) Stem cell memory T cell proportions among CD4+ and CD8+ expanded T cells; n=5, medians with range, Friedman test, α=0.05, * for $p<0.05$.



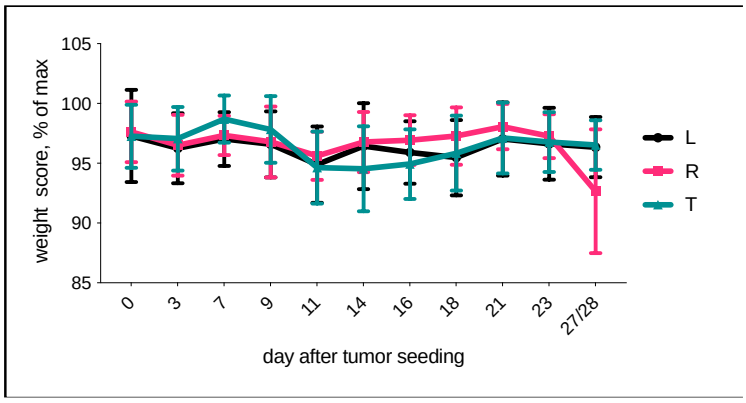
Supplemental Figure 2. NK cell enrichment during PBMC culture in the presence of IL-15. (A) Representative plot showing enrichment of CD56+ positive T cells in an EBV-CTL product. (B) The purity (percentage of $CD3^{+}CD56^{-}$ among live cells) of resulting products. (C) Proportions of T_{SCM} populations.



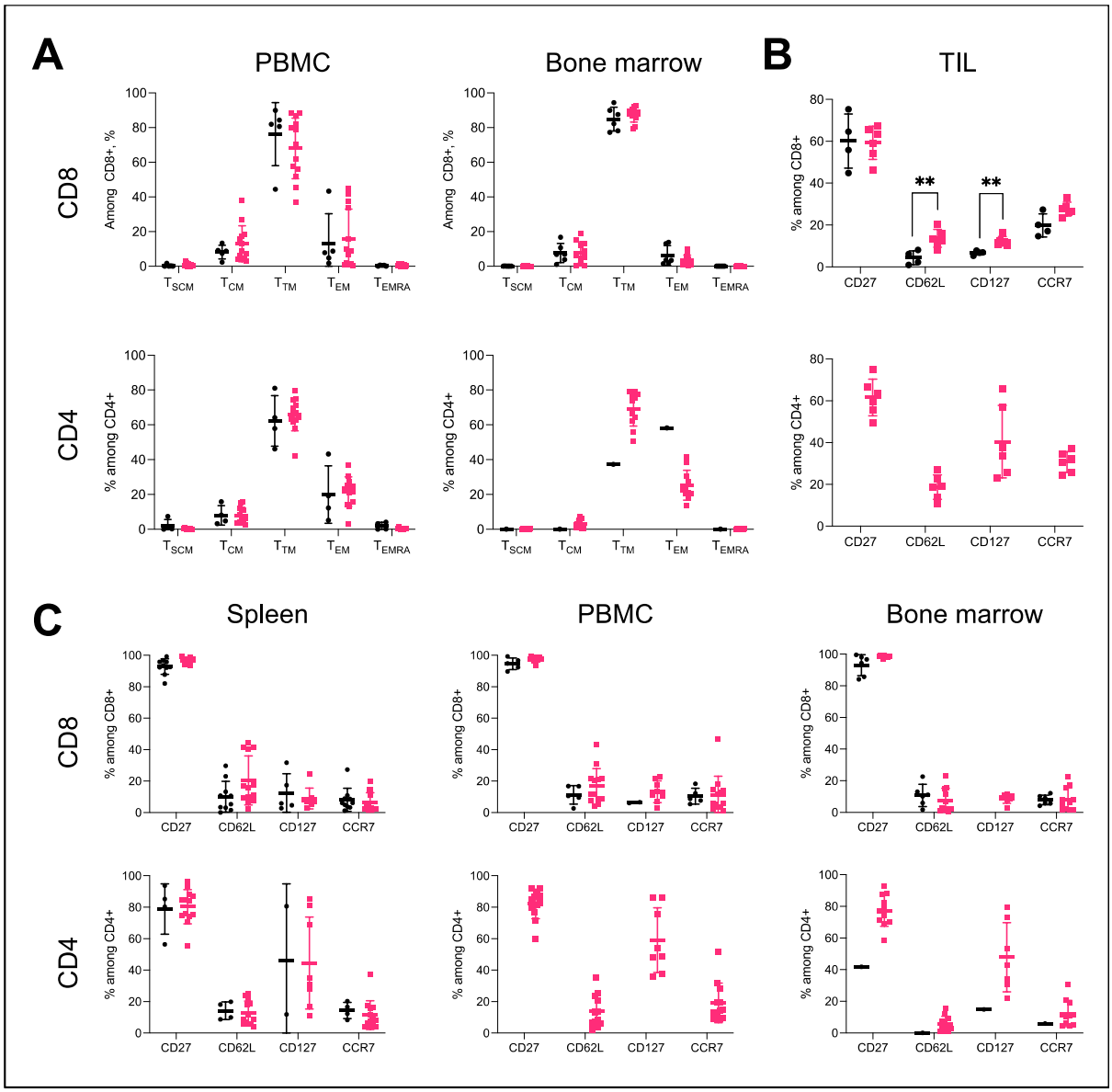
Supplemental Figure 3. General characteristics of EBV-CTL products. (A) Proportions of MHC class I-peptide specific CD8+ T cells measured by respective multimer staining. N=4, medians. (B) Total cell expansion folds, and (C) CD4+ and CD8+ proportions of EBV-CTL products; n=5, medians with range.



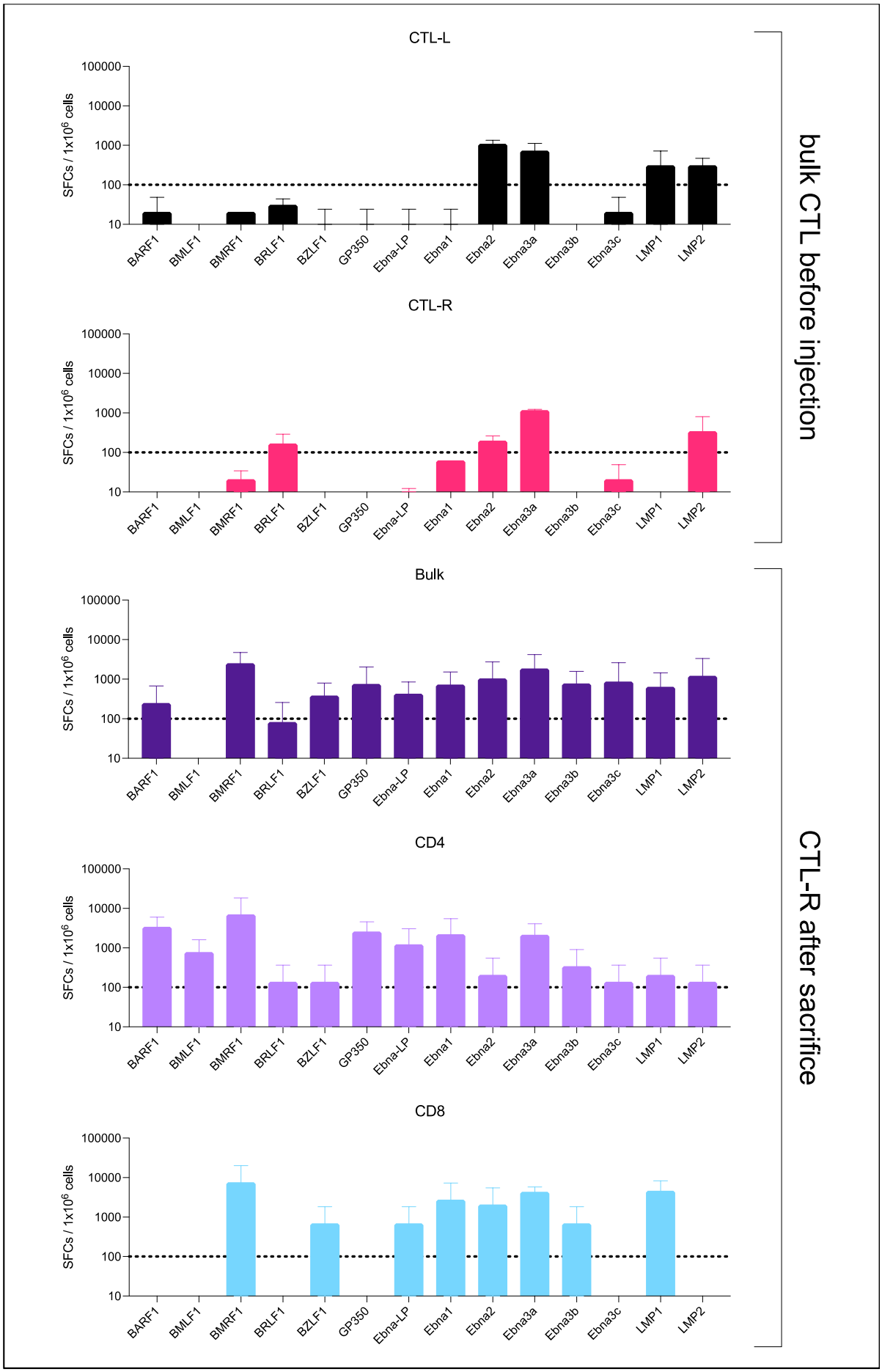
Supplemental Figure 4. General details from *in vivo* experiments. (A) spleen weights. (B) human CD45+, CD19+ vs CD3+, CD4+ vs. CD8+ proportions in bone marrow. (C) Dynamic of CD4+ and CD8+ proportions changes over the experimental time course. For A-C, mixed effects analysis was used.



Supplemental Figure 5. Weight loss in the CTL-R groups and associated parameters. Weight score dynamics during in vivo experimentations, shown means with SD, mixed effects analysis (no sigificannce).



Supplemental Figure 6. Memory phenotyping of different organs from *in vivo* experiments. CTL-L groups are shown in black circles, and CTL-R – in pink squares. Only samples with >100 ecennts / population are shown. (A) Memory T cell populations in PBMCs and bone marrow. Expression of separate memory markers in TILs (B) and organs (C).


CTL-L
CTL-R
Bulk
CD4
CD8
SFCs / 1x10^6 cells
100000
10000
1000
100
10
BARF1
BMLF1
BMRF1
BRLF1
BZLF1
GP350
Ebna-LP
Ebna1
Ebna2
Ebna3a
Ebna3b
Ebna3c
LMP1
LMP2
bulk CTL before injection
CTL-R after sacrifice

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Supplemental Figure 7. Quantitative details of specific response of bulk CTL-L and CTL-R cultures before injection vs CTL-R bulk, CD4+ and CD8+ splenocytes after sacrifice to stimulation with single-EBV protein antigen pools measured by ELISpot (based on mean data from all available samples collected from one experiment / one donor).

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CHAPTER II. Developing a method of precise transgene integration into PBMC-derived EBV-CTLs

The following manuscript is a preprint submitted to *Nature Communications.*

A method for polyclonal antigen-specific T cell-targeted genome editing (TarGET) for adoptive cell therapy applications

Darya Palianina*[1], Raphaël B. Di Roberto*[2], Rocío Castellanos-Rueda[2,3], Fabrice Schlatter[2], Sai T. Reddy[2] and Nina Khanna[1,4]†

[1]Department of Biomedicine, University and University Hospital of Basel, 4056 Basel, Switzerland

[2]Department of Biosystems Science and Engineering, ETH Zürich, 4058 Basel, Switzerland

[3]Life Science Zurich Graduate School, Systems Biology, ETH Zürich, University of Zurich, 8057 Zürich, Switzerland

[4]Divsion of Infectious Diseases and Hospital Epidemiology, University Hospital of Basel, 4031 Basel, Switzerland

*Co-equal contributors

†To whom correspondence should be addressed. Hebelstrasse 20, 4056 Basel, Switzerland; Tel: +41 61 328 73 25; Email: nina.khanna@usb.ch

ABSTRACT

Adoptive cell therapy of donor-derived, antigen-specific T cells expressing native T cell receptors (TCRs) is a powerful strategy to fight viral infections in immunocompromised patients. Determining the fate of T cells following patient infusion hinges on the ability to track them *in vivo*. While this is possible by genetic labeling of parent cells, the applicability of this approach has been limited by the non-specificity of the edited T cells.

Here, we devised a method for CRISPR-targeted genome integration of a barcoded gene into Epstein-Barr virus-antigen-stimulated T cells and demonstrated its use for exclusively identifying expanded virus-specific cell lineages. Our method facilitated the enrichment of antigen-specific T cells, which then mediated improved cytotoxicity against EBV-transformed target cells. Single-cell and deep sequencing for lineage tracing revealed the expansion profile of specific T cell clones and their corresponding gene expression signature. This method has the potential to enhance the traceability and the monitoring capabilities during immunotherapeutic T cell regimens.

INTRODUCTION

Adoptive cell transfer of donor-derived antigen-specific T cells expressing native T cell receptors (TCRs) with defined specificities is an attractive immunotherapy strategy or clinical indications where polyclonality is beneficial. T cell therapies against cancer based on engineered TCRs or chimeric antigen receptors (CARs) typically only target a single antigen, reducing their applicable scope and making them vulnerable to relapses via antigen escape [2]. In contrast, a polyclonal and polyspecific T cell population can target multiple antigens, potentially enhancing the overall effectiveness of an adoptive cell therapy [3, 4]. The feasibility of this strategy has been demonstrated with virus-specific polyclonal T cells enriched from seropositive donors via stimulation with genetically-modified or Epstein-Barr virus (EBV)-transformed antigen-presenting cells (e.g., lymphoblastoid cell lines, or LCLs) [5] or rapidly expanded from peripheral blood mononuclear cells (PBMCs) using peptide pools as stimuli [4], [6], (manuscript in preparation). In this approach, single cell antigen specificity and phenotype characterization can be assessed prior to transfer through methods such as flow cytometry, ELISPOT and TCR RNA- or transcriptome-sequencing. These assessments become especially important during treatment. Beyond monitoring needs, the ability to identify the most therapeutically-relevant clones and phenotypes is of significant interest, particularly for long term efficacy. Recently, it was shown that CAR T cells can persist in patients as many as 10 years after infusion [7]. While CAR T cells are readily identifiable, non-engineered therapeutic T cells are difficult to distinguish from naïve T cells. Genome-based lineage tracing of adoptively transferred lymphocytes has been proposed for facilitating follow-up studies [8, 9]. For example, LCL-stimulated EBV-specific cells transduced with the *neo*-containing G1Na vector could be traced up to 9 years after adoptive transfer [10, 11]. However, the use of

retroviral vectors is associated with safety risks [12] due to the largely random nature of vector integration into the genome. Targeted gene editing by CRISPR/Cas9 is a superior approach and has been successfully used to knock out genes connected to exhaustion and checkpoint inhibition (e.g., PD-1) [13] or resist administered immunosuppressants (e.g., tacrolimus) [14]. However, this approach has limitations, particularly for integrating a gene of interest, known as homology-directed repair (HDR). HDR is cell-cycle dependent and restricted to actively dividing cells [15]. To date, CRISPR-based HDR approaches in T cells have relied on strong and nonspecific activation through anti-CD3 antibodies or coated beads. This approach is not compatible with a polyclonal T cell therapy where only target-specific cells are desired. Here, we describe a novel approach for targeted CRISPR/Cas9-based genome editing and lineage tracing of virus-specific T cells. Notably, our approach combines autologous peptide presentation for T cell stimulation and editing, as well as the use of a barcoded GFP cassette library to enable the detailed characterization of clonal expansion. Using antigen-presenting cells and T cells directly from donor-derived PBMCs we generated a pool of uniquely barcoded EBV-specific T cells. By leveraging the cell cycle dependence of HDR, we used GFP integration as a marker of EBV specificity for enrichment by fluorescence-activated sorting (FACS). Sorted GFP-positive populations were devoid of unreactive cells as shown by single-cell RNA sequencing. This high purity resulted in an increased EBV-specificity and cytotoxicity against target cells (EBV-LCLs). Our method has a range of scientific and clinical applications: e.g., the possibility for sophisticated follow up after adoptive transfer on a single cell level, lineage tracing, the specific integration of therapy-enhancing genes such as a safety switch [16] or cytokines [17].

RESULTS

Library design and peptide-based T cell expansion

In order to fluorescently label and barcode reactive T cells in a single step, we designed an AAV vector encoding 1) inverted terminal repeats (ITRs); 2) homology arms for targeted insertion into the *CCR5* safe harbor locus [18]; 3) the cytomegalovirus (CMV) constitutive promoter; 4) the GFP open reading frame (ORF) ; and 5) a 9-nucleotide barcode (Fig. 1a). Although the diversity of our library could theoretically encompass 262 144 unique barcodes, we restricted its size to 50000 colony-forming units.

Sequencing of this cloned library identified 36030 unique barcodes (Fig. 1b) and no major bias (Fig. 1c). The repair template library was packaged into AAV6 capsid commercially and subsequently used for HDR following transfection.

To expand EBV-specific T cells from human PBMCs, we adapted an established protocol for rapid expansion of virus-specific cytotoxic T cells (CTLs) [19] and used the PepTivator EBV Consensus peptide pool as stimulus for display by native monocytes [4]. This mixture covers 41 lytic and latent EBV antigens. It was previously shown that HDR is generally restricted to the S/G2 phases of the cell [20]. Thus, proliferative activated T cells will preferentially undergo HDR following genome editing. We hypothesized that a population of pepmix-activated virus-specific T cells could be selected on the basis of successful HDR editing. To identify the optimal time-point for gene editing, we characterized the T cell proliferation and activation profiles of PBMCs from two healthy EBV seropositive donors with whole-cell staining and intracellular cytokine staining (ICC) every second day following EBV pepmix re-stimulation. No proliferation was observed among bulk T cells (Supplementary Fig. 1a) and EBV-responsive T cells (Supplementary Fig. 1b) by day 3, while daughter cells were present at day 5 and an abundant fraction of these were EBV-specific T cells. This lag between stimulation and expansion provided us with an opportune window for transfection. By transfecting prior to exponential cell expansion, we aimed to edit as many parent cells as possible. As such, we opted to transfect our barcoded library during this lag time, i.e., on day 3.

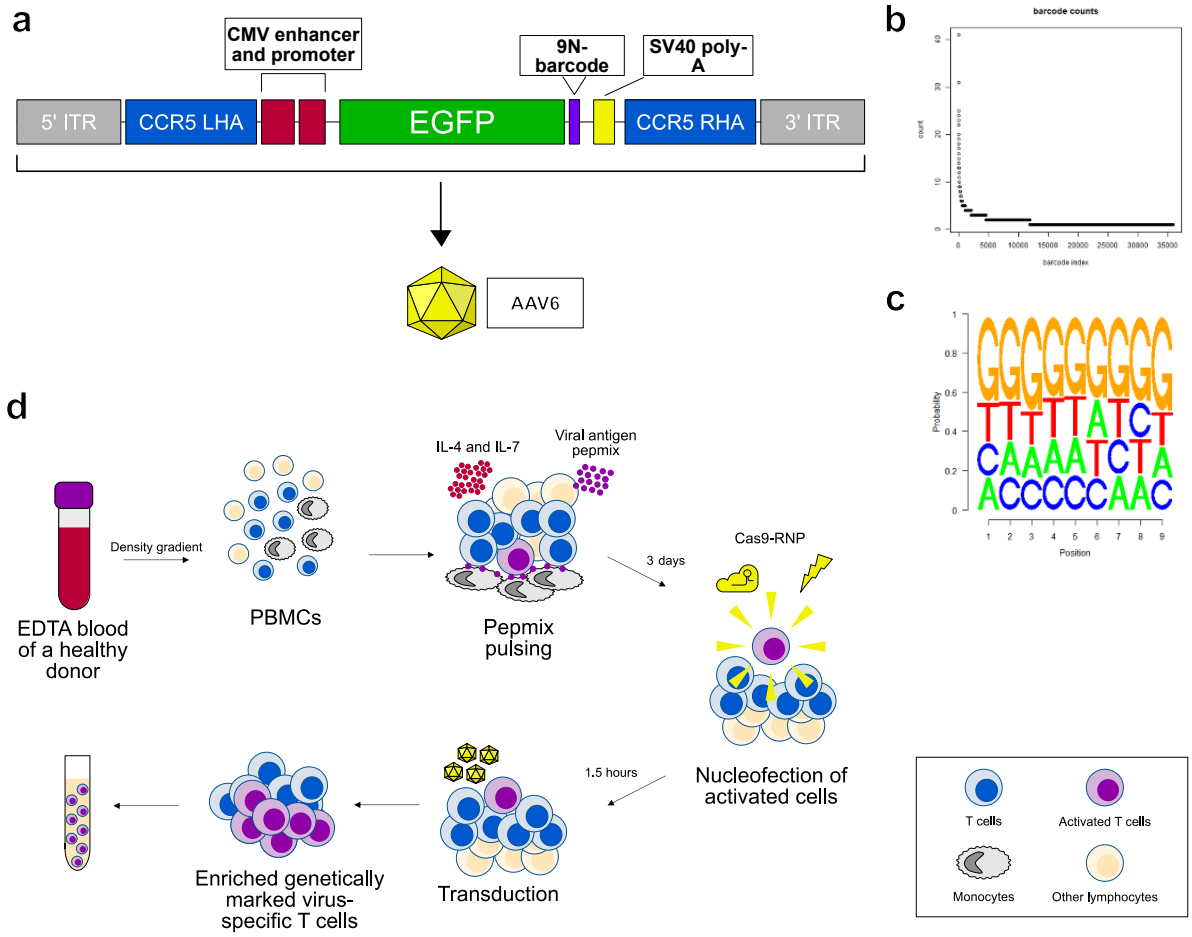

Fig. 1: Library cloning and genome editing procedure. a, Library vector and repair template DNA design. Next generation sequencing (NGS) library analysis: **b**, sequencing visualization and **c**, sequence logo plots of the cloned library; **d**, Schematic of the cell culture and gene editing procedures. ITR – inverted terminal repeat, LHA and RHA – left and right homology arms, respectively; CCR5 - C-C chemokine receptor type 5, CMV – cytomegalovirus, SV40 polyA – simian vacuolating virus 40 polyadenylation signal, AAV6 – adeno-associated virus serotype 6, EDTA - ethylenediaminetetraacetic acid, PBMCs – peripheral blood mononuclear cells; RNP – ribonuclear protein.

Efficient transduction of peptide-stimulated T cells

In order to induce the genomic integration of a library in EBV-specific T cells, we devised the following strategy (Figure 1d). Following PBMC isolation from a healthy donor, cells were pulsed with EBV-pepmix or stimulated with anti-CD3/CD28 dynabeads in the presence of IL-4/IL-7. On day 3, cells were transfected with CRISPR/Cas9 ribonucleoprotein (RNP) and transduced with AAV6 particles carrying the barcoded GFP library. An RNP-only sample was included to serve as an HDR-negative control. On day 10, we analyzed cell type counts as well as GFP positivity. All samples including AAV-transduced were highly CD3+-enriched, confirming the

efficiency of the pepmix and cytokines conditions for T cell enrichment (Supplementary Fig. 2). Cells transduced with the library showed GFP expression in both pepmix-stimulated and CD3/CD28 dynabeads-stimulated cells (Fig. 2a). Editing efficiency was donor-specific, ranging from 6.8% up to 25.0% for pepmix-stimulated cells and from 16.6% to 45.1% for bead-stimulated ones. For pepmix-stimulated product, we observed a higher proportion of GFP-positive T cells within the CD8+ population (median 12.5%) compared to those within the CD4+ one (median 5.7%), and we observed a similar trend for bead-activated T cells (medians 40.8% for CD8+ and 17.8% for CD4+). We also saw enrichment of CD8+ T cells in the AAV-transduced GFP-positive EBV-activated T cells compared to the bulk transduced ones (pepmix-stimulated but untransfected) product ($p<0.05$, 2-way ANOVA) (Fig. 2b).

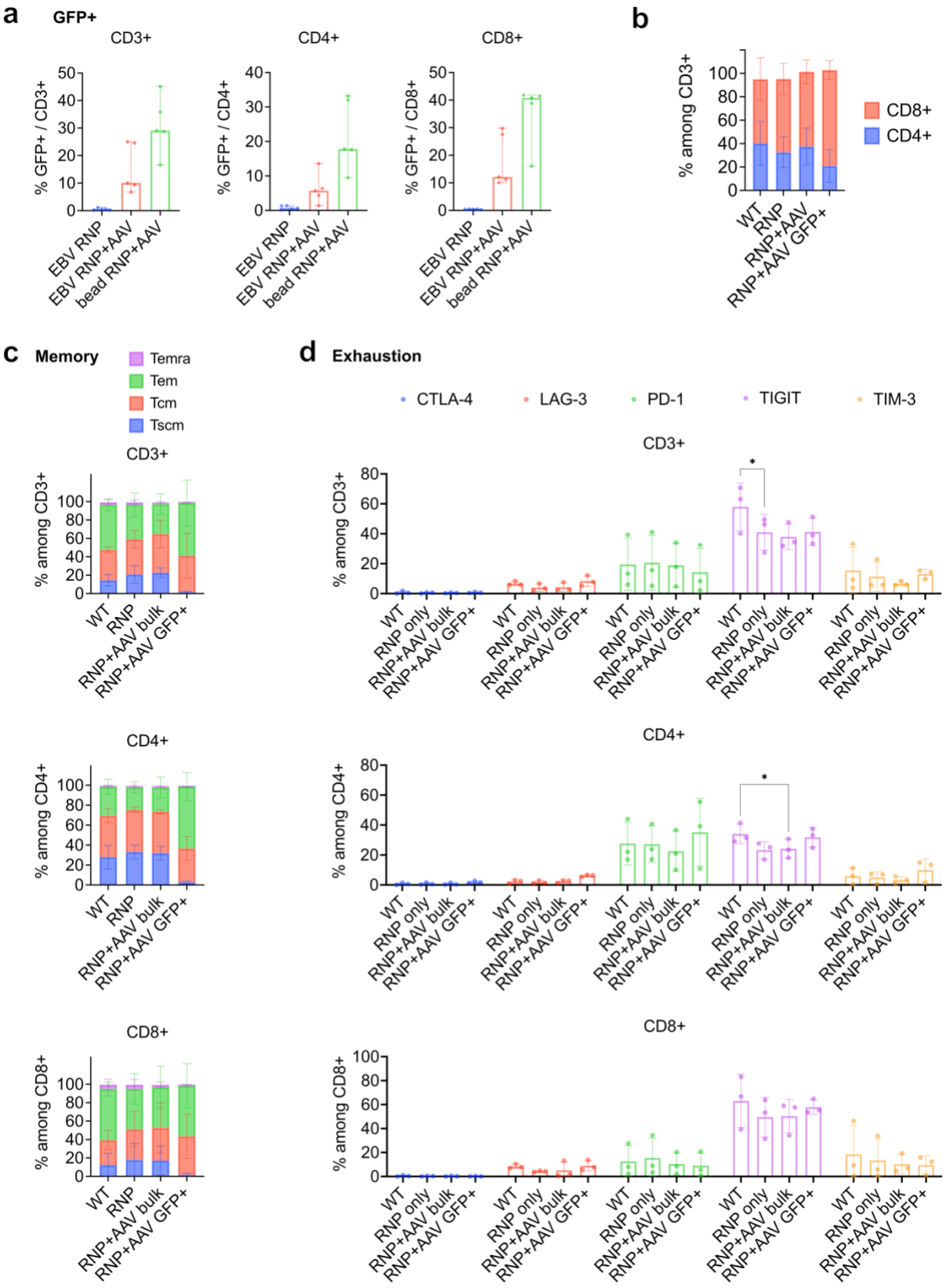

Fig. 2: Transduction efficiencies and phenotype differences between expanded cells. a, Transduction efficiencies for pepmix-stimulated vs. anti-CD3/CD28 dynabeads-stimulated T cells for bulk CD3+, CD4+ and CD8+ cells, respectively; n=5, shown medians with range. **b**, CD4 vs. CD8

proportions within different populations of expanded pepmix-stimulated T cells; n=5, shown means with SD. **c**, memory phenotypes and **d**, exhaustion marker expression of expanded pepmix-stimulated T cells among WT, RNP-only transfected and transduced bulk CD3+, CD4+ and CD8+ cells; n=3. WT stands for wild type. Asterisk represent statistically significant differences ($p<0.05$, 2-way ANOVA). ANOVA – analysis of variance, EBV – Epstein-Barr virus, WT – wild type, RNP – ribonucleoprotein, AAV – adeno-associated virus; Temra – terminally differentiated, Tem – effector memory, Tcm – central memory, Tscm – stem cell memory T cells.

Next, we analyzed the memory phenotype of the generated EBV-CTLs (Fig. 2c). While untransfected samples showed an even mixture of stem cell memory ($T_{SCM)}$, central memory (T_{CM}) and effector memory cells (T_{EM}) with only a small minority of terminally differentiated (T_{EMRA}) cells, we observed a depletion of T_{SCM} in GFP-positive T cells which comprised almost exclusively T_{CM} and T_{EM}. This effect could be explained by low initial number of early-differentiated (T_{SCM} -like) EBV-CTLs in PBMCs due to EBV re-activation [21]. Alternatively, early differentiated EBV-specific T cells might not be activated enough to enable HDR. Generally, CD4+ cells had a less differentiated phenotype compared to CD8+ in all conditions except among those GFP+-gated. Interestingly, among CD4+ GFP+ cells, there was a significantly higher proportion of T_{EM} compared to bulk transduced cells ($p<0.05$, 2way ANOVA).

We then assessed the expression of several exhaustion markers: CTLA-4, LAG-3, PD-1, TIGIT and TIM-3 (Fig. 2d). CTLA-4 was almost absent in all conditions, LAG-3 and TIM-3 were expressed at very moderate levels, slightly more among CD8+ cells compared to CD4+. PD-1 was overall also low but more present in CD4+ populations decreasing in AAV-transduced cells. On the contrary, TIGIT was expressed at high levels in CD8+ cells but less abundant in CD4+, decreasing further with both AAV transduction and RNP-only transfection. The decrease of TIGIT in transfected cells could be due to the death of exhausted cells following the transfection procedure.

Together, these results indicate that unique expansion and genome editing protocol efficiently integrated GFP in a population of activated T cells and did not markedly interfere with cell phenotype.

HDR-based sorting enriches for EBV-CTLs and improves their anti-EBV response

In order to measure the EBV specificity and activation potential of the transduced bulk and GFP-positive cells, we re-stimulated expanded cells with pepmix and analyzed the expression of key cytotoxic T cell markers such as CD107a (LAMP-1), Granzyme B, IFNγ and TNFα using flow cytometry. While RNP-only transfected and bulk AAV-transduced cells did not show elevated cytotoxic marker expression compared to untransfected, within the GFP-positive T cell populations we saw elevated production of most markers (CD107a, IFNγ and TNFα) corresponding to at least a 2-fold increase in EBV specificity for CD8+ cells and 4-fold for CD4+ cells compared to wild type (Fig. 3a).

In order to assess target-specific functionality, we sorted GFP-positive and GFP-negative fractions of transduced EBV-CTLs and assessed their *in vitro* cytotoxicity against autologous EBV-transformed LCL and compared it to that of the other samples (Fig. 3b). Although we observed a slight increase of cytotoxicity in the RNP-only samples as well as the GFP-negative sorted fractions, this was less significant than that of the sorted GFP-positive cells highlighting the efficacy-enhancing potential of HDR-based selection.

These findings show that the designed HDR-based selection of EBV-CTLs leads to an increased antigen specificity and target-specific toxicity.

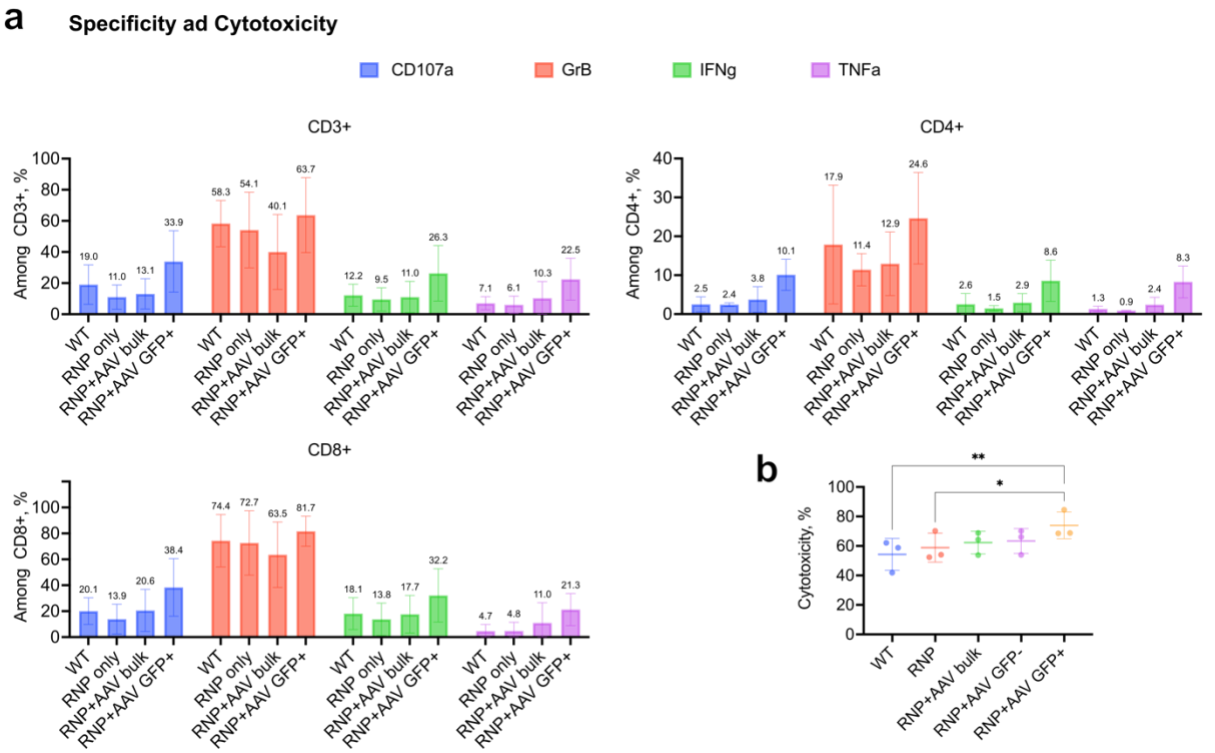

Fig. 3: Specificity and functionality of pepmix-stimulated and expanded transduced T cells. a, Production of cytotoxicity markers and cytokines (CD107a, Granzyme B, IFNγ and TNFα) among bulk CD3+, CD4+ and CD8+ populations in response to EBV-pepmix-restimulation, n=3, means with standard deviation (SD). **b,** 6-hour cytotoxicity assay against autologous EBV-transformed LCLs (effector/target = 20:1), means of triplicates with SD for 3 donors, 2way ANOVA mixed effects analysis, **=0.0043, *=0.0405, α=0.05. ANOVA – analysis of variance, EBV – Epstein-Barr virus, WT – wild type, RNP – ribonucleoprotein, AAV – adeno-associated virus, GrB – granzyme B.

GFP barcoding and selection provide expansion and enrichment statistics, respectively

Ten days following peptide pulsing, we sequenced 38 908 cells across two donors and the two sorting conditions (GFP-positive and GFP-negative) from which 27 283 had a properly annotated TCR (Fig. 4a). Single cell sequencing provided us with three layers of data for both edited and unedited T cells: 1) TCR clonal identity; 2) GFP barcode clonal identity; and 3) Gene expression (transcriptome) profiles. Among all cells, 295 unique TCR clonotypes appeared at least three times. One highly represented clonotype, representing 45% and 65% of the GFP-positive and -negative datasets for Donor 2, respectively, was omitted for TCR identity analysis as a likely indiscriminately-expanding clone. Of the remaining clonotypes, none were shared between donors, while V and J gene usage diversity was also distinct (Fig. 4b).

We next compared the GFP-positive and -negative sorted samples. Clonotype overlap between samples was high (Fig. 4c), while CDR3 length distribution were a close match (Fig. 4d, Supplementary Fig. 3a). To better distinguish between clonotypes, we investigated post-GFP integration fold-expansion (cell proliferation after day 3) and fold-enrichment (selection efficiency). For expansion, we performed a lineage tracing analysis through deep sequencing of the GFP gene to specifically link cell and GFP barcodes. We obtained GFP barcodes for 44% of the sequenced GFP-positive cells, representing 1491 unique barcodes. Only a minor fraction of these (2%) were associated with more than one TCR clonotype (Fig. 4e) and were excluded from subsequent analyses. Using the ratio of GFP barcodes to cell barcode, we calculated the mean fold-expansion of 209 individual T cell clonotypes (Fig. 4f). While the highest expansion was 49-fold (with one GFP barcode), the middle 50% of clones ranged between 1- and 3-fold. Interestingly clonotypes with the highest post-GFP integration fold expansion did not correlate with the clonotypes that had overall the highest number of cells, revealing interesting clonotype expansion dynamics.

In addition to fold-expansion, we calculated fold-enrichment for the 170 clonotypes that were assigned to a GFP-barcode and had cells in both GFP-negative and -positive samples, based on their enrichment across samples (Fig. 4g). Selection on this basis resulted in enrichment as high as 43-fold, or in depletion as high as 12-fold. Fold-expansion and fold-enrichment showed a significant correlation ($P < 0.0001$) though perhaps driven by a handful of clonotypes (Supplementary Fig. 3b).

We submitted the beta chains' V gene, J gene and CDR3 for TCRs with barcoded GFP to TCRex, a tool designed for querying TCR identity in public databases (TCRex (biodatamining.be)). Fourteen clones within eleven clonotypes were classified as EBV-specific, three of which showed enrichment and expansion both above one (Table 1). Of these, two are perfect matches by CDR3β to dominant clones highlighted in previous work [22-24], while clonotype 110 is a close match (Levenshtein distance of 3). EPLPQGQLTAY and GLCTLVAML correspond to peptides from BMLF1 and BZLF1 lytic EBV proteins while HPVGEADYFEY (EBNA1) and IVTDFSVIK (EBNA4) correspond to the latent ones. Overall, we have high confidence that we identified multiple EBV-specific TCRs for which we have lineage tracing data.

Table 1: EBV-specific TCR clones within GFP-positive and barcoded dataset as predicted by TCRex

TRBV gene	CDR3 beta	TRBJ gene	Enrichment	Expansion	Epitope	TCRex score
TRBV10-03	CATGTGDSNQPQHF	TRBJ01-05	0.76	2.13	EPLPQGQLTAY	0.99
TRBV03-01	CATSTGDSNQPQHF	TRBJ01-05	1.86	1.86	EPLPQGQLTAY	0.99
TRBV14	CASSQSPGGIQYF	TRBJ02-04	1.81	1.00	GLCTLVAML	0.99
TRBV09	CASSARSGELFF	TRBJ02-02	0.19	2.00	HPVGEADYFEY	0.99
TRBV11-02	CASSWGGGSNYGYTF	TRBJ01-02	0.83	6.60	IVTDFSVIK	0.97
TRBV10-03	CAAGTGDSNQPQHF	TRBJ01-05	0.76	2.13	EPLPQGQLTAY	0.95
TRBV20-01	CSARDRGIGNTIYF	TRBJ01-03	1.21	1.00	GLCTLVAML	0.95
TRBV03-01	CASATGDSNQPQHF	TRBJ01-05	1.86	1.86	EPLPQGQLTAY	0.92
TRBV02	CASSASSGGYYNEQFF	TRBJ02-01	0.55	3.00	IVTDFSVIK	0.89
TRBV02	CASSEYAGGYYNEQFF	TRBJ02-01	0.55	3.00	IVTDFSVIK	0.80
TRBV07-08	CASSLGQAYEQYF	TRBJ02-07	1.65	5.10	GLCTLVAML	0.78
TRBV02	CASTQSAGGFYNEQFF	TRBJ02-01	7.24	1.60	IVTDFSVIK	0.74
TRBV10-03	CASGTGPDSNQPQHF	TRBJ01-05	0.20	1.00	EPLPQGQLTAY	0.66
TRBV07-06	CASSLEPGRNEKLFF	TRBJ01-04	0.62	2.30	IVTDFSVIK	0.64

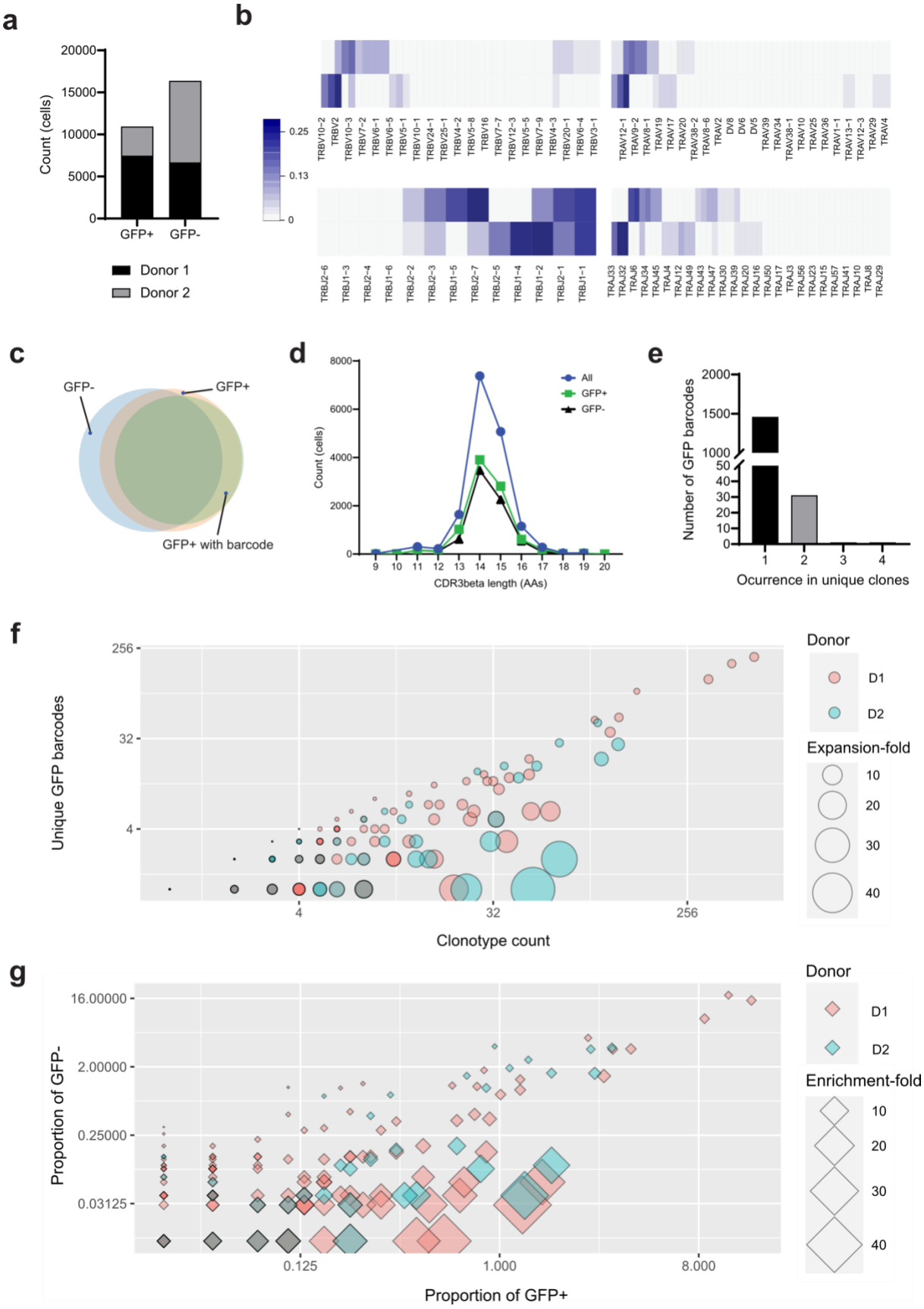

Fig. 4: Lineage tracing and enrichment analysis of single-cell sequencing data reveals highly-expanded and highly-enriched TCR clones. a, Sequenced cell counts and their sample and donor origin. Each donor was used in a single expansion, genome editing, sorting and sequencing workflow.

Cells were sorted for the presence (GFP+) or absence (GFP-) of GFP fluorescence. **b,** Heatmaps comparing the sequenced alpha and beta TCR chains and their V- and J-gene usage for each donor. Donors showed markedly different gene usage profiles. **c,** Venn diagram of the membership of TCR clonotypes across GFP-positive and GFP-negative samples. The high overlap between the samples enabled the downstream calculation of enrichment statistics. **d,** Distributions of the CDR3 beta lengths for GFP+ and GFP- samples. The distributions do not differentiate between samples. **e,** Frequencies of identical GFP barcodes found in more than one T cell clone, based on TCR identity. The vast majority of GFP barcodes were associated with only a single clone, confirming that the barcode library was sufficient. **f, and g,** Scatter plots of the fold-expansion and fold-enrichment respectively for individual clonotypes. Fold-expansion was calculated from the ratio of GFP barcodes to clonotype count. Fold-enrichment was calculated from the ratio of proportions within libraries, from GFP- to GFP+. Grey data points show perfect overlap across donors. Both statistics enable clonotype comparisons.

Single-cell transcriptome sequencing confirms the enrichment of reactive T cell phenotypes in GFP-positive sorted cells

Using single-cell transcriptomics, we explored the phenotypic landscape of EBV-CTLs. Unsupervised cell clustering divided all cells into 13 main clusters (Fig. 5a-b and Supplementary Fig. 4a-c). With few exceptions, CD4/CD8 identity, cell cycle phase, cytotoxicity and memory markers were the main drivers of cluster separation. CD8 clusters 0,1,2,3,5,7 and 8 describe a homogeneous population of activated cytotoxic CD8 cells enriched in the expression of NKG7, GZMK, GZMA, GZMH, PRF1, HLA-DRA, and EOMES. Clustering resolves cycling cells (clusters 1 and 2), non-proliferative CD27/CCL4/CCL5-high and GZMB/LAG3-high cells (clusters 0 and 3 respectively), glycolytic cells (cluster 5) and apoptotic cells (cluster 8). Cluster 4 is a CD4-enriched cluster of moderately proliferative cells presenting an activated phenotype and retaining the expression of memory markers such as TCF7, LEF1 and CD7. T reg CD4 cells are found in cluster 9 enriched in FOXP3/ ILR2A and lastly, cluster 6 describes a CD4/CD8 population of resting cells enriched in memory and resting cell markers such as IL7R, CCR7 and TCF7 which present a phenotype of unreactive T cells. Clusters 10, 11 and 12 show small populations of NK and B cells remaining from the initial whole-PBMC population.

The detection of GFP transcripts at single cell level was used to confirm the correct CRISPR-Cas9 genome integration of the GFP-Barcode transgene (Fig. 5c-d). About 45% of GFP-positive sorted cells showed detectable GFP transcript, compared to just 2% for the GFP-negative sample. Moreover, 87% of the GFP-positive sorted cells that were assigned to a correctly annotated GFP barcode by deep sequencing also expressed GFP transcripts, further demonstrating the accuracy of our GFP barcode readout.

When comparing the enrichment of GFP-positive and -negative cells across clusters, GFP-positive cells were strongly depleted in cluster 6 (unreactive T cells) and enriched in activated T cell clusters 1 through 5 (Fig. 5c and Supplementary Fig. 5a-b). In addition, we saw different patterns of enrichment when looking at the expansion of TCR clonotypes within these two sample groups. Cells sorted for absence of GFP were enriched for non-expanded (only one cell per clone) and highly expanded (more than 200 cells per clone) clonotypes as opposed to GFP sorted cells, which were enriched in highly expanded and also moderately expanded clonotypes (5-200 cells per clone; Fig. 5e-f). Cluster enrichment across TCR expansion bins and the top expanded clonotypes showed that overlooking moderately expanded clonotypes restricted the diversity of T cell phenotypes (Supplementary Fig. 6a-b). On the other hand, our results showed that this could be avoided by using our targeted GFP-positive selection; while most TCR expanded clonotypes clustered around the same phenotypes, the expansion of our GFP-barcode was distributed more homogeneously across activated T cell phenotypes (Supplementary Fig. 7).

These results illustrate how our method can be more effective in identifying highly but also moderately expanded reactive T cells across any activated phenotype for both the CD4 and CD8 compartments.

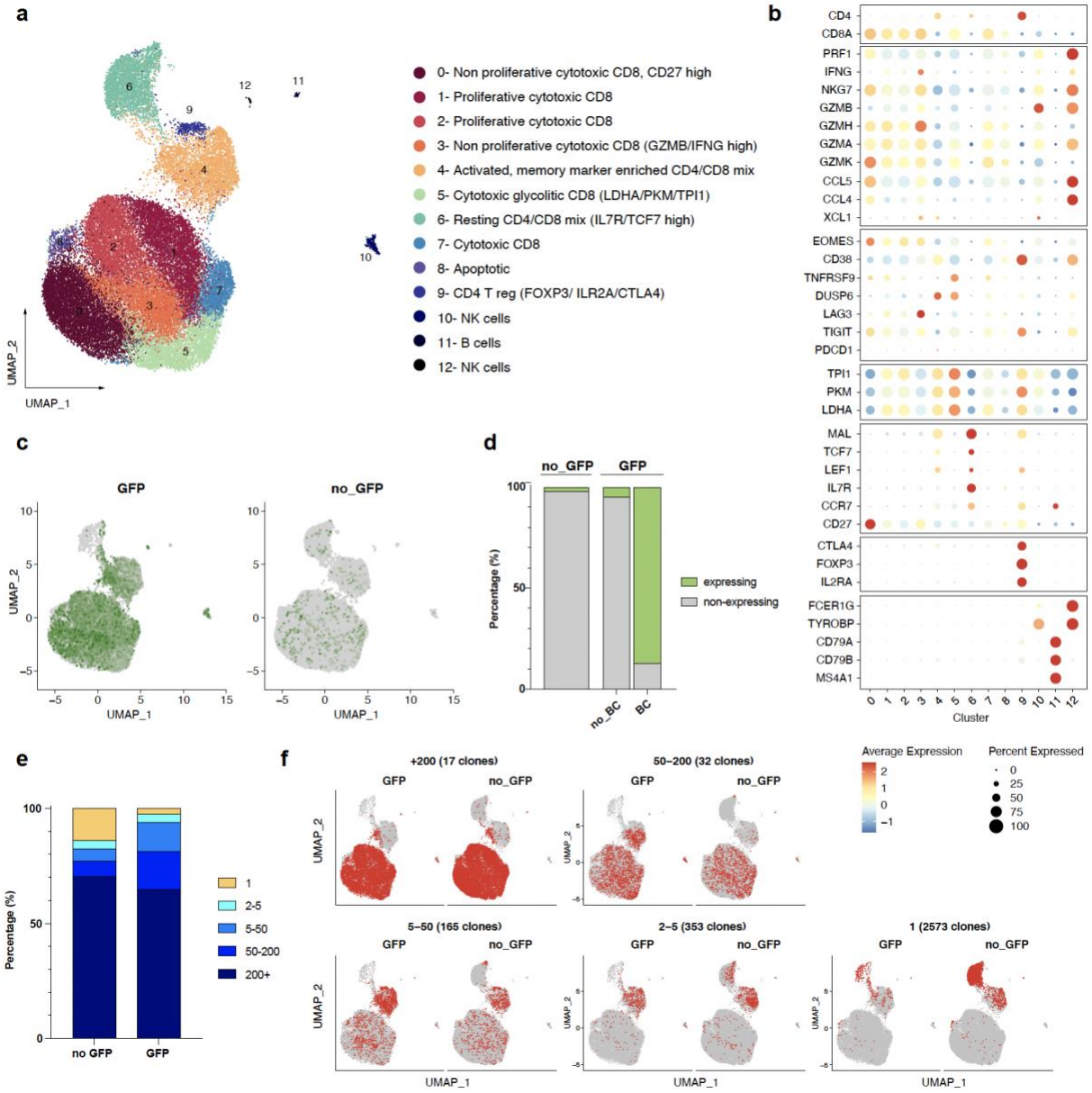

Fig. 5: Single cell transcriptomics and TCR sequencing reveals a broader enrichment of EBV reactive T cell phenotypes in GFP sorted cell samples. a, UMAP embedding and unsupervised cell clustering of 38908 EBV pulsed T cells. **b,** Dot plot showing the expression of a selection of T cell marker genes across clusters found in A. **c,** Feature plots showing the distribution of GFP expression across cells from GFP positive and GFP negative sorted samples. **d,** Enrichment of GFP positive and GFP negative sorted sample groups in GFP expressing cells. GFP positive sample group is further divided into cells that did or did not have a correctly annotated GFP-barcode. **e,** Enrichment of GFP positive and GFP negative sorted sample groups in 5 different TCR clonotype expansion bins. **f,** Projection of cells from 5 different TCR clonotype expansion bins on to the transcriptomic UMAP embedding. Cells from GFP positive and GFP negative sorted sample groups are shown in separate UMAP plots. BC – barcode.

DISCUSSION

Adoptive T cell therapy is a highly versatile treatment option due to the involvement of T cell immunity in a variety of indications such as autoimmunity [25], blood [26] and solid[27] cancers, infectious diseases [28, 29] and diabetes [30], to name but a few. While CAR-T cells are becoming a standard-of-care treatment for some hematological malignancies, patients with other challenging indications would benefit from alternative options with enhanced efficacy and persistence or with a broader targeting spectrum such as those afforded by the use of isolated antigen-specific T cells with native TCR, as shown for virus-associated malignancies [18].

Here, we present an efficient and polyvalent method of targeted gene delivery into antigen-specific T cells using a CRISPR protocol adapted to the use of peptide antigens as HDR-enabling stimuli in contrast to the commonly used nonspecific anti-CD3/CD28 stimulation of T cells. Although we focused on EBV-CTLs as a proof of concept, we note that this method does not depend on the specifics of this model, and can therefore also be applied to other antigen targets such as anti-tumor WT1-reactive T cell enrichment for anti-leukemic activity [31].

The use of genome editing for cell engineering offers notable advantages, in particular the precision of DNA construct integration. This ensures minimal disruption of cell function, as evidenced by our post-transfection phenotypic analysis, as well as long-term lineage tracing. While a typical weakness of CRISPR-induced HDR lies in its efficiency, we were able to achieve rates of integration suitable for a substantial DNA barcode library. Coupled with the permanence of genome editing, DNA barcodes may soon become standard procedure in cell therapies [32], making next-generation sequencers a likely soon-to-be essential tool in the clinic.

In order to assess the quality of the barcoded and selected EBV-CTLs, we combined our methodology with scRNA-seq, another tool that is revolutionizing cell engineering and immunotherapies [33]. Single-cell barcode sequencing, coupled with TCR clonotype information, provided an unprecedented level of detail on clonal expansion. In addition, scRNA-seq can link lineages to specific T cell phenotypes. The heterogeneity of stimulated T cell populations is essential to the development of effective immunity, and our genome editing protocol does not interfere with phenotype diversity. For instance, beyond the cytotoxic potential of CD8 T cells, it has been clearly shown that CD4+ T cells are crucial for sustaining anti-viral memory and effector functions [34, 35]. We observed that antigen-specific T cell stimulation combined with genome editing-based selection enabled the enrichment of EBV-CTLs with both CD4 and CD8 populations showing increased production of CD107a and cytotoxic molecules such as Granzyme B, IFNγ and TNFα among GFP-positive cells. Memory composition is another critical parameter of an effective therapeutic T cell product [36]. Early differentiated memory phenotypes such as stem cell memory and central memory are superior in the sustaining long-term anti-tumor responses [37, 38]. Generally, we had high proportions of central memory population among the GFP-positive cells and a good enrichment of memory

markers in the transcriptomics of the GFP-sorts. Interestingly, we observed a decrease of naïve-like/stem cell memory like CD62L+CD45RA+ population in contrast to bulk transduced or wild type cells which could be explained either by a slower activation of early-differentiated cells compared to central and effector memory cells and as a result lower level of HDR, or by initially low amount of EBV-specific T cells among early-differentiated cells due to a high frequency of EBV (CTL-cell-controlled) reactivation in humans [21]. Functionally, we noted that GFP-positive sorted T cells exhibited enhanced antigen specificity and improved cytotoxicity against autologous EBV-transformed LCLs.

Our work constitutes the first instance of the precise introduction of a genetic marker targeting selected donor-derived antigen-specific T cells. The method and these data combined should help establish the next generation of cell therapies combining *in-vitro* and *in-vivo* lineage tracing and the functional enrichment of antigen-specific T cells.

METHODS

Plasmid library construction

The barcoded GFP library was encoded in a plasmid constructed in two steps. First, the pCMV-GFP and homology arms were designed *in silico* and synthesized externally (Twist Bioscience). Second, the GFP was barcoded using oligonucleotide F1(RB203)* (Supplementary Table 1) with 9 degenerate “N” nucleotides and flanking regions homologous to the end of the GFP open reading frame and the start of the polyA signal. The oligonucleotide was used with primer R1(RB202)* in a NEBuilder assembly reaction (NEB). The resulting plasmid was transformed in electro-competent *E. coli* DH5α cells which were then grown in Luria-Bertani broth with 50 µg/ml ampicillin. An aliquot was plated to assess the transformation efficiency.

Peripheral blood mononuclear cell (PBMC) extraction and cell culture.

EDTA blood collected from adult healthy donors was used for peripheral blood mononuclear cell (PBMC) extraction. The study was approved by the Ethical Committee of Northwestern and Central Switzerland (PB_ 2018-00081), and written informed consents were obtained. PBMCs were isolated as previously published[39]. All cells were cultured at 37°C, 5% CO2.

T cells were cultured in cytotoxic T cell line medium (CTLm) composed of RPMI (Gibco), 5% human serum and 10,000 U/mL Penicillin-Streptomycin (ThermoFisher). PBMCs were stimulated with either anti-CD3/CD28 Dynabeads (ThermoFisher) according to manufacturer’s instructions or with EBV pepmix (PepTivator EBV Consensus peptide pool (Miltenyi Biotec)), at a final concentration of 60 pmol/peptide/mL in CTLm supplemented with 400 U/mL IL-4 and

10 ng/mL IL-7 (R&D Systems) for three days. After that, cells were washed, transfected and cultured n CTLm with cytokines or cultured without transfection.
EBV-transformed LCLs were generated using the B95.8 EBV strain as previously published [40].

Cell proliferation assay

$1.5x10^7$ PBMCs were stained with CellTrace™ Violet (CTV) Cell Proliferation Kit according to manufacturer's protocol, stimulated with the EBV pepmix and cultured in 6-well GRex plates (Wilson Wolf) and cultured for 9 days. Every second day starting day 3 of culture cells were gently resuspended and a fraction of cells was taken for immunocytochemistry and cell proliferation tracing by flow cytometry.

Genome editing of EBV-specific T cells

PBMCs were genome-edited using a combination of CRISPR/Cas9 ribonucleoprotein (RNP) and adeno-associated viral particles (AAV) after three days of culture with or without stimulation. The RNP was assembled by first duplexing the CRISPR RNA (crRNA, sequence TGACATCAATTATTATACAT CGG [41]) and trans-activating CRISPR RNA (trcrRNA) (IDT) through co-incubation at 95°C for 5 minutes and cooling to room temperature. The duplexed RNA molecules were then complexed with 25 µg (153 pmol) of Cas9 protein (IDT) at room temperature for 20 minutes. The AAV particles were produced externally (Vigene Biosciences) by packaging the repair template DNA encoding the pCMV-barcoded GFP construct in a AAV6 capsid. From the PBMC cultures, cells in suspension were gently extracted without a detaching agent. The culture wells, which retained adherent monocytes, were gently washed and topped with serum-free CTL and set aside during the transfection procedure. Suspension cells were centrifuged to remove the culture medium and resuspended in 100 µL P3 nucleofection buffer (Lonza), to which 6.5 µL of RNP were mixed in. Cells were transferred to nucleocuvettes and shocked using a 4D-Nucleofector (Lonza) with protocol EO-115. Cells were then gently diluted in 600 µL of warm serum-free CTL medium. After 30 minutes, the transfected cells were placed in their original well after emptying them again without detaching monocytes. After two hours of incubation, 20 µL of AAV particles at $2.25x10^{13}$ particles/mL (for a target MOI of $2x10^5$ particles/cell) were added to the cultures. After 24 hours, the cultures were diluted 1:1 with human serum-supplemented CTL medium.

Fluorescence activated cell sorting (FACS) of GFP+ cells

Expanded EBV-stimulated and transduced T cells were sorted based on GFP fluorescence after 10 days of culture. Cells in suspension were gently extracted without a detaching agent and centrifuged to remove the culture medium. Cells were then washed in DPBS (Gibco),

sorted using SH800 cell sorter (Sony Biotechnology) into CTL medium. For specificity and cytotoxicity analysis, cells were recovered for three days in CTLm supplemented with IL-4 and IL-7.

Staining for flow cytometry analysis of surface markers

Cells were stained with Zombie Aqua viability dye (Biolegend) in PBS, washed in FACS buffer and stained with the cocktail of monoclonal antibodies for CD3-BUV395 (clone UCHT1), CD4-BUV496 (SK3), CD8-BUV805 (SK1), TIM-3-BV480 (7D3), PD1-BB700 (EH12.2H7) (all BD Biosciences); CD45RA-APC (MEM-56, Thermo Fisher Scientific); CD45RO-Alexa Fluor 700 (UCHL1), CD62L-BV650 (SK11), CD27-BV421 (M-T271), CTLA-4-BV785 (L3D10), LAG-3-BV711 (11C3C65, Biolegend) and TIGIT-BV605 (A15153G, Biolegend).

Intracellular cytokine staining (ICC)

Cells in a pure CTLm as a negative control and cells stimulated with EBV pepmix were seeded into a U-bottom 96-well plate containing pure CTLm as a negative control, or CTLm with 500x-diluted pepmix, respectively. Cross-linked costimulatory anti-CD28/CD49d monoclonal antibodies (BD Biosciences), 1 µg/ml each, and anti-CD107a-BV510 (H4A3, Biolegend) were added, and cells were incubated at 37°C, 5% CO2 for 1 hour. Next, cell transport was blocked by 10 µg/ml Brefeldin A (Sigma). 5-hour incubation was followed by intracellular staining for flow cytometry analysis.

Cells were stained for viability with Zombie UV dye (Biolegend) according to manufacturer's instructions. Next, cells were washed with FACS buffer (2% sterile filtered FBS and 0.1% NaN3 in PBS), stained with surface monoclonal antibodies (all from BD Biosciences) for CD3-BUV395 (UCHT1), CD4-BUV496 (SK3), CD8-BUV805 (SK1) in FACS buffer, washed, fixed with fixation buffer (Biolegend), washed with permeabilization buffer (Biolegend) and stained for 30 min with the cytokine antibodies for (all Biolegend): IFNγ-APC/Cy7 (B27), TNFα-PE/Cy7 (MAb11) and Granzyme B-PE/Cy5 (QA16A02).

Cytotoxicity assay

T cells were incubated with autologous LCLs (Effector:Target = 20:1) for 5 h 30 min, stained for apoptosis with CellEvent Caspase-3/7 (Thermo Fisher), incubated for additional 30 min, washed in PBS, stained for dead cells with Zombie Aqua (Biolegend), then stained for CD3+ and CD19+ in FACS buffer and analyzed by flow cytometry. LCLs incubated without T cells were used as a control. The analysis was performed as previously published [4]. The formula used to define cytotoxicity was: % specific lysis = 100 - ([Vtest/Vcontrol]*100), where V is percentage of viable cells (double-negative for ZombieAqua and CellEvent).

Flow cytometry analysis

Samples were acquired on Cytek Aurora using SpectroFlo software. Data were processed using FlowJo.

Statistical analysis of flow cytometry data

Data were analyzed in Prism (GraphPad) using ANOVA or 2way ANOVA via statistical methods whichever were applicable.

Genomic PCR

Genomic DNA from 10^4 to 10^5 T cells was was extracted using QuickExtract buffer (Lucigen). The resulting product was used as a DNA template for a first PCR amplification reaction using primers F2(RB198)* and R2(RB199)* primers (Supplementary Table 1). The 3 kbp product was extracted by gel agarose electrophoresis and used as template for a second PCR amplification using primers F3(RB214)* and R3(RB215)*. The final amplimers were purified and sequenced externally by Illumina paired-end sequencing (GENEWIZ).

Single-cell sequencing

Single-cell sequencing was done according to the 10X Genomics pipeline and the manufacturer' instructions as previously described [42]. Briefly, for each donor, 20000 GFP-expressing cells and 20000 GFP-negative cells were sorted as described above and processed for single-cell sequencing using a Chromium Next GEM Single Cell 5' Library & Gel Bead Kit v1.1, a Chromium Next GEM Chip G Single Cell Kit and a Chromium Controller. The gene expression (GEX) and the fragmented TCR VDJ targeted enrichment libraries were prepared using a Chromium Single Cell 5' Library Construction Kit and a Chromium Single Cell V(D)J Enrichment Kit, Human T Cell. For GFP targeted enrichment, the primers F4(RB200)* and R4(RB222)* were used in a PCR amplification reaction. The resulting product was used as template for a second PCR amplification using an indexing primer and primer R5(RB201)*. All libraries were indexed using primers from a Chromium i7 Multiplex Kit and sequenced by the Genomics Facility Basel using an Illumina Novaseq and a single lane of a S4 flow cell.

Analysis of scRNA-seq GEX data

The raw scRNA-seq data was aligned to the human genome (GRCh38) using Cell Ranger (10x Genomics, version 6.0.0). In the first place a custom reference human genome, incorporating the GFP gene, was created using the *mkref* function, then the *count* function was used to obtain the raw gene expression matrix. Downstream analysis was carried out using the Seurat R package (version 4.0.1). As quality control, cells presenting low and high number

of detected UMIs (200 < nFeature_RNA < 7,000) and high percentage of mitochondrial genes (Percentage_MT < 20% of total reads) were removed. In addition, TCR genes were removed to avoid clonotype from guiding the subsequent clustering.

After QC a total of 38908 cells were used for downstream transcriptomic analysis. All samples were merged, normalized and scaled using 2000 variable features (GFP gene was removed to avoid its influence in downstream clustering analysis) while also regressing out cell cycle scores. Dimensionality reduction was done using the *RunPCA* function and batch effect was removed by performing harmony integration. Finally unsupervised cell clustering and differential gene expression was used to find marker genes used for cluster annotation. Results were then visualized using UMAP dimensionality reduction and ggplot2 R package.

Paired TCR repertoire analysis

Raw TCR scRNA-seq data was aligned to the VDJ-GRCh38-alts-ensembl (5.0.0) using Cell Ranger (10x Genomics, version 3.1.0). As quality control, only cells retaining a productive alpha and a productive beta chain were used. Downstream analysis was done using the R programming language and common packages (code available upon request). Cluster definition was performed as previously described by [42] and the comparisons of V and J gene usage was done using the package bcRep [43].

Analysis of scRNA-seq GFP barcode data

The GFP barcodes were linked to single cells through the 10X Genomics barcode. Where two GFP barcodes were identified (likely bi-allelic integration), they were concatenated and treated as one. For EBV-specificity predictions using TCRex, V- and J-gene information, along with CDR3 beta sequences, were queried against all available EBV epitopes. The output was then re-linked to clonotype identity.

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AUTHOR CONTRIBUTIONS

D.P., R.B.D.R, S.T.R. and N.K. designed the study; D.P., R.B.D.R. and R.C.R. performed experiments; R.C.R. and F.S. analyzed the sequence data. D.P., R.B.D.R. and N.K. discussed results. D.P., R.B.D.R. and R.C.R. wrote the manuscript with input and commentaries from all authors.

COMPETING INTERESTS

There are no competing interests to declare.

DATA AVAILABILITY

The raw FASTQ files from deep sequencing that support the findings of this study will be deposited (following peer-review and publication) in the Sequence Read Archive (SRA) with the primary accession code(s) <code(s) (https://www.ncbi.nlm.nih.gov/sra)>. Additional data that support the findings of this study are available from the corresponding author upon reasonable request. The raw and processed sc-RNAseq data generated in this study will be deposited in the Gene Expression Omnibus under accession number ---.

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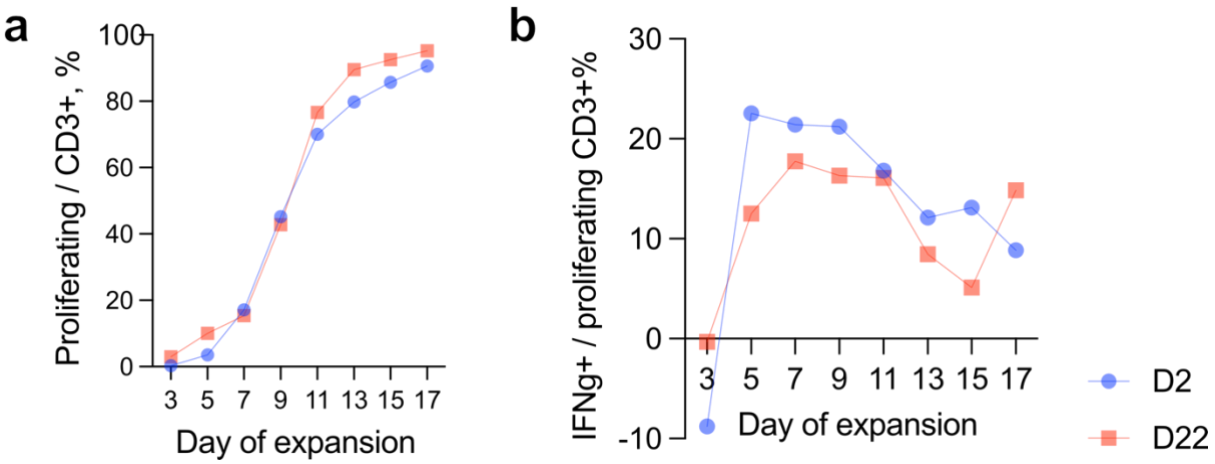
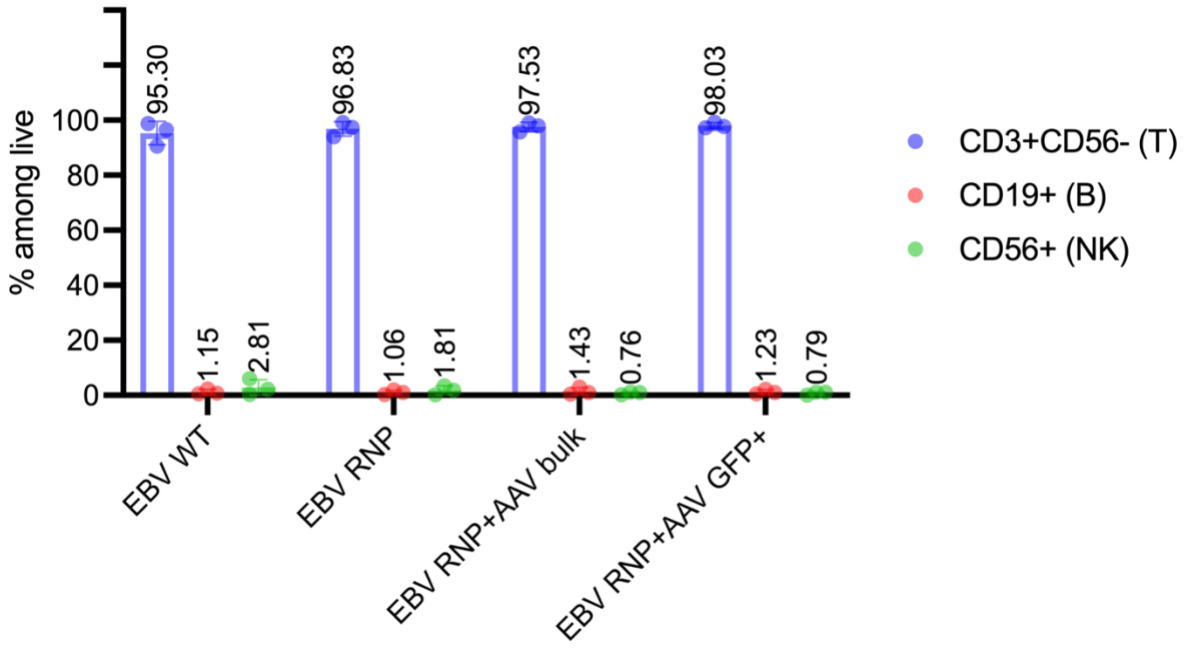
SUPPLEMENTARY MATERIALS

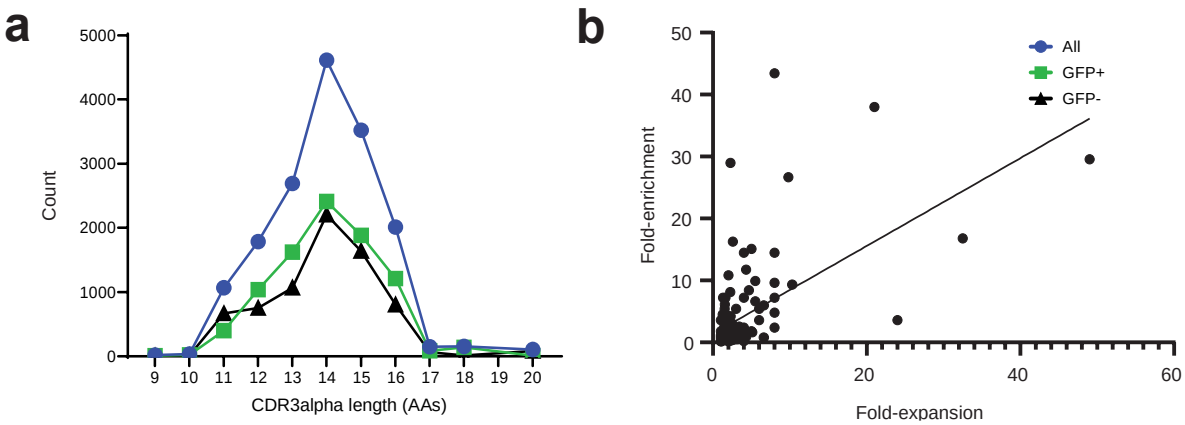
Supplementary Figures



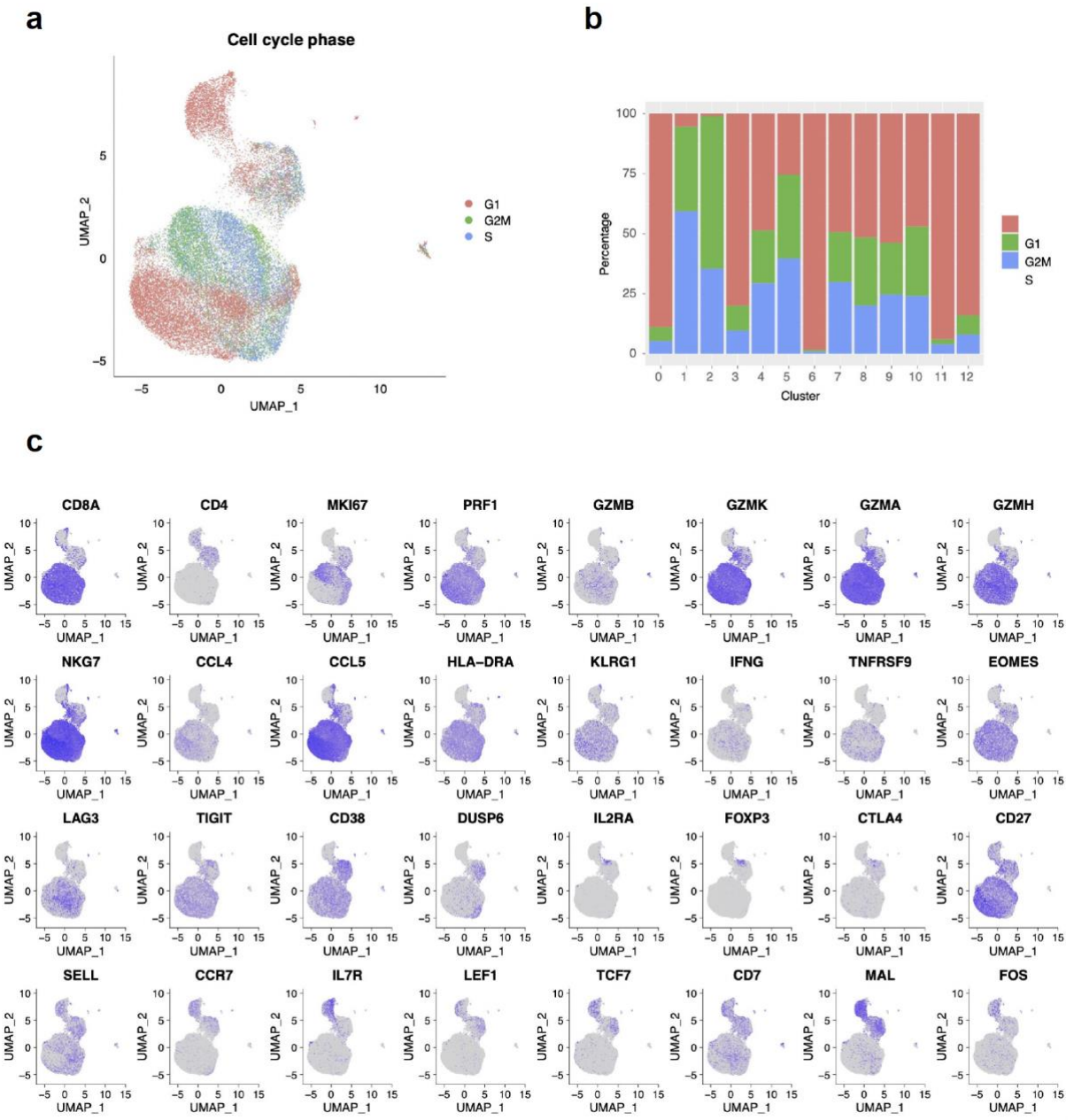
Suppementary Fig.1: EBV-specific T cell expansion detected by cell proliferation assay. **a**, dynamic of overall proliferation of CD3+ cells (proliferating cells = cells proliferated at least once). **b**, dynamic of EBV-specific T cells identified by immunocytochemistry following EBV pepmix re-stimulations.



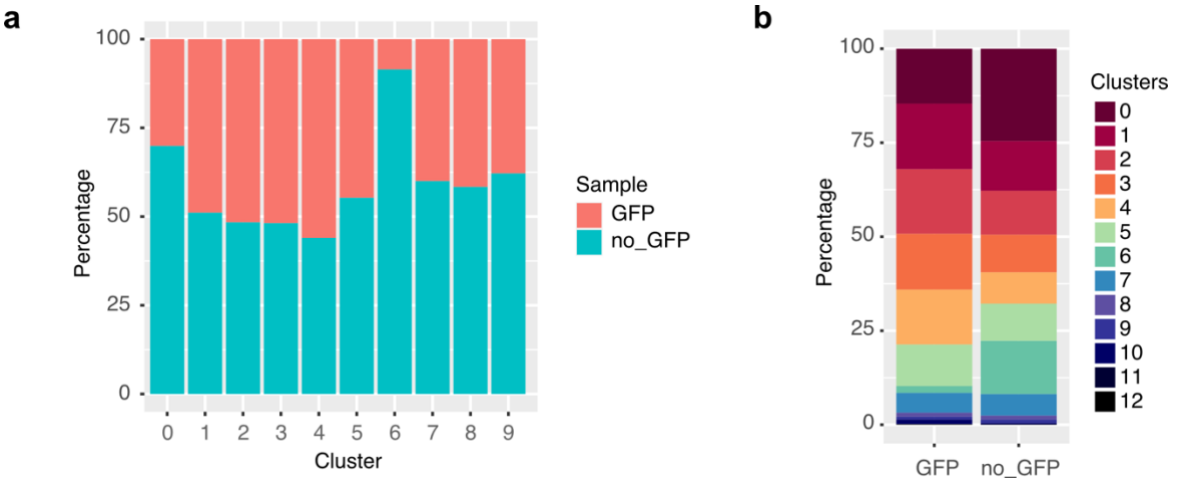
Supplementary Fig. 2: The purity of expanded EBV-CTLs.



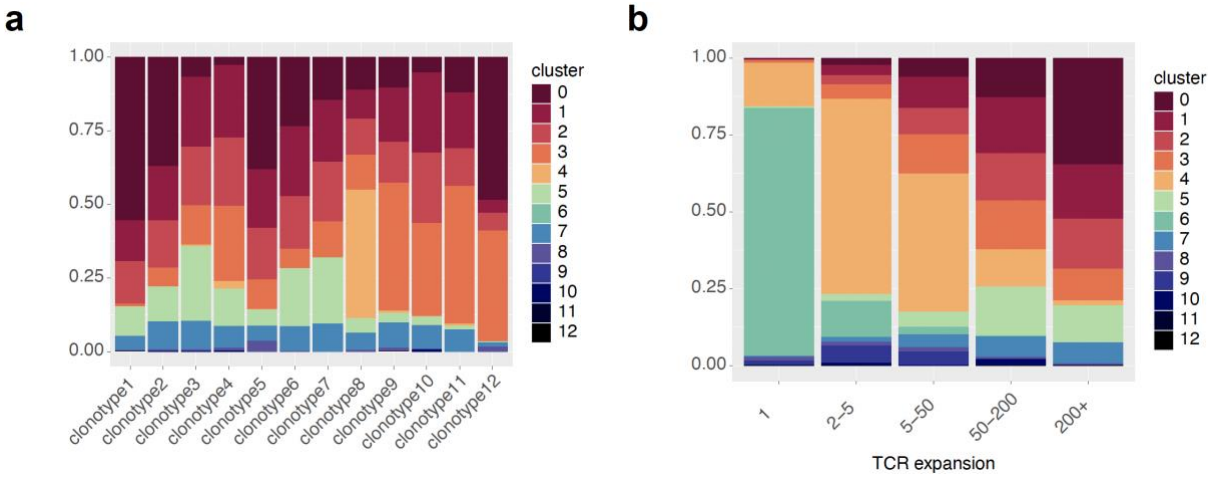
Supplementary Fig. 3: a, Distributions of the CDR3 alpha lengths for GFP+ and GFP- samples. Like the distributions of the CDR3 beta lengths, they do not differentiate between samples. **b,** Scatter plot of the fold-expansion and fold-enrichment values for all GFP+ clonotypes with barcodes. A linear regression of the data reveals a statistically significant correlation ($P < 0.0001$, $R^2 = 0.34$), indicating that a greater fold-expansion of the T cell clone is linked to a greater fold-enrichment in the GFP+ subset.



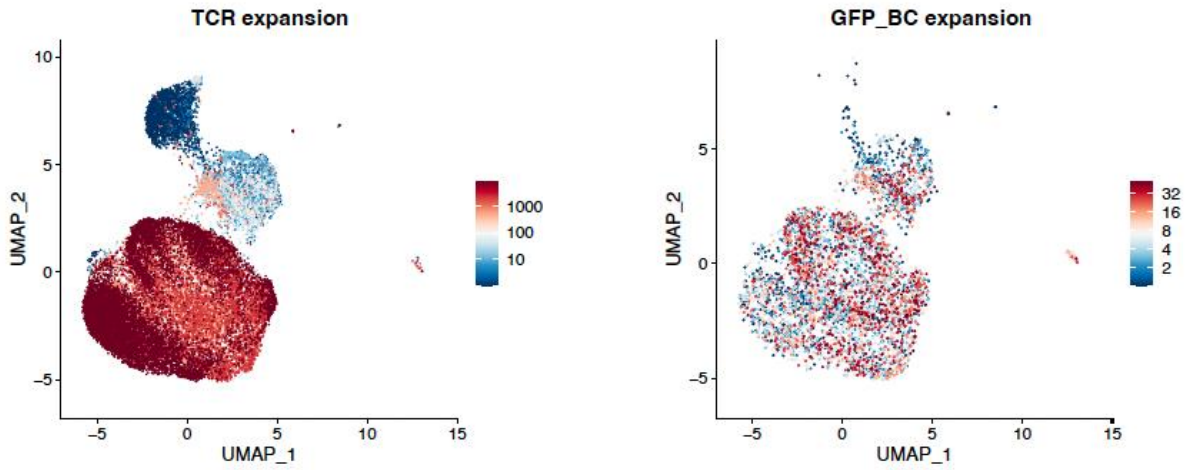
Supplementary Fig. 4: Gene expression profiles of EBV pulsed T cells. a, UMAP plot depicting cell cycle phase prediction for each single cell and **b,** abundance of each cell cycle phase within different clusters. **c,** projection of differential marker expression.



Supplementary Fig. 5. Cluster distribution within samples. a, Relative distribution of each cluster between GFP and no_GFP samples. **b,** distribution of clusters within each sample.



Supplementary Fig. 6. Cluster distribution across clonotypes with different expansion rates. a, Relative distribution of each cluster between the top 12 most expanded clonotypes. **b,** Distribution of clusters within clonotypes belonging to 5 different expansion bins.



Supplementary Fig. 7. Distribution of TCR and GFP-barcode clonotypes according to the level of expansion.

Supplementary Tables

Supplementary Table 1:

RB202	GFP_end_R	TTACTTGTACAGCTCGTCCATGCCG
RB203	BAR_MCS_F	NNNNNNNNNNNNGAATTCGATATCAAGCTTGTCGACC
RB198	CCR5_LHA_start_F	TTCTTTGTGGGCTAACTCTAGCGTC
RB199	CCR5_RHA_end_R	GGCCAAAGAATTCCTGGAAGGTGTT
RB214	EGFP-C_Illu	CCCTCCTTTAATTCCCCATGGTCCTGCTGGAGTTCGTG
RB215	EBF-rev_Illu	GAGGAGAGAGAGAGAGGTGGTTTGTCCAAACTCATC

CONCLUSIONS AND OUTLOOK

Different strategies might be implicated to improve adoptive cell therapies. In my work, I aimed at improving particularly Epstein-Barr virus-specific T cell therapies.

The first question I addressed was the possibility of **EBV-specific stem cell memory T cell enrichment from PBMCs for ACT application**. The method that has been so far utilized to generate EBV-specific T_{SCM} only include priming of naïve cells by EBV-peptide-loaded DCs or EBV-LCLs. Although they demonstrated an improved anti-tumor cytotoxicity in mice, such approach is difficult to transfer into clinical setting due to a complex manufacturing procedure, and a simpler T_{SCM} generation method is required. I hypothesized that EBV-specific T_{SCM} can be readily enriched from EBV-seropositive donor-derived PBMCs and can restore T cell immunity to EBV more efficiently than conventional long-term stimulated and expanded EBV-CTLs.
The work described in chapter I demonstrates for the first time that rapid expansion from PBMCs stimulated by an EBV antigen pool, cultured with IL-4 / IL-7 and supplemented with TWS119 yields promising EBV-specific T_{SCM}-enriched CTLs with favorable properties for VST, such as early differentiated memory composition, low exhaustion, better tumor infiltration, efficient CD4+ and CD8+-mediated cytotoxicity, long-term persistence potential, and broader antigen specificity. The method and these data together should help to establish the next generation of unmodified antigen-specific cell therapies beyond EBV indications. The clonal diversity of a CTL-R response remains to be investigated in the upcoming clinical trial.

Another strategy I explored is the possibility of EBV-specific T cell identification upon the adoptive transfer. There is no genetic marker that would allow to readily distinguish infused cells from the patient's own cells except some HLA diversity (and only in case of allogeneic transfer). Previously, genetic markers were introduced in virus-specific T cells only once using retroviral vectors but the use of such vectors is associated with safety risks. I hypothesized that CRISPR/Cas9 so far used to insert transgene only in bulk T cells can be directed to edit antigen-stimulated and activated virus-specific T cells. In chapter II, I showed a **novel approach of polyclonal antigen-specific T cell-targeted genome editing (TarGET)** for adoptive therapy applications utilizing T cells with native TCRs. In essence, we established a method of **transgene insertion into**

the genome of T cells that were *specifically* activated and thus select antigen-specific T cells (on the example of EBV-specific T cells) using a transgene and trace lineages using DNA barcodes. We characterized the resulting products by immune phenotyping and deep TCR and single-cell RNA sequencing and showed that our method helps to enrich polyclonal functional EBV-specific T cells with an improved target cytolytic activity. Precise genome insertion and the exploitation of a safe harbor locus helps to minimize safety concerns. We used GFP as a transgene in our work however for potential clinical application safer options should be considered, e.g., truncated CD19-CAR or better a therapeutically relevant marker, e.g., cytokine production or resistance to immune suppression. Moreover, this method can be used beyond EBV- or virus-specific T cell modification. Self-cancer-specific (TAA-specific, e.g., WT-1-specific) T cells can be enriched in a similar way.

The TarGET method applied to the protocol of T_{SCM}-enriched EBV-CTL expansion can provide further insights into the dynamic of EBV-specific lineage expansion and function in response to the PTLD model *in vivo*. In future, TarGET has the potential to enhance the traceability and the monitoring capabilities during immunotherapeutic T cell regimens.

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Я таксама хачу падзякаваць самое сябе, за ўнутраны моц і вытрымку, за тое, што навучылася даваць рады апатыі ды адчаю і дасягаць пастаўленных мэтаў.

APPENDIX I. Clinical trials with EBV-specific T cell therapies

Table I. Published clinical studies and case reports of EBV-specific T cell therapy. Partially included in [1].

Diagnosis (PTLD/viremia/ primary immunodeficiency)	Number of patients (only receiving EBVSTs)	Type of transplant	Indication (prophylaxis/preemptive or treatment)	Specificity (only EBV?)	Generation method (cell culture, vector/peptide) IFN-g	Source (HCT donor or third party)	Cell dose	GVHD	Outcomes	Reference
PTLD	33	HSCT	Prophylaxis	EBV	Ex vivo expansion with LCLs	3rd	$2x10^7/m^2$	No	ND – 33/33	Rooney 1998, Houston [2, 3]
PTLD	6	HSCT	Prophylaxis	EBV	Ex vivo expansion with LCLs	HSC donors	$10x10^7/m^2$ 2-4 infusions	1/6	ND – 5/6 PD – 1	Gustafsson 2000, Stockholm [4]
PTLD	7	SOT	Prophylaxis	EBV	Ex vivo expansion with LCLs	3rd party	$2x10^7$ CTL/m^2 (multiple infusions)	No	ND 5/7 NR 2/7	Comoli 2002, Pavia [5]
PTLD	13	HSCT	Prophylaxis	EBV, Adv	Ex vivo expansion with LCLs	HSC donors	$5x10^6$-$1.35x10^8/m^2$	No	ND – 9/13	Leen 2009, Houston [6]
PTLD	101	HSCT	Prophylaxis	EBV	Ex vivo expansion with LCLs	HSC donors	$10x10^7/m^2$ (2x or 4x)	16/101	ND – 101/101	Heslop 2010, Houston [7]
PTLD	3	HSCT	Prophylaxis	AdV, EBV, CMV, BKV, HHV6	Rapid expansion	HSC donors	$0.5x10^7$-$2x10^7/m^2$;	2/3 - TAM (not related to VSTs)	n/a	Papadopoulou 2014, Houston [8]
PTLD	10	HSCT	Prophylaxis	EBV, CMV, Adv, VZV	Ex vivo expansion with DCs	HSC donors	$2x10^7/m^2$	3/10	ND – 10/10	Ma 2015, Sydney [9]
PTLD	11	HSCT	Prophylaxis	EBV, CMV, BKV, Adv, VZV, Influenza,	Ex vivo expansion with DCs	HSC donors	$2x10^7/m^2$	4/11	ND – 11/11	Gottlieb 2021, Sydney [10]

				Aspergillus fumigatus						
PTLD	5	HSCT	Treatment	EBV	DLIs	HSC donors	0.12-1x10^6/kg	3/5	CR – 5/5	Papadopoulos 1994, NY [11]
PTLD	1	HSCT	Treatment	EBV	Ex vivo expansion with LCLs	HSC donor	1st - 8.2x10^6, 2nd – 1x10^6 + 1.26 ´ 107 (unstimulated lymphocytes)	no	NR – 1/1	Imashuku 1997, Kyoto [12]
PTLD	6	HSCT	Treatment	EBV	Ex vivo expansion with LCLs	3rd	2x10^7/m^2	No	CR – 3/6	Rooney 1998, Houston [2]
PTLD	5	HSCT/SOT	Treatment	EBV	Ex vivo expansion with LCLs	3rd	1x10^6/kg	No + graft improvement in 3 cases	CR – 3/5, NR – 2/5	Haque 2002, Edinburgh [13]
PTLD	33	HSCT/SOT	Treatment	EBV	Ex vivo expansion with LCLs	3rd	2x10^6/kg	No	CR – 14/33, PR – 3/33, NR – 16/33	Haque 2007, Edinburgh [14]
PTLD	5	HSCT	Treatment	EBV	Ex vivo expansion with LCLs	HSC donors	0.5×10^6 cells/kg (multiple infusions)	no	CR – 5/5	Comoli 2008, Pavia [15]
PTLD	2	CB HSCT	Treatment	EBV	Ex vivo expansion with LCLs	3rd	10x10^6/kg, multiple infusions	No	CR – 2/2	Barker 2010, NY [16]
PTLD	13	HSCT	Treatment	EBV	Ex vivo expansion with LCLs	HSC donors	10x10^7/ m^2 (2x or 4x)	5/13	CR – 11/13, PD – 2/13	Heslop 2010, Houston [7]
PTLD	6	HSCT	Treatment	EBV	IFNg capture	HSC donors	0.3-7.4x10^6 (single infusions)	No	CR – 3/6, NR – 1/6, PD – 2/6	Moosmann 2010, Munich [17]
PTLD	1	HSCT	Treatment	EBV	Pentamer selection	3rd party	1st - 1.1x10^4/kg,	No	CR – 1/1	Uhlin 2010, Stockholm [18]

							2^{nd} – $2x10^4$/kg			
PTLD	19	HSCT	Treatment	EBV	Ex vivo expansion with LCLs	HSC donors (14) or 3^{rd} (5)	$1x10^6$/kg weekly for 3 weeks	No	CR – 13/19, NR – 6/19	Doubrovina 2012, NY [19]
PTLD	30	HSCT	Treatment	EBV	DLIs	HSC donors	0.2-$1x10^6$/kg once	5/30	CR – 22/30	Doubrovina 2012, NY [19]
PTLD	10	HSCT	Treatment	Adv, EBV, CMV	Rapid expansion	3rd	$5x10^6$-$2x10^7/m^2$	1/10	CR – 8/10, PR – 1/10, NR – 1/10	Gerdemann 2013, Houston [20]
EBV viremia and / or PTLD	10	HSCT	Treatment	EBV	IFNg capture	HSC donors	148-53796/kg	1/10	CR – 6/10, PR – 1/10, NR – 3/10	Icheva 2013, Tübingen [21]
PTLD	8 (4 with EBV infection / reactivation)	HSCT	Treatment	AdV, EBV, CMV, BKV, HHV6	Rapid expansion	HSC donors	$0.5x10^7$-$2x10^7/m^2$;	1/11 (improved).	CR – 4/4 (EBV)	Papadopoulou 2014, Houston [8]
PTLD	10	HSCT	Treatment	EBV	Ex vivo expansion with LCLs	3^{rd} party	1-$2x10^6$/kg	no	CR – 8/10, PD – 2/10	Vickers 2014, Aberdeen [22]
PTLD	77	HSCT	Treatment	EBV	DLI (62) or Ex vivo expansion with LCLs (15)	Donor DLI, autoCTL	DLI $2x10^7$/kg, CTL $1x10^6$/kg, 1-8 doses	DLI: 35/62 CTL: 5/15	CR – 70/77	Jiang 2016, Guangzhou [3]
PTLD	2	HSCT	Treatment	EBV	IFNg capture	3rd	1.8-$2.3x10^4$/kg	No	CR – 2/2	Kállay 2018, Budapest [23]
CNS PTLD	1	SOT	Treatment	EBV	IFNg capture	3^{rd} party	$2.5x10^4$	No	CR – 1/1	Schultze-Florey 2018, Hannover [24]
PTLD	48	HSCT/SOT	Treatment	EBV	Ex vivo expansion with LCLs	3^{rd} party	1-$2x10^6$ /kg	2/48	CR – 18/48, PR – 10/48,	Kazi 2019, Aberdeen [25]

									NR – 20/48	
PTLD	46	HSCT (33), SOT (13)	Treatment	EBV	Ex vivo expansion with LCLs	3rd party	3 infusions	1/46	HSCT: CR – 19/33, PR – 3/33, NR – 1/33, PD – 9/33. SOT: CR – 2/13, PR – 5/13, NR – 1/13, PD – 5/13	Prockop 2019, New York [26]
PTLD	1	HSCT	Treatment	EBV (LMP-1)	Rapid expansion	HSC donor	8.94×10^8 (5 infusions)	1/1	CR	Hong 2020, Anhui [27]
PTLD	1	HSCT	Treatment	CMV, EBV, AdV	Ex vivo expansion with DCs	3rd party	$2x10^7/m^2$ (1 infusion – for EBV)	no	1/1 - CR	Di Ciaccio 2020, Sydney [28]
EBV replication	1	HSCT	Treatment	EBV	Ex vivo expansion with LCLs	HSC donor	0.5×10^6 cells/kg (multiple infusions)	no	1/1 - CR	Comoli 2008, Pavia [15]
PID	16	HSCT	Prophylaxis	CMV, EBV, AdV, HHV, BK	Rapid expansion / Ex vivo expansion with LCLs / streptamer selection	HSC donors / 3rd-party / Cord blood	$5x10^6$ to $1.35x10^8$ /m2	1/16	ND – 16/16	Naik 2016, Houston-London-Washington-Newcastle [29]
PID with viral replication or EBV-LPD	20	HSCT	Treatment	CMV, EBV, AdV, HHV, BK	Rapid expansion / Ex vivo expansion with LCLs / streptamer selection	HSC donors / 3rd-party / Cord blood	$5x10^6$ to $1.35x10^8$ /m2	3/20	CR – 11/20, PR – 2/20, PD – 7/20	Naik 2016, Houston-London-Washington-Newcastle [29]

PID, lymphoblastic leukemia, Crohn disease, Systemic Lupus Erythematosus	11	No HCT setting	Treatment	EBV	Ex vivo expansion with LCLs	Unrelated	1-2x10^6 /kg	No	CR – 5/11, PR – 2/11, NR – 4/1	Kazi 2019, Aberdeen [25]
Refractory infection after HSCT/SOT or PID	13	HSCT / SOT/ -	Treatment	ADV, CMV, EBV (2) or BKV	IFNg capture	HSC donors / 3rd-party	0.5x10^4 /kg for HLA mismatched related donors, 2.5x10^4 /kg for matched related donors (up to 5 infusions)	1/13	CR – 10/13, PR – 2/13, not yet evaluated – 1/13	Flower 2020, NY [30]
PID	2	No HCT setting	Treatment	EBV	Rapid expansion	3rd-party	5×10^7 cells/m^2	No	CR – 1/2, PR – 1/2	Rubinstein 2019, Ohio [31]
EBV-lymphoma	29	No HCT setting	Adjuvant therapy	EBV	Ex vivo expansion with irradiated DCs	Patient	4x10^7-5x10^8/m2	No	28/29 – no relapse	Bollard 2014, Houston [32]
EBV-lymphoma	19	HSCT	Prophylaxis	EBV	Ex vivo expansion with LCLs	HSC donors	2x10^7-1.2x10^8/m^2	2/19	ND – 15/19, PD – 4/19	McLaughlin 2018, Washington [33]
Primary CNS B-cell lymphoma	1	HSCT	Treatment	EBV	Ex vivo expansion with LCLs	Patient's cells	2x10^6/kg 7 infusions + 2 additional infusions	no	CR	Wynn 2005, Machester [34]

							after recurrence			
EBV-lymphoma	21	No HCT setting	Treatment	EBV		Patient	$4x10^7$-$5x10^8$/m2	No	CR – 11/21, PR – 2/21, NR – 8/21	Bollard 2014, Houston [32]
EBV-lymphoma	11	SOT/CBT/auto PBSC / -	Treatment	EBV	Ex vivo expansion with LCLs	3rd party or auto PBSC	$5x10^6$/kg (2 infusions)	no	CR – 3/11, PR – 1/6, PD – 6/11, n/a – 1/11	Gallot 2014, Nantes [35]
EBV CNS lymphoma	2	SOT	Treatment	EBV	Activation marker selection with subsequent ex vivo expansion	3rd party	$2x10^7/m^2$ (4 infusions)	no	CR – 2/2	Law 2021, Brisbane [36]
EBV-associated non-haematopoietic sarcoma	1	SOT	Treatment	EBV	Ex vivo expansion with LCLs	3rd party	$1\text{-}2x10^6$/kg	no	PR – 1/1	Vickers 2014, Aberdeen [22]
EBV-associated NK/T lymphoma	1	HSCT	Treatment	EBV	Rapid expansion with irradiated DCs	3rd party	$2x10^7/m^2$	No	PD – 1/1	Withers 2017, Sydney [37]
EBV lymphoma	7	HSCT	Treatment	EBV	Ex vivo expansion with LCLs	HSC donors	$2x10^7$-$1.2x10^8/m^2$	1/7	PR – 2/7, NR – 1/7, PD – 4/7	McLaughlin 2018, Washington [33]
Various virally caused diseases	11	HSCT	Treatment	Adv, EBV, CMV	Ex vivo expansion with LCLs	HSC donors	5x106-1x108/m2	No	CR – 1/11, PR – 2/11 (others - ???).	Leen 2006, Houston [38]
Adv/CMV/EBV viremia	7	CBT	Treatment	EBV, CMV, Adv	Ex vivo expansion with LCLs	CB	$5x10^6$-$1x10^7/m^2$	2/7	ND – 4/7, PR – 1/7, PD – 2/7	Abraham 2019, Washington [39]
Viral reactivation	24	HSCT	Prophylaxis	CMV, EBV, Adv	Streptamer selection	3rd party	0.4–$26.5x10^6$	2/24	EBV: ND – 5/6,	Roex 2019, Leiden [40]

									PTLD – 1/6	

Abbrevations:
CR – complete remission
PR – partial response
ND – no disease
NR – no response
PD – progressive disease

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