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CHAPTER 1

General introduction

1 VIRAL INFECTIONS IN IMMUNOCOMPROMISED PATIENTS

Viruses are pathogens that only contain a protein coat and a core of genetic material and that need a host-cell in order to replicate(1). Depending on the type of virus, the viruses are released when the host cell dies or they leave infected cells by budding from the membrane without directly killing the cell. The antiviral immune response can be divided into an early (first ~7 days), non-specific phase involving innate immune mechanisms and an antigen-specific phase involving adaptive immunity by T and B cells. Plasma cells originate from B cells and produce virus-specific antibodies that play a role in neutralizing free viral particles, while virus-specific T cells are essential to suppress virus replication by eliminating virus infected cells. This antiviral cellular immunity can be hampered in immunocompromised patients, like patients undergoing allogeneic stem cell transplantation (alloSCT) as treatment for hematopoietic malignancies(2, 3). Prior to alloSCT, these patients receive a conditioning regimen consisting of chemotherapy, irradiation and/or immune suppressive antibodies to eradicate malignant cells, prevent graft rejection and allow replacement of patient hematopoiesis by donor hematopoiesis following infusion of the stem cell graft(4-6). The main beneficial effect of alloSCT is mediated by donor-derived alloreactive T cells directed against antigens expressed on hematopoietic cells that genetically differ between donor and recipient(5, 7). This T-cell response, resulting in elimination of hematopoietic cells from the recipient, including the malignant cells, is known as the graft-versus-leukemia (GVL) effect. Since after alloSCT normal hematopoiesis is of donor origin, such donor-anti-patient T-cell responses can cause complete elimination of patient hematopoiesis without causing pancytopenia. However, donor-derived T-cell responses can also be directed against polymorphic antigens presented by non-hematopoietic healthy cells in the tissues and organs of these patients, which can lead to graft-versus-host disease (GVHD)(8, 9). The major challenge in the field of alloSCT is to find a balance between the prevention of GVHD, while maintaining strong GVL responses and protective immunity against pathogens. Strategies to prevent or treat GVHD rely on treatment of patients with profound immune suppression following alloSCT or by depleting T cells from the stem cell grafts(7, 10-12). These interventions can lead to profound impairment of cellular immunity resulting in lack of control of viral infections. T-cell depletion (TCD) from the graft also increases the incidence of relapse of the malignancy. To restore the GVL response, TCD alloSCT can be followed by a postponed administration of donor lymphocyte infusion (DLI) which may also restore viral immunity(10, 13). However, during the interval between alloSCT and DLI, and during immune suppressive treatment for GVHD, patients experience a period of profound and prolonged T-cell deficiency in which they are at risk for developing infectious complications. The major viral pathogens causing morbidity and mortality after alloSCT (and recipients of solid-organ transplantation) are the common viruses

cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human adenovirus (AdV) that persist in the patient after primary infection and are able to reactivate from their latent state(14).

LATENT VIRUSES AND ABSENCE OF ANTIVIRAL IMMUNITY

CMV, EBV and AdV are causing frequent problems in immunocompromised patients because these viruses cannot be completely cleared from the host after primary infection, even by individuals with a healthy immune system. These viruses therefore persists in specific cells after primary infection, but remain under control of the anti-viral immune system. However, such viruses reactivate and cause morbidity and mortality when previously infected individuals become immunocompromised. There are three types of persistent virus-infections that are defined as latent, chronic and slow infection(15). CMV, EBV and AdV persist as latent viruses, characterized by the ability of the virus to remain dormant within cells of the host, while remaining undetectable in peripheral blood. Latency is generally maintained by specific viral genes. Expression of such latency-associated genes keeps the viral genome from being digested by cellular ribosomes. Another mechanism of a virus to remain latent, is to inhibit recognition by the immune system through downregulation of Human Leukocyte Antigen (HLA)-class-I or inhibiting the apoptotic pathway of a cell. During these latent infections, the viral genome may be either stably integrated into the cellular host DNA or maintained episomally(16). From the latency state of infection, the virus can reactivate to start lytic replication again. Many different factors such as trauma, infection caused by other pathogens, various stress factors, menstruation, medication and various illnesses can result in the reactivation of latent viruses in healthy individuals(15, 17). Contrary to healthy individuals, reactivations in patients receiving alloSCT or solid-organ transplantation cannot be controlled due to the absent functional antiviral cellular immunity(18).

CMV

CMV is a beta-herpesvirus, and the largest known human herpesvirus. The virus has linear double-stranded DNA enveloped by a matrix with a lipid bilayer that contains viral glycoproteins(19). After primary infection, CMV infects and replicates in a wide variety of cells, including epithelial cells of gland and mucosal tissue, smooth muscle cells, fibroblasts, macrophages, dendritic cells, hepatocytes and vascular endothelial cells(20). CMV remains latent in hematopoietic progenitor cells and cells of the myeloid lineage (e.g. $CD14^{pos}$ monocytes)(21). It was estimated that 83% of the general population worldwide have had a primary infection with CMV(22). This can be measured by CMV-specific antibodies in peripheral blood and individuals are then considered as

CMV seropositive(22). In Western Europe and the United States 45% to 60% of alloSCT recipients are seropositive for CMV and therefore at risk for endogenous reactivation of latent CMV infection(23-25). Around 80% of these CMV-seropositive patients will encounter a CMV reactivation after alloSCT(26). Primary CMV infection in these patients can also occur, but this is uncommon. In the absence of adequate immunological control by CMV-specific memory T cells, CMV reactivation can progress to CMV disease, which is characterized by potentially fatal organ involvement, such as CMV pneumonia, colitis or encephalitis(27). Availability of antiviral agents like ganciclovir, foscarnet, cidofovir and letermovir(28) have contributed to a significant reduction in CMV-related morbidity and mortality. However, administration of these drugs has only a temporary effect(29), and a functional antiviral immunity is needed to control these viruses.

EBV

EBV is a human gamma-herpesvirus, that is composed of double-stranded DNA and enveloped by a nucleoplasmid and matrix with glycoproteins(30, 31). Approximately 90% of adults have experienced primary infection with this virus during childhood(30). In immunocompetent individuals, active EBV infection usually resolves without treatment and only results in mild symptoms, followed by lifelong persistence of the EBV virus in B cells and pharyngeal epithelial cells as a latent infection(31). In healthy individuals, upon infecting naïve B cells or epithelial cells, EBV first enters the immunogenic latency phase III where EBV expresses all viral proteins (e.g. EBV Nuclear Antigen 1-3 (EBNA1-3) and latent membrane proteins (LMP) 1 and 2)(32). This results in the activation of the naïve B cell, followed by entrance to the second latency phase (II) with a restricted gene expression of only EBNA1, LMP1 and LMP2. This induces the activated B cell to differentiate into a memory B cell, resulting in the establishment of the latency phase I, where only EBNA1 and BARF1 RNAs are expressed(33). In patients after alloSCT, donor-derived B cells transferred with the graft are the primary source for EBV reactivations. Although residual patient-derived B cells that survived the conditioning regimen can also be a source for EBV reactivations, this is less frequent(34). Similarly to CMV, in the absence of adequate immunological control by EBV-specific memory T cells, EBV reactivation can progress to uncontrolled proliferation of EBV-infected B cells, leading to potentially fatal post-transplant lymphoproliferative disease(35).

The inability to control EBV reactivations can also occur as a very rare complication in healthy individuals, often referred to as chronic active EBV (CAEBV) infection(36). CAEBV infection may then progress to the development of a broad range of malignancies of lymphoid origin, including Burkitt lymphoma, Hodgkin lymphoma (HL), B-, T- and natural killer- (NK) cell lymphomas and diffuse large B-cell lymphoma (DLBCL), as well as epithelial malignancies like nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC) (37, 38). These different EBV-associated malignancies are all associated with a specific

latent phase of EBV. Each latency phase (I, II or III) is associated with differently expressed genes, with latency type I expressing the least genes and type III the most genes(38-40).

AdV

AdV consists of a group of double-stranded non-enveloped linear DNA viruses that are composed of a protein capsid containing 240 hexon and 12 penton components with a nucleoprotein core that contains the DNA viral genome and internal proteins(41). There are currently 66 serotypes described that can be grouped into six subgroups (A-F)(42). However, all serotypes express the Hexon protein, which contains generic antigenic components common to all adenoviral species(43). Symptomatic AdV infections are most common in children, with a peak incidence between the ages of 6 months and 5 years, which mainly affects the respiratory, ocular, skin, and gastrointestinal tract(44, 45). Following primary infection, AdV viral DNA has been detected in mucosal lymphocytes, lung, upper airway, and also in cells of the gastrointestinal tract, showing that AdV is capable of establishing a latent infection(44). AdV reactivations in recipients of alloSCT are most often seen in pediatric patients (20%-26%) and less often in adults (9%)(44, 46). Since AdV mainly resides in mucosal lymphocytes and lung/intestinal-tissue as latent infection, it can be argued that reactivations in recipients of alloSCT mainly originates from the patient.

HOST DEFENSE MECHANISMS AGAINST VIRUSES

Antigen processing, presentation and recognition

After a virus infects a cell, virus-derived antigens can be endogenously processed and presented to T cells, whereby virus-derived proteins within the cell become ubiquitinated, marking them for proteasomal degradation(47). Proteasomes then break the proteins up into smaller peptides of varying length. In humans, HLA-class-I molecules are responsible for presenting these intracellular peptides on the surface of the infected cell. Most of the nucleated cells in the human body express HLA-class-I molecules on their cell-surfaces(48, 49). HLA-class-I molecules are heterodimers that consist of an α and β_2-microglobulin (B2M) chain. Only the α chain is polymorphic and the α_1 and α_2 domains fold to make up a groove for peptides to bind. Peptides of a length of 8-12 amino-acids are suitable for fitting within the peptide binding region of these HLA-class-I molecules(48). Binding by a peptide stabilizes the HLA-class-I complex, allowing it to be transported intracellularly to the cell surface of the infected cell(49). Cytotoxic T cells express the co-receptor cluster of differentiation 8 (CD8) that, together with their T-cell receptor (TCR), can bind specifically to a peptide-loaded HLA-class-I molecule on the surface of a cell. Since there are many different virus-derived peptides that can be presented by HLA molecules, T cells express different TCRs, each with a different

specificity that can contribute to the anti-viral response. Upon successful binding, cytotoxic T cells release granzymes and perforins that kill these infected cells (**Figure 1**; top panel. On-target reactivity). Additionally, cytotoxic T cells also release cytokines such as IFN-γ, TNF-α, and TNF-β, which contribute to the host defense in several ways(50). For example, IFN-γ directly inhibits viral replication, but also induces the increased expression of HLA-class-I and HLA-class-II in infected cells(50, 51).

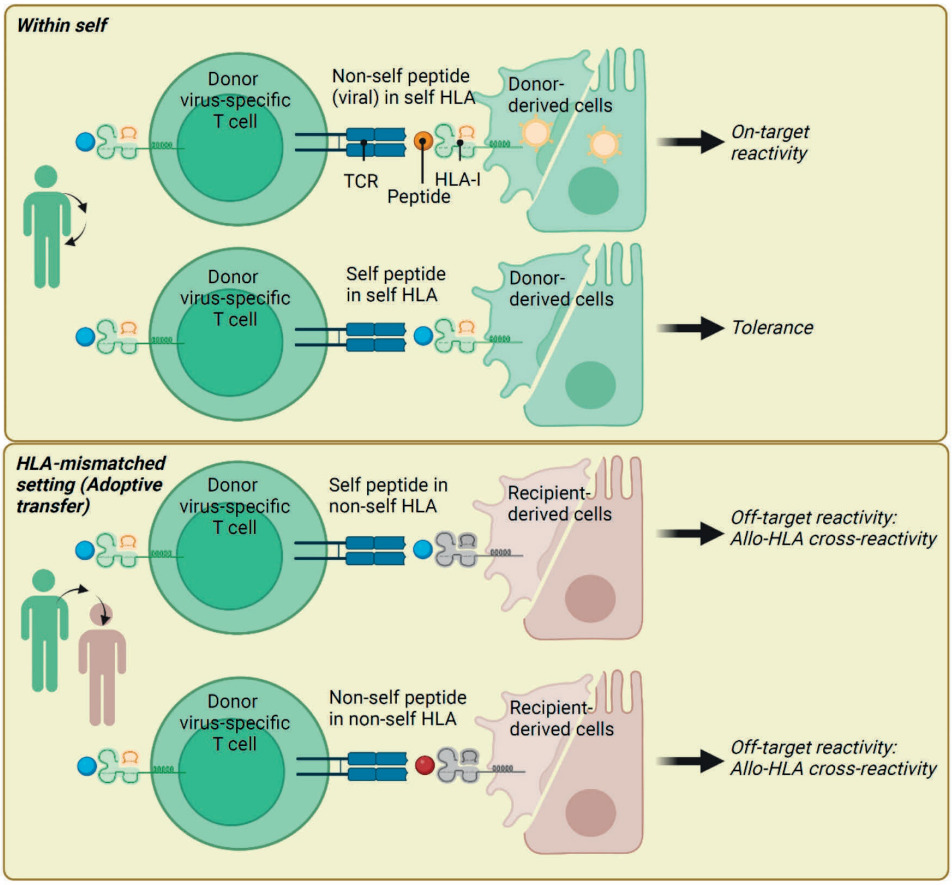

Figure 1. Peptide-HLA complexes as targets for T cells expressing a virus-specific TCR in an HLA-mismatched setting. Top panel; Non-self peptides (e.g. viral peptides) recognized in the context of donor-derived HLA molecules (self) induces on-target reactivity by donor-derived T cells expressing a virus-specific TCR, while donor-derived peptides (self) presented by donor-derived HLA molecules (self) induce tolerance. **Bottom panel**; When donor-derived T cells that express a virus-specific TCR are adoptively transferred to an HLA-mismatched individual, donor-derived T cells with a virus-specific TCR can additionally recognize either self or non-self peptides presented by non-self (HLA-mismatched) HLA molecules, leading to off-target reactivity.

HLA-class-II molecules are also heterodimers, but consist of two homogenous chains (α and β) that are both polymorphic(48). In this case, both the α and the β chain make up a groove for peptides to bind, but the peptide-binding groove is open at both ends(52). Generally, longer peptides can be presented by HLA-class-II molecules, compared to HLA-class-I molecules. HLA-class-II molecules are involved in the "exogenous pathway of antigen processing and presentation" and are used to present peptides derived from proteins that the cell has endocytosed. In this case, the proteins are degraded by acid-dependent proteases in endosomes. These molecules are mostly highly-expressed on antigen presenting cells (APCs) like dendritic cells (DCs), B cells, monocytes/macrophages and other cells of hematopoietic origin, but can also be upregulated in other cells in the presence of inflammation(49). After successful transportation to the cell surface, helper T cells recognize these peptides presented by HLA-class-II molecules with their TCR and their co-receptor CD4. Helper T cells can aid in the response mediated by cytotoxic T cells by releasing cytokines such as IFN-γ and Interleukin 4 (IL-4)(53). Additionally, helper T cells can also aid in the humoral-response by inducing class-switching of B cells or stimulating B cells into proliferation by producing cytokines like IL-4, IL-6, IL-7 and IL-10(54).

Polymorphisms of HLA

HLA-class-I molecules can be subdivided in HLA-A, HLA-B and HLA-C molecules. In total, more than 10.000 different HLA-class-I alleles have been identified, but the majority of those are rare variants. The most frequent HLA alleles in the Netherlands, and in the general Caucasian population, are HLA-A*01:01 (17.5%), HLA-A*02:01 (29.2%), HLA-B*07:02 (15.1%), HLA-B*08:01 (12.8%), HLA-C*07:01 (15.7%) and HLA-C*07:02 (16.8%)(55). Each individual can express up to two different HLA-class-I molecules of each group (HLA-A,-B and-C), depending on the genotype of their parents. Based on the extensive polymorphisms of HLA genes, it is very unlikely that two randomly selected individuals will express the same HLA molecules. The polymorphic properties of the HLA molecules allow for presentation of different peptides by each of these HLA molecules to the immune system, resulting in high probability that at least one of the HLA molecules in an individual can present a virus-derived peptide successfully to our immune system. HLA polymorphism is most likely the result of an evolutionary benefit, and heterozygosity may double the antigen presenting potential of each individual. Each HLA molecule has preferences regarding the type of peptides it can present. For example, peptides that can be presented by HLA-A*02:01 predominantly require a Leucine (L) on position 2 and Leucine (L) or Valine (V) on position 9, while peptides presented by HLA-B*07:02 require a Proline (P) on position 2(56). Such positions are called anchor positions and HLA molecules can be grouped together based on such anchor positions in so called HLA superfamilies(57).

HLA-class-II molecules can be subdivided in HLA-DR, HLA-DP and HLA-DQ molecules. Because HLA- HLA-class-II α and β chains are both highly polymorphic, and are both responsible for which peptides can be presented, the presented peptidome is highly diverse between individuals. In total, around 200 different HLA-class-II α chains and 5.000 different HLA-class-II β chains have been identified.

T-CELL DEVELOPMENT

TCR-gene recombination

T cells need a very diverse TCR repertoire in order to bind to all potential pathogen-derived peptides that can be presented by the different HLA molecules. This involves a complex process known as TCR-gene recombination. First, the precursors of T cells are produced from a lymphoid progenitor stem-cell in the bone marrow and reach the thymus via the blood, thus becoming thymocytes. In the thymus they further mature and will express TCR-alpha (α) and TCR-beta (β) chains by random rearrangement of different germline elements. The number of different TCRs that can be generated has been estimated to be between 10^{15}-10^{20} (58). The reason for this diversity is because both the α and the β chains have highly variable sequences as a result of recombination. For the TCRβ-chain, recombination of 1 of 48 functional T-cell Receptor Beta Variable (TRBV), 1 of 2 functional T-cell receptor Beta Diversity (TRBD) and 1 of 12 T-cell Receptor Beta Joining (TRBJ) gene segments leads to a V-D-J reading frame(59). The TCRα-chain is generated by a similar recombination process with the exception of a diversity gene, resulting in a V-J reading frame(60). Insertion of template-independent nucleotides between the recombined segments (junctional regions) results in a significant increase in variability(61). The sequence around these junctions encodes for the Complementary Determining Region 3 (CDR3), a loop that reaches out and interacts with the peptide embedded in the HLA molecule, together with the loops of the CDR1 and CDR2 regions which are fixed within the TRBV germline sequence(62, 63). Thymocytes that have acquired TCRs will then undergo thymic selection.

Thymic selection

After thymocytes express TCRs, they will first undergo positive thymic selection in the thymic cortex. Positive selection is also the stage where thymocytes undergo lineage commitment (helper T cells or cytotoxic T cells). Thymocytes are first double positive for CD4 and CD8 and depending on the HLA-class they recognize, they differentiate into $CD8^{pos}$ thymocytes if they recognize an HLA-class-I molecule or $CD4^{pos}$ thymocytes when they recognize an HLA-class-II molecule(64). In order to be positively selected, the TCRs of thymocytes will have to bind to one of the HLA-class-I or class-II molecules loaded with a so called “self peptide”, presented by thymic epithelial cells. During positive selection

thymocytes are selected with a TCR able to bind (self-peptide loaded) HLA-class-I or class-II molecules with at least a weak affinity(65, 66) Thymocytes that are unable to bind to either HLA-class-I or class-II molecules might be harmful and recognize non-HLA structures. Such thymocytes will not receive a signal to proliferate and therefore start apoptosis(65, 66). Thymocytes that were positively selected and underwent lineage commitment then migrate to the thymic medulla and will undergo negative selection. During negative selection, all thymocytes with a too high affinity for binding self-peptides presented by HLA-class-I or class-II molecules that are presented by APCs in the thymic medulla will undergo apoptosis(66). This process eliminates T cells that may cause auto-immune disorders. This results in the indirect selection of thymocytes that do not bind to self-peptides presented by HLA-class-I or class-II molecules with high enough affinity to cause activation once they are mature (**Figure 1**; top panel. Tolerance). After this maturation process of positive and negative selection, thymocytes enter the peripheral blood and are then considered mature naïve (non-experienced) cytotoxic T cells ($CD8^{pos}$) or naïve helper T cells ($CD4^{pos}$).

Formation of a virus-specific T-cell response: naïve, effector and memory

During a primary viral infection, a small proportion of the naïve T-cell repertoire has specific TCRs for the presented antigens and is activated and differentiates into functional effector T cells, which can control the initial viral infection. Activation of virus-specific naïve T cells requires the induction of two signals(67, 68). Signal 1 is generated after high enough affinity interaction of the T-cell receptor (TCR) with a virus-derived peptide presented by HLA-class I, whereas signal 2 is generated via an interaction between co-stimulatory molecules (e.g. CD80 and CD86) on the APC and receptors for these molecules (e.g. CD28) on the T cell(68). Only a small fraction of the naïve T cells expresses TCRs that are specific for peptides from the invading virus (signal 1) and will differentiate and expand into large numbers of effector T cells that will fight the virus. After successful clearance and control of the virus, contraction of the virus-specific T-cell population takes place whereby most of the effector T cells die via apoptosis and a small part will survive as protective memory virus-specific T cells(69). The memory virus-specific T cells persist in low frequencies as central memory T cells with the capacity to self-renew and differentiate, and as effector memory virus-specific T cells that can quickly control the subsequent infection with the same virus(70, 71). These persisting central memory virus-specific T cells replenish the effector memory virus-specific T-cell pool for the next reactivation/antigen encounter. Therefore, reactivations in patients receiving alloSCT or solid-organ transplantation are sometimes ineffectively controlled due to the absence of functional central memory and effector memory virus-specific T cells.

CONTROL OF VIRAL REACTIVATIONS IN IMMUNOCOMPROMISED PATIENTS

Reactivation of latent viral infections can be a life-threatening complication in patients during the early immune compromised phase after alloSCT. In these patients, the memory virus-specific T cells that are responsible for control of reactivations are either deleted due to the T-cell depletion in TCD alloSCT, or suppressed by immunosuppressive agents. Eventually a new primary virus-specific T-cell response will be induced from the donor SCT graft, but this takes time. Although the availability of antiviral agents such as ganciclovir, foscarnet, cidofovir and letermovir(28) has contributed to a significant reduction of CMV-related morbidity and mortality, administration of these drugs is limited by hematological and renal toxicity, and subsequent viral reactivations and refractory disease are commonly observed(72). During the immune compromised phase after TCD alloSCT, administration of unmodified DLI can be given that may also restore viral immunity(10, 13). However, early DLI can also result in detrimental GVHD since inflammatory conditions and the presence of patient-derived hematopoietic APCs can provoke a profound alloreactive donor-derived T-cell response(4, 5, 73). Selection and transfer of virus-specific T-cell populations from the donor is a desirable strategy for these immunocompromised patients, as such cells would control the virus but have limited alloreactivity. However, this strategy can only be implemented if the donor is seropositive (i.e. has generated a virus specific memory T-cell response).

Virus-specific T-cell therapy: isolation methods

Different methods for the isolation of virus-specific T cells from seropositive donors have been developed and translated into good manufacturing practice (GMP) compliant procedures. One of the first approaches was the repetitive stimulation of peripheral blood with viral antigens *in vitro* followed by long lasting expansion in the presence of interleukin-2 (IL-2), resulting in enrichment for virus-specific T cells due to preferential expansion of the activated virus-antigen-specific T cells(74-76). However, the *in vivo* efficacy and long-term survival of these enriched virus-specific T cells after administration was disappointing and was attributed to the abrupt withdrawal of IL-2 in combination with phenotypical changes of T cells during the *in vitro* culture period(77, 78). Further efforts have been made to develop methods to directly isolate $CD4^{pos}$ and/or $CD8^{pos}$ virus-specific T cells followed by short time culturing or direct infusion without *in vitro* expansion. These T cells are supposed to proliferate more efficiently under physiological conditions *in* vivo compared to T cells that were cultured *in vitro* with IL-2. In these cases, peripheral blood of virus seropositive donors was stimulated with viral antigens and activated virus-specific T cells were isolated using GMP-compliant procedures based on an activation-induced effect, such as the secretion of a cytokine (e.g. IFN-γ) or the upregulation of an activation marker on the cell-surface (e.g. CD137)(79-86). Another

approach that allows for direct isolation without *in vitro* culture, is using peptide-HLA-class-I multimers (tetramers) or peptide-HLA-class-I *Strep*tamers (GMP grade) that can be used for isolation of single-peptide-specific T-cell populations. These techniques allow isolation of T cells based on the specificity of their TCR and are independent of cytokine production or activation marker expression. However, these approaches require knowledge of defined viral peptides restricted to prevalent HLA molecules and are not available for the isolation of $CD4^{pos}$ T cells due to the lack of functional HLA-class-II multimers. Although $CD4^{pos}$ helper T cells are thought to contribute to *in vivo* survival, persistence and function of $CD8^{pos}$ CMV or EBV-specific T cells, T-cell products with only $CD8^{pos}$ CMV or EBV-specific T cells also showed promising results regarding T-cell expansion and clinical outcomes. Furthermore, it has been suggested that a minimum of 250-5000 virus-specific T cells/kg body weight of the patient may be sufficient for virus control, encouraging the direct adoptive transfer without *in vitro* expansion(87-89). These studies show that the generation of CMV, EBV and AdV-specific T-cell products is feasible and administration is safe without coinciding GVHD. To study efficacy of transferred virus-specific T cells, it is important to determine the fate of the individual transferred T-cell populations. However, it is difficult to unequivocally distinguish progeny of the transferred T-cell products from recipient- or stem-cell graft-derived T cells that survived T-cell depletion during conditioning or stem-cell graft manipulation. Another reason that efficacy of such products remains difficult to prove is the lack of randomized controlled, prospective clinical trials. The first multi-national clinical phase-III trial TRACE (**TR**ansfer of **A**denovirus, **C**ytomegalovirus and **E**pstein-Barr virus-specific T cells) aims to prove efficacy and safety of adoptive T-cell transfer in immunocompromised individuals, but is currently still recruiting and estimated to finish in December 2024.

Virus-specific T-cell therapy: Source

Different sources can be used to isolate virus-specific T cells, each with their own benefits and drawbacks. In all cases, a virus seropositive donor source is essential to isolate memory virus-specific T cells from, since naïve virus-specific T cells are difficult to isolate and the effectiveness of adoptively transferred naïve virus-specific T cells against virus-infected cells *in vivo* is limited. (90). When donors are seropositive, memory virus-specific T cells can be isolated from a related or unrelated SCT donor. The unrelated HLA-matched stem cell donor is most often the source of *in vitro* isolated virus-specific T cells. Safety and feasibility of adoptive transfer of such memory virus-specific T-cell products in alloSCT patients with coinciding viral clearance could be demonstrated in multiple clinical studies by different groups, including our department(80, 84, 91-93). Adoptive transfer of haplo-identical-derived (related SCT donor, partially HLA matched) memory virus-specific T-cell products in alloSCT patients also resulted in viral clearance in most of the patients, but some patients developed coinciding acute grade II GVHD (81, 94, 95).

In the field of solid organ transplantation, autologous peripheral blood from the patient is often the only available source for the isolation of virus-specific T cells and can be used when the patient is seropositive. In this setting, memory virus-specific T cells are present in the patient, but are often suppressed by immunosuppression and/or exhaustion. Additionally, cells of the recipient (patient) and cells of the donor-derived organ are often only partially HLA-matched. Therefore, virus-specific T-cell products need to be directed against viral peptides that are presented by HLA molecules that are shared between patient and donor for broad efficacy of the virus-specific T cells against infected cells of the patient as well as the graft. Due to ongoing immunosuppression, these cells will most likely only have a temporary effect when adoptively transferred. However, multiple studies did show potential efficacy by decrease in viral loads using this approach(96-98). Genetic modification strategies, like introduced resistance to calcineurin to mitigate the immunosuppressive effects, have been explored in mice and showed promising effects that can possibly be applied in future human strategies to achieve long-term antiviral protection in these patient groups receiving continuous immune suppression(99).

When a seropositive HLA-matched donor is not available, a third option for heavily immune compromised patients (especially after hematopoietic stem cell transplantation or for recipients of solid organs) is the adoptive transfer of memory virus-specific T cells from a third party seropositive donor. A third party source would allow for rapid intervention to restore antiviral immunity in patients for whom there is no easy access to memory virus-specific T cells. Such virus-specific T-cell products from third party donors could be directly administered when stored as T-cell biobank and administered as off-the-shelf product(100). However, it remains very difficult to match the third-party-derived off-the-shelf product for HLA with the recipient and/or hematopoietic stem cell donor. The difficulty is that such T-cell biobanks are limited in size and the T-cell products are often generated against immunogenic peptides restricted to only common HLA-molecules. Some studies have generated and treated patients that were expressing frequently occurring HLA molecules, like HLA-A*01:01, HLA-A*02:01 and HLA-B*07:02, which allowed a sufficient HLA-class-I match with patient and recipient(101). However, most of the studies only rely on high coverage (i.e. patients expressing one of these highly frequent Caucasian-related HLA molecules) of the off-the-shelf third party products, and patients that are treated are often only partially HLA-class-I matched and matching for HLA-class-II is not performed(100, 102, 103). In these studies only low rates (~5%) of off-target toxicity/de novo GVHD were observed in stem cell recipients that were treated with partially HLA-matched virus-specific T cells. Although potential efficacy and safety was demonstrated in some of these studies(18), there are also concerns regarding potential rejection of the infused products as demonstrated by a phase I/II clinical study by Neuenhahn et al., where survival/persistence was only demonstrated for adoptively transferred virus-specific T cells of the original stem cell donor (8/8 HLA-

matched), but not for virus-specific T cells derived from third-party donors with a higher degree of HLA-mismatch(103). Another approach for third-party-derived virus-specific T-cell products would be to generate products on demand from a large virtual bank of HLA-typed healthy donors. Several blood banks or registries contain large numbers of HLA typed healthy individuals who are willing to donate peripheral blood mononuclear cells and from whom the viral serostatus is known or can easily be determined. This would allow for the "best-possible" HLA match between the third-party-derived virus-specific T cells and the recipient within a short time window.

As an alternative approach, genes encoding TCRs specific for CMV, EBV or AdV-derived antigens may be transferred into appropriate T-cell populations. In this strategy, donor or patient-derived T-cell populations are equipped with a TCR of defined specificity using short-term *in vitro* procedures, and the redirected cells are infused to provide control of viral reactivations. This approach would be extremely useful as therapy for virus-associated malignancies, like those reported for EBV(92, 104, 105).

OFF-TARGET REACTIVITY MEDIATED BY ALLO-HLA CROSS-REACTIVITY

HLA disparity between patient and stem-cell or third party donor can lead to unwanted reactivity (alloreactivity) of donor-derived T cells against mismatched HLA molecules expressed by the patient-derived cells, leading to GVHD. Third party donor-derived T cells can furthermore be alloreactive against the cells from the stem cell graft. Finally, the newly donor-derived hematopoietic system can reject the third-party virus-specific T cells. In the last case, such rejection prevents toxicity, but it also diminishes the protection mediated by the third-party derived T cells. Alloreactive T cells have not been negatively selected during thymic selection against the mismatched HLA molecules of the recipient and therefore are able to respond to peptides presented by allogeneic HLA molecules. It was recently demonstrated that between 0.5% and 6% of the TCR-repertoire is able to respond to different HLA-mismatched stimulators(106). This reactivity by these alloreactive T cells was shown to be mediated by both naïve and memory T-cell populations(107-109). This shows that for instance TCRs of memory T cells that are specific for a pathogen-derived peptide, additionally have the capacity to also recognize (different) peptides presented in allo-HLA molecules. Thus, virus-specific T cells can also cross-react with allo-HLA- via the same TCR complex(107, 109). This type of allogeneic recognition is also referred to as allo-HLA cross-reactivity. Since memory T cells lack the requirement for co-stimulation, allo-HLA cross-reactivity by memory T cells can be triggered by non-professional antigen presenting cells. Thus, when third party donor-derived memory virus-specific T cells are not fully HLA-matched with the recipient, this

can lead to off-target reactivities directed against the HLA-mismatched patient-derived cells (**Figure 1**: bottom panel). Additionally, in the setting of solid organ transplantation, allo-HLA cross-reactivity mediated by recipient-derived virus-specific T cells can also be a trigger of graft rejection, as shown by the association between viral reactivation, virus-specific T-cell expansion and graft rejection in recipients of solid organs(110-112). It would be useful if we could predict which non-matched HLA molecules are recognized by third party donor-derived virus-specific T cells upon adoptive transfer. This would allow selection of specific donors, specific T-cell populations or TCRs with a low likelihood of exerting off-target reactivity. This has remained difficult due to the large variety of TCRs that can be expressed by the virus-specific T cells, each with a risk of mitigating off-target reactivities, which reduces the chance to find pattern.

Thus far, recurrent off-target reactivity towards the same non-matched HLA molecule was only found for T-cell populations isolated from different individuals that expressed the exact same TCR (public TCR)(109, 113, 114). A classic and well-characterized example of a public allo-HLA cross-reactivity has been demonstrated by Burrows and colleagues for virus-specific T cells that recognize the EBV-EBNA3a antigen-derived FLRGRAYGL (FLR) peptide in the context of HLA-B*08:01(114). A fraction of this population with a TCR named LC13 was shown to also cross-react with allogeneic HLA-B*44:02(114, 115). Individuals who harbor both HLA-B*08:01 and HLA-B*44:02 do not contain LC13 expressing EBV-EBNA3a-FLR-specific T cells due to thymic negative selection for self-tolerance for HLA-B*44:02, while retaining HLA-B*08:01-restricted EBV-EBNA3a-FLR specificity(115). This example shows that heterozygosity for certain HLA alleles makes it difficult to observe patterns of all-HLA cross-reactivity. In general, heterozygosity will induce more tolerance which potentially results in less allo-HLA cross-reactivity. However, due to the polymorphism of HLA molecules, this tolerance might not be equal for every heterozygous HLA combination, as indicated by the allo-HLA-B*44:02 cross-reactive EBV-specific T cells expressing the LC13 TCR. Additionally, each virus-specific T-cell population can express different TCRs each cross-reacting with a potentially different HLA molecule. So far, allo-HLA cross-reactivities could only be predicted when virus-specific T-cell populations contained a dominant public TCR like the example with LC13. In depth characterization of the TCR-repertoires of virus-specific T-cell populations would allow identification of new public TCRs that could be assessed for their safety and allo-HLA cross-reactivity. Depending on the recognition pattern, virus-specific T-cell populations could be selectively depleted or selected based on their TCR-variable domain, to allow adoptive transfer with a low risk of off-target reactivities. Thus far, no other patterns have been identified that influence the risk of off-target reactivities.

AIMS OF THE THESIS

Virus-specific T cells play a key role in the control of viral-reactivations in healthy individuals and this cellular immunity is impaired in patients receiving alloSCT. In the period around the transplantation, donor-derived T cells are either depleted or suppressed to reduce the risk of GVHD. However, in the absence of donor-derived T cells, latent viruses such as CMV, EBV and AdV can reactivate and remain uncontrolled and at the same time the curative GVL effect is abrogated. Therefore, the major challenge in the field of alloSCT is to find a balance between the GVL effect, protection against viruses and GVHD. The research described in this thesis focusses on the options to control for viral reactivations using adoptive transfer of virus-specific T cells or TCRs and the risks associated with this.
To establish treatment efficacy of adoptive transfer of stem-cell donor-derived virus-specific T cells, it is important to determine the fate of the individual transferred T-cell populations. However, it is difficult to unequivocally distinguish progeny of the transferred T-cell products from recipient- or stem-cell graft-derived T cells that survived T-cell depletion during conditioning or stem-cell graft manipulation. In ***chapter 2***, we aim to track SCT-donor-derived virus-specific T cells that were prophylactically infused in patients after alloSCT. Using mRNA sequencing of the TCRβ-chains of the individual virus-specific T-cell populations within these T-cell products, we are able to track the multiple clonal virus-specific subpopulations in peripheral blood and distinguish recipient- and stem-cell graft-derived virus-specific T cells from the progeny of the infused T-cell products.

For some patients, there is no easy access to memory virus-specific T cells from the stem cell donor. A third party source would allow for rapid intervention to restore antiviral immunity in these patients. However, third-party-derived T-cell products are likely to be only partially HLA-matched with the patient. In ***chapter 3***, we study the risks for off-target reactivity of T-cell products derived from third-party donors and whether these can be predicted based on specificity, HLA-restriction or HLA-background. We use third-party donor-derived CMV, EBV and AdV-specific T cells as model to investigate this by *in vitro* stimulation assays using an EBV-transformed lymphoblastoid cell-line (EBV-LCL) panel covering 116 allogeneic HLA-molecules. The off-target reactivity, mediated by allo-HLA cross-reactivity, is confirmed using HLA-class-I and HLA-class-II negative K562 cells that are retrovirally transduced with single HLA-class-I alleles of interest.

To decrease the risk of off-target reactivities mediated by third-party-derived T-cell products, virus-specific T-cell populations could be enriched for T cells that express TCRs that are safe or have a limited off-target reactivity. T-cell populations that are known to express public TCRs could be used. In ***chapter 4***, we quantitively analyze the TCR-repertoires of CMV, EBV and AdV-specific T cells from healthy individuals, and determine the magnitude, defined as prevalence within the population and frequencies within

individuals, of public TCRs and TCRs that are highly-similar to these public TCRs. Because the T cells from such virus-specific memory TCR-repertoires are the result of successful control of the virus in these healthy individuals, these public and highly-similar TCRs may be attractive candidates for immunotherapy in immunocompromised patients that lack virus-specific T cells to control viral reactivation.

TCRs that are highly-similar to public TCRs, with only minor variations in amino-acids on specific positions in the CDR3 region, are frequently found. However, the degree of freedom at these positions is not clear. Therefore, in ***chapter 5***, we use the HLA-A*02:01-restricted EBV-LMP2^{FLY}-specific public TCR as model and systematically replace the amino-acid at the highly-variable position 5 in the CDR3β sequence of this public TCR with all 20 possible amino-acids to investigate whether specific rules apply to this highly-variable position.

TCR-gene transfer could be an approach that would allow for rapid intervention to restore antiviral immunity in patients for whom there is no virus-seropositive stem cell donor available. However, this approach could also be used for patients with virus-associated malignancies. In ***chapter 6,*** we aim to isolate HLA-A*01:01-restricted EBV-LMP2-specific T cells and their TCR to treat patients with EBV-associated latency type II/III malignancies who are HLA-A*01:01 positive. These patients can benefit from such products, since no T cells recognizing any EBV-derived peptide in this common HLA allele have been found thus far. Additionally, we aim to optimize the functionality of primary T cells transduced with HLA-A*01:01–restricted EBV-LMP2–specific TCRs by knocking out the endogenous TCRs of primary T cells (ΔTCR) using CRISPR-Cas9 technology. Such TCRs can potentially be used in future TCR gene therapies to treat LMP2-expressing EBV-associated latency type II/III malignancies.

In ***chapter 7*** the results of this thesis are summarized and discussed, conclusions based on the results of this thesis are drawn and new research questions and ideas are proposed.

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BENEFITS

CHAPTER 2

Tracking the progeny of adoptively transferred virus-specific T cells in patients post-transplant using T-cell receptor sequencing

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ABSTRACT

Adoptive cellular therapies with T cells are increasingly used to treat a variety of conditions. For instance, in a recent phase I/II trial, we prophylactically administered multi-virus-specific T-cell products to protect recipients of T-cell depleted allogeneic stem-cell grafts against viral reactivations. To establish treatment efficacy, it is important to determine the fate of the individual transferred T-cell populations. However, it is difficult to unequivocally distinguish progeny of the transferred T-cell products from recipient- or stem-cell graft-derived T cells that survived T-cell depletion during conditioning or stem-cell graft manipulation. Using mRNA sequencing of the TCRβ-chains of the individual virus-specific T-cell populations within these T-cell products, we were now able to track the multiple clonal virus-specific subpopulations in peripheral blood and distinguish recipient- and stem-cell graft-derived virus-specific T cells from the progeny of the infused T-cell products. We observed in vivo expansion of virus-specific T cells that were exclusively derived from the T-cell products with similar kinetics as the expansion of virus-specific T cells that could also be detected before the T-cell product infusion. Additionally, we demonstrated persistence of virus-specific T cells derived from the T-cell products in most patients who did not show viral reactivations. This study demonstrates that virus-specific T cells from prophylactically infused multi-antigen-specific T-cell products can expand in response to antigen encounter in vivo and even persist in the absence of early viral reactivations.

INTRODUCTION

The use of adoptive T-cell therapies has increased both in frequencies as in options in the last few decades(1). Although these strategies show promising results, it is currently difficult to adequately follow the fate of these products in the patient. *In vivo* tracking and tracing of the progeny from the infused T-cell products is vital in the assessment of treatment efficacy. For instance, when administered products fail to achieve expected results, it is important to determine whether this might be explained by lack of persistence. Likewise, selective *in vivo* expansion of the transferred T cells in response to antigen encounter would be an argument to support the case that engrafted T cells helped to elicit the clinical effect. Chapuis et al. recently tackled this by tracking adoptively transferred T-cell products using T-cell receptor (TCR) deep sequencing(2).

Patients that underwent allogeneic stem-cell transplantation (alloSCT) are (temporarily) immune-compromised and reactivations of cytomegalovirus (CMV), Epstein-Barr virus (EBV) and adenovirus (AdV) are frequently seen in these patients. The adoptive transfer of human leukocyte antigen (HLA)-matched virus-specific T cells from the original stem-cell donor can reduce viral infection and reactivation risks in these patients(3). Safety and feasibility of adoptive transfer of donor-derived virus-specific memory T-cell products has been demonstrated in multiple phase I/II clinical studies by different groups, including ours(4-12). However, persistence of the virus-specific T cells could not be attributed unequivocally to the transferred T-cell product, as T cells with the same antigen-specificity might already have been present in the patient or derived from the stem-cell graft. Furthermore, the currently used techniques (i.e. peptideMHC-tetramer staining, marker-gene analysis and/or Elispot) allow only for the detection of frequencies higher than ~0.1% within total peripheral blood mononuclear cells (PBMCs)(13-18). More sensitive and specific detection methods are required to *in vivo* track individual T-cell populations derived from the T-cell products.

In a recent phase I/II trial in our department, 24 patients who received a T-cell depleted alloSCT were treated post-transplant with a prophylactic infusion of a stem-cell donor-derived multi-(virus) antigen-specific T-cell product, containing $CD8^{pos}$ T cells directed against CMV, EBV and/or AdV antigens(19, 20). The aim was to prevent uncontrolled viral reactivations in these patients. The T cells from the products had to persist long enough without the presence of viral antigen to be able to prevent or control the viral reactivation. Safety and feasibility of this approach and appearance in peripheral blood (PB) of virus-specific T cells as detected by conventional peptide-MHC-tetramer staining were demonstrated. However, we could not determine whether these virus-specific T cells were derived from the infused product and whether virus-specific T cells persisted in those patients in which the frequencies of such cells were below the detection

threshold of conventional peptideMHC-tetramer staining.

In this study, we aimed to track prophylactically administered virus-specific T cells by using high-throughput T-cell receptor (TCR)-sequencing of the infused T-cell products and PBMC samples of the patients after administration. We analyzed in detail how many and which T cells persisted and expanded after administration of the products. Using the TCR-sequencing technology, we were able to distinguish the virus-specific T cells that were already present before infusion of the product from persisting or expanding T cells that were exclusively derived from the product.

MATERIALS AND METHODS

Collection of patient and donor material

After informed consent according to the Declaration of Helsinki, PBMCs were isolated from alloSCT patients and their stem-cell donors by standard Ficoll-Isopaque separation and stored in the vapor phase of liquid nitrogen(20). The patients and donors had been included in a previous single center, phase I/II study exploring the safety, feasibility and first evidence of efficacy of prophylactic infusion of multi-antigen specific T cells to prevent complications early after T-cell depleted alloSCT(20) (T Control, EudraCT-number 2014-003171-39). Clinical results can be found in the original paper(20). In the current analysis, patients and donor-derived T-cell products were numbered identical to the phase I/II study(20). See also supplementary table 1 for relevant patient and donor characteristics.

Generation of peptide-MHC tetramers

All viral peptides were synthesized in-house using standard Fmoc chemistry. Recombinant HLA-A*01:01, HLA-A*02:01, HLA-A*24:02, HLA-B*07:02, HLA-B*08:01 heavy chain and human β2m light chain were in-house produced in Escherichia coli. MHC-class-I refolding was performed as previously described with minor modifications(21). Major histocompatibility complex (MHC)-class-I molecules were purified by gel-filtration using FPLC. Peptide-MHC(pMHC) tetramers were generated by labeling biotinylated pMHC-monomers with streptavidin-coupled phycoerythrin (PE; Invitrogen, Carlsbad, USA), allophycocyanin (APC, Invitrogen). Complexes were stored at -80 °C. Formation of stable pMHC-monomers was performed using UVexchange technology(22) and according to a previously described protocol(23).

Generation of multi-antigen-specific T-cell products and isolation of single-antigen-specific T-cell populations

T-cell products were generated using the MHC-I-Streptamer isolation technology, as

previously described(19, 20). In short, isolation complexes (MHC-I-Streptamers) were generated per target-antigen T-cell specificity. For every patient, MHC-I-Streptamers for 4 HLA*02:01-restricted viral antigens (2 CMV, 1 EBV, and 1 AdV) were pooled. In addition, depending on HLA-type of patient/donor and regardless of donor CMV, EBV, and AdV serostatus, MHC-I-Streptamers for peptides presented in HLA-A*01:01, -A*24:02, -B*07:02, and/or -B*08:01 were added to this pool (**Table 1**). The pool of MHC-I-Streptamers was incubated with 2×10^9 donor-derived PBMCs and MHC-I-Streptamer-bound cells were isolated using a CliniMACS Plus instrument (Miltenyi Biotec, Bergisch Gladbach, Germany) under GMP conditions. Five percent of the cells from all products, except products U (3%) and Y (4%), were non-specifically expanded using 800ng/ml phytohemagglutinin with autologous PBMCs as feeder mixture, as previously described(20), cryopreserved and used for in-depth analysis in this study. In order to isolate single-antigen-specific T-cell populations from the expanded T cells from the products, cells were first incubated with pMHC-tetramer complexes for 30 min at 4°C followed by incubation with fluorescein isothiocyanate (FITC)-labeled CD8 (BD) antibodies at 4°C for 30 min. PeptideMHC-tetramer^{pos} virus-specific T cells were bulk Fluorescence Activated Cell Sorted (FACS) for each specificity and directly lysed. Sorting was performed on a FACS ARIA (BD) using Diva software (BD). For the generation of peptide-MHC-I-Tetramers, see supplementary material and methods.

Table 1. PeptideMHC-streptamers used for the generation of multi-virus-specific T-cell products

Virus	Protein	Peptide	HLA-restriction	Specificity included in T-cell product manufacturing for this # patients
CMV	pp50	**VTE**HDTLLY	HLA-A*01:01	3
	pp65	**NLV**PMVATV	HLA-A*02:01	20
	IE1	**VLE**ETSVML	HLA-A*02:01	20
	pp65	**QYD**PVAALF	HLA-A*24:02	6
	pp65	**TPR**VTGGGAM	HLA-B*07:02	7
	IE1	**QIK**VRVDMV	HLA-B*08:01	5
EBV	BMLF1	**GLC**TLVAML	HLA-A*02:01	20
	EBNA3A	**RPP**IFIRRL	HLA-B*07:02	7
	BZLF1	**RAK**FKQLL	HLA-B*08:01	5
AdV	HEXON	**TDL**GQNLLY	HLA-A*01:01	3
	E1A	**LLD**QLIEEV	HLA-A*02:01	20
	HEXON	**TYF**SLNNKF	HLA-A*24:02	6
	HEXON	**KPY**SGTAYNAL	HLA-B*07:02	7

Abbreviations: CMV, Cytomegalovirus. EBV, Epstein Barr virus. AdV, Adenovirus

TCRβ-library preparation

TCRβ-sequences were identified using ARTISAN PCR adapted for TCR PCR as previously described(24-26). Ten µl (~1µg) of mRNA per sample was mixed with TCRβ constant region-specific primers (1µM final concentration) and SmartSeq2modified template-switching oligonucleotides (SS2m_TSO; 0.2µM final concentration) and denatured for 3 minutes at 72°C. After cooling, cDNA was synthesized for 90 minutes at 42°C with 170U SMARTscribe reverse transcriptase (Takara, Clontech) in a total volume of 20µl containing 1.7U/µl RNasin (Promega), 1.7mM DTT (Invitrogen, Thermo Fisher Scientific), 0.8mM each of high-purity RNAse-free dNTPs (Invitrogen, Thermo Fisher Scientific) and 4µl of 5x first-strand buffer. During cDNA synthesis, a non-templated 3'polycytosine terminus was added, which created a template for extension of the cDNA with the TSO(24). PCR (2min at 98°C followed by 40 cycles of [1s at 98°C, 15s at 67°C, 15s at 72°C], 2 min at 72°C) of 5µl of cDNA was then performed using Phusion Flash (Thermo Fisher Scientific) with anchor-specific primer (SS2m_For; 0.4µM final concentration) and each (0.4µM final concentration) of the nested primers specific for the constant regions of TCRβ constant 1 and TCRβ constant 2. Both forward and reverse PCR primers contained overhanging sequences suitable for barcoding. Amplicons were purified and underwent a second PCR (2min at 98°C followed by 10 cycles of [1s at 98°C, 15s at 65°C, 30s at 72°C], 2 min at 72°C) using forward and reverse primers (0.5µM final concentration) with overhanging sequences containing identifiers (sequences of 6 base-pairs) and adapter sequences appropriate for Illumina HiSeq platforms or Novaseq. See supplementary tables 2 and 3 for primer and identifier sequences, respectively. Total mRNA (10µl) was extracted from T cells from 20 unsorted products and 81 pMHC-tetramer^{pos} CD8^{pos} T-cell populations directly after FACSorting using magnetic beads (Dynabead mRNA DIRECT kit: Invitrogen, Thermo Fisher Scientific) (**Supplementary Figure 1A**). To investigate whether T cells with TCRβ-sequences detected in the T-cell products were present in PB of the patients after administration, we isolated primary CD8^{pos} T cells from follow-up PB samples with magnetic activated cell sorting (MACS) using CD8 T-cell isolation kits with LS columns from Miltenyi Biotec (Bergisch Gladbach, Germany). Peripheral blood samples at moment of product infusion contained a median of 110*10^6 CD8^{pos} T cells/L (Inter quartile range IQR: 47-509, **Supplementary Figure 2A**). After CD8 enrichment all PB samples contained a median of 81% CD8^{pos} cells (IQR: 61%-92%, **Supplementary Figure 2B**) PB samples for follow-up were taken every 2 weeks until 8 weeks after T-cell product infusion and every 4 weeks thereafter until 6 months after alloSCT(20). Total mRNA (10µl) was extracted from 109 CD8^{pos} populations isolated from follow-up PB samples of the patients (**Supplementary Figure 1B**), containing a median of 0.3*10^6 cells (IQR 0.16-0.66*10^6 cells, **Supplementary Figure 2C**). The samples from each patient were sequenced separately. Unique identifiers were used for the PCR products of each virus-specific T-cell population, unsorted T-cell product and each monitoring sample (see **Supplementary Tables 2 and 3** for primer and identifier sequences, respectively).

These amplicons with identifiers were purified, quantified and pooled into one library per patient for paired-end sequencing of 125bp on an Illumina HiSeq4000 or Novaseq 6000. The samples of each patient were separately sequenced on different chips to obtain enough reads per sample (oversampling). Each $CD8^{pos}$ PB follow-up sample contained a median of $8.5*10^6$ reads (IQR 5.5-12.8*10^6 reads, **Supplementary Figure 2D**). Because of the oversampling this resulted in a median of 28 reads per cell per sample (IQR 14-46 reads per cell, **Supplementary Figure 2E**). Deep sequencing was performed at GenomeScan (Leiden, The Netherlands) and almost all reads contained a Phred quality score above Q30 (median 92%, IQR 90%-93%). One-third of a lane (~100.000.000 reads) was used per library while the other lanes were used for other projects that did not contain TCR-sequences. Raw data was de-multiplexed and aligned to the matching T-cell receptor beta variable (TRBV), diversity (TRBD), joining (TRBJ) and constant (TRBC) genes. In total, a median of 33% (IQR 22%-49%) of all reads could successfully be aligned, **Supplementary Figure 2F**). CDR3β-sequences were built using MIXCR software using a bi-directional approach (5'-3' and 3'-5' read)(27). MIXCR corrects for PCR and sequencing errors (https://mixcr.readthedocs.io/en/master/). CDR3β-sequences that were present in multiple T-cell populations with different specificities as a result of FACS contamination, were annotated to the T-cell population that contained this sequence more than 10-fold compared to the other T-cell populations with different specificities (Representative example, **Supplementary Figure 3**). An exception was made for dominant T-cell populations that contained one or two CDR3β-sequences that were present at high frequencies (>40%) within this T-cell population and at high frequencies within the total product. Such sequences contaminated T-cell populations that were of low frequency within the product. This was only the case for CDR3β-sequences specific for CMV-$pp65^{TPR}$, CMV-$pp50^{VTE}$, EBV-$EBNA3A^{RPP}$, and EBV-$BZLF1^{RAK}$ from products F, T, Y and V, respectively. In these cases, a 5-fold difference in frequency compared to T-cell populations with other specificities was used for annotation.

Target-antigen-specific immune reconstitution

Absolute numbers of circulating $CD3^{pos}$/$CD8^{pos}$ T cells per liter blood were determined on fresh blood by flow cytometry. Absolute numbers of circulating $CD4^{pos}$ T cells ($CD45^{pos}CD3^{pos}CD4^{pos}$), $CD8^{pos}$ T cells ($CD45^{pos}$ $CD3^{pos}CD8^{pos}$), B cells ($CD45^{pos}CD3^{neg}CD19^{pos}$) and NK cells ($CD45^{pos}CD3^{neg}CD16/CD56^{pos}$ cells) were determined as part of routine clinical evaluation on fresh venous blood using BD TruCount Tubes (BD), following the manufacturer's instructions. Samples were stained with APC-labeled CD3 (BD), FITC-labeled CD4 (BD), PE-labeled CD8 (BD), PerCP-labeled CD45 (BD) or with FITC-labeled CD3 (BD), PE-labeled CD16 (BD), APC-labeled CD19 (BD), PerCP-labeled CD45 (BD) and PE-labeled CD56 (BD). Cells were measured on a FACS Canto and analyzed using Diva Software. Frequencies of target-antigen-specific T cells were determined based on the percentages of target-antigen-specific TCR-nucleotide-sequences from each $CD8^{pos}$

populations isolated from a follow-up sample. Absolute numbers of target-antigen-specific T cells per liter were calculated by multiplying the percentages of target-antigen-specific TCR-nucleotide-sequences, with the absolute numbers of $CD3^{pos}CD8^{pos}$ T cells per liter blood.

Detection limit and cut-off value

A median of $3.7*10^5$ (range $0.6\text{-}74*10^5$) and a median of $3.6*10^5$ (range $1.2\text{-}47*10^5$) $CD8^{pos}$ T cells were MACS isolated from PB for each patient before infusion of the T-cell product and from subsequent monitoring samples, respectively. In theory we could detect the amplified reads from one T cell. One T cell would correspond with a median frequency of 0.0037% and 0.0036% for samples before and after infusion of the product, respectively. Due to this detection limit, we used a cut-off whereby sequences were analyzed that occurred at frequencies above 0.001%, while sequences below this frequency were not analyzed.

RESULTS

Annotation and quantification of virus-specific T-cell receptor sequences from the T-cell products

In a recent phase I/II trial, 24 stem-cell donor-derived multi-(virus)antigen-specific T-cell products, each containing $CD8^{pos}$ T-cell populations specific for several CMV, EBV and AdV antigens, were generated and prophylactically administered to the respective patients early after T-cell depleted alloSCT(20). All donors were EBV seropositive, 13 donors were CMV seropositive, whereas the serostatus for AdV was not determined (Supplementary table 1). All products contained EBV-specific T cells, all but one contained AdV-specific T cells, whereas CMV-specific T cells were only detectable in products from CMV-seropositive donors. We aimed to track the *in vivo* fate of the transferred virus-specific T cells through high-throughput TCR-sequencing of virus-specific T cells from the products and of $CD8^{pos}$ T cells in PB samples of the patients taken at different times after infusion of the products. In order to track these T cells, TCR-sequences had to be correctly allocated to the virus-specific T-cell populations that were present in each product. To this end, T-cell products were first polyclonally expanded *in vitro* to have sufficient T cells to sort each virus-specific T-cell population and to allocate the TCR-sequences to the specific populations (for a schematic overview see: **Supplementary Figure 1A**). Insufficient PB samples were available from two patients, which were therefore excluded from analysis. Additionally, T cells of two T-cell products could not be expanded *in vitro* and thus the patients that had received the corresponding T-cell products were also excluded from analysis. From 20 out of the initial 24 products, a median of $0.29*10^6$ T cells were expanded to a median of $21.9*10^6$ T cells (**Figure 1A**). From these expanded

fractions, we separately isolated all the different virus-specific T-cell populations that were present in each product(20) by FACS using pMHC-tetramers and performed TCR-sequencing of the CDR3β-regions of each isolated virus-specific T-cell population. Unsorted fractions of the expanded T-cell products were also sequenced in parallel to quantify the different virus-specific TCRs present in the products (**Supplementary Figure 1A**). In total, 97.5% (range 81%-100%) of the TCR-sequences from the sorted virus-specific T-cell populations could be detected in the unsorted bulk products (data not shown). To investigate the distribution of the different specificities in each product, we analyzed the frequencies of the TCR-sequences for each target-antigen of each virus. In total, from the 13 products that were generated from CMV-seropositive donors, TCRs of 32 CMV-specific T-cell populations were annotated (**Figure 1B**). From the 20 products (all of which were generated from EBV-seropositive donors), TCRs of 31 EBV-specific T-cell populations were annotated (**Figure 1C**). Finally, we could annotate the TCRs of a total of 22 AdV-specific T-cell populations from 17 expanded products (**Figure 1D**), whereas 2 expanded products yielded insufficient numbers of AdV-specific cells after expansion for analysis.

To investigate how many different clonal virus-specific T-cell populations from each product we could potentially track *in vivo*, we quantified the different TCR-nucleotide sequences for each virus-specific T-cell population within the T-cell products. The CMV-, EBV-, or AdV-specific T-cell populations from the products contained a median of 30 (range 1-79), 34 (range 5-140) and 20 (range 2-74) different TCR-nucleotide sequences, respectively (**Figures 1E, 1F and 1G**). The majority of virus-specific TCR-nucleotide sequences were found at low frequencies (between 0.001% and 0.1%) in these expanded T-cell products (**Supplementary Figure 4**).

T cells with TCR-nucleotide sequences found in the T-cell products could be identified in patients with and without detectable viral-loads

To investigate whether virus-specific T cells from the infused T-cell products could be found back in PB samples of the patients, we sequenced the TCRs of $CD8^{pos}$ T-cell fractions from PB samples taken at various timepoints after infusion of the T-cell products (see Supplementary Figure 2 for sample quality assessments). These TCR-nucleotide sequences were then compared with the TCR-nucleotide sequences that were present in the T-cell products (for a schematic overview see: **Supplementary Figure 1B**).

We previously illustrated that despite T-cell depletion of the graft, part of the donor-derived T-cell compartment can survive this procedure(28, 29). This implies that TCRs detected in post-infusion samples may not necessarily be derived from the infused products, but may have already been introduced in the patients with the stem-cell grafts. We therefore also analyzed PB samples that were obtained from patients before infusion

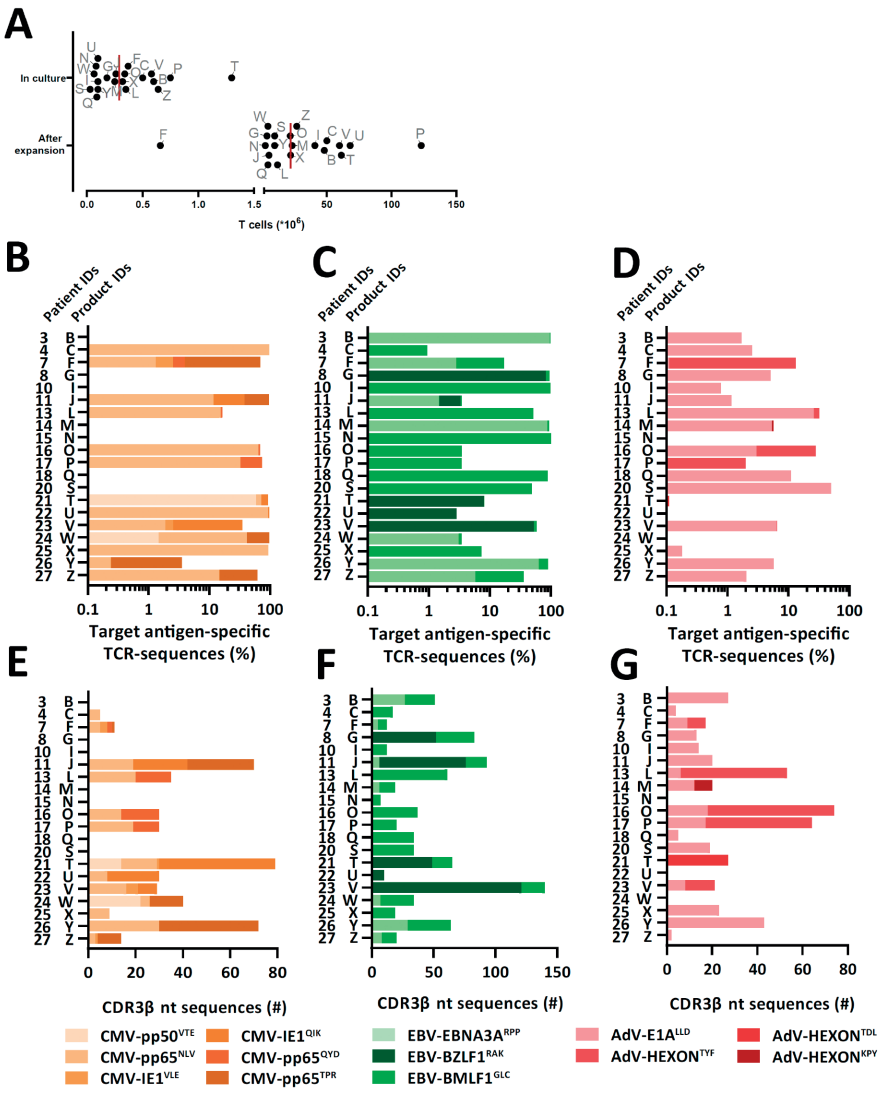

Figure 1. Annotation and quantification of virus-specific TCR-sequences from the T-cell products.
T cells from 20 T-cell products were successfully expanded. The different virus-specific T-cell populations were FACSorted from these expanded products into separate pure populations using pMHC-tetramers followed by direct mRNA isolation and TCR-sequencing of the CDR3β-regions. The remaining unsorted T cells from the T-cell products were sequenced in parallel to quantify the TCRs that were present in the products. **A)** The numbers of T cells from the products that were put in culture and the cell numbers after expansion are shown. The red-lines represent medians. **B, C and D)** Shown are the frequencies of CMV **(B)**, EBV **(C)** and AdV **(D)** –specific TCR-nucleotide sequences (CDR3β-sequences) that were present in the target-antigen-specific T-cell products. The sum of all target-antigen-specific TCR-nucleotide sequences were set to 100%. The different virus-specificities are shown as stacked-columns for each product. **E, F and G)** The number of different CDR3β-sequences are shown that were specific for CMV (**E**), EBV **(F)** and AdV **(G)**-derived antigens for each product.
Abbreviations: CMV, Cytomegalovirus. EBV, Epstein Barr virus. AdV, Adenovirus. CDR3, complementary determining region 3. nt, nucleotide

of the products. In figure 2 we separately depicted the virus-specific TCR-nucleotide sequences present in the products that could be found in patient PB samples only after infusion (orange: CMV, green: EBV and red: AdV), both before and after infusion (light orange: CMV, light green: EBV and light red: AdV), or could not be found after infusion (white) (**Figures 2A, 2B and 2C**). In addition, we determined whether the diversity (number of different TCR-nucleotide-sequences) correlated with viral reactivation as determined by presence of viral-load in PB.

In 9 patients the presence of some CMV-specific T-cell populations could not unequivocally be ascribed to the infused T-cell product, as the same sequences were also found in PB samples taken before infusion (**Figure 2A**, light orange bars). However, many CMV-specific TCR-nucleotide sequences were only found in PB samples of the 13 patients after infusion, strongly indicating that they originated from the infused products (**Figure 2A,** orange bars). Such TCRs were found both in patients with or without viral-load after infusion and a higher number of different TCRs (diversity) were found in samples from patients with CMV reactivations than in samples from patients without CMV reactivations following infusions, indicating *in vivo* expansion in response to the virus (**Figure 2D**). Similar results were obtained for the EBV-specific T-cell populations (**Figure 2B**). In 13 patients, a number of EBV-specific TCR-nucleotide sequences were found both before and after infusion. Conclusions about their origin from the infused products (light green bars) could therefore not be made. However, in 16 patients TCR-nucleotide sequences of EBV-specific T cells could be tracked in PB samples only after infusion of the products (green bars) and these EBV-specific TCRs were more abundant in patients with EBV reactivations than in those without (**Figure 2E**). In 2 patients without EBV reactivation, EBV-specific TCR-nucleotide sequences from the infused products could not be detected in the PB samples. Finally, while PB samples from 6 patients contained AdV-specific TCR-nucleotide sequences that were present also in the patients prior to infusion, in 8 patients AdV-specific TCR-nucleotide sequences were found in samples only after infusion of the products (**Figure 2C**, red bars). In PB samples of the single patient with a single positive AdV viral-load, no AdV-specific TCRs could be tracked back. These data show that after infusion of the multi antigen-specific T-cell products, in most patients high frequencies of virus-specific TCRs could be identified that were derived from the infused T-cell products. As measured by frequencies of virus-specific TCRs, significantly larger proportions of the T-cell products were found back in patients with viral reactivations compared to patients without viral reactivations, indicating that these virus-specific T cells from the T-cell products contributed to the anti-viral immune response.

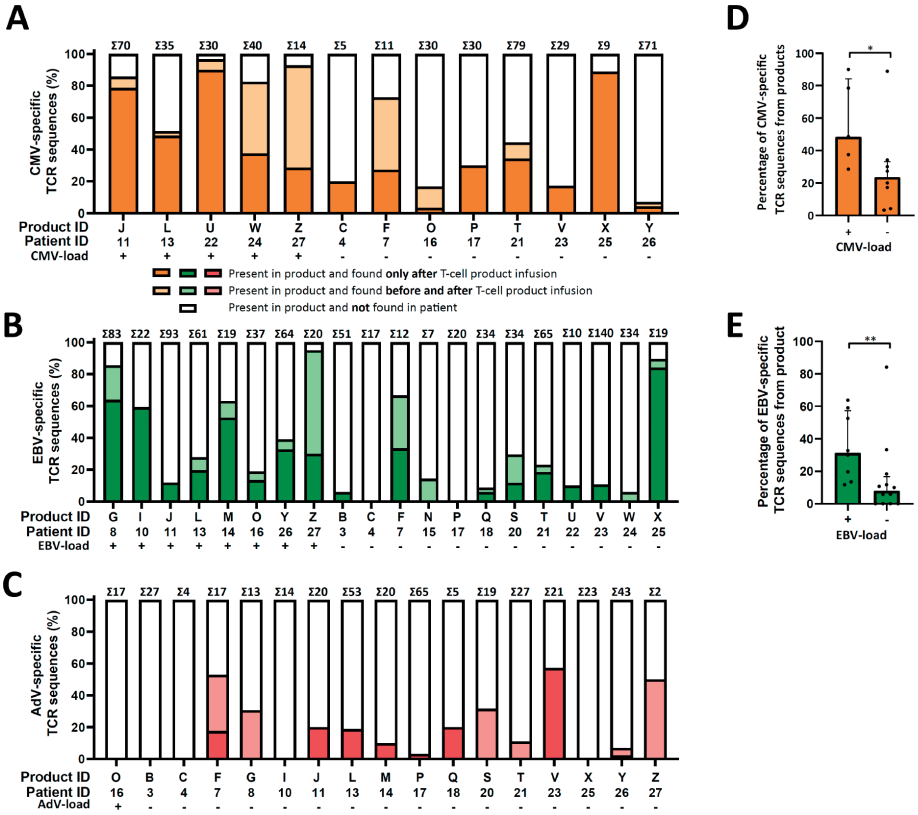

Figure 2. Virus-specific T cells with TCR-nucleotide sequences found in the T-cell products, could be identified in patients with and without detectable viral-loads. The total numbers (Σ) of different virus-specific TCR-nucleotide sequences are shown for each product (above each bar). Patients are grouped according to detectable viral-loads after infusion of the T-cell product. **A, B and C**) Shown are the percentages of different CMV- (**A**) , EBV- (**B**) and AdV- (**C**) specific TCR-nucleotide sequences that could be found back only after infusion of the products (color-scale), be detected before and after infusion (light-color-scale) or not detected (white). **D and E)** The numbers of different TCR-nucleotide sequences that were found only after infusion, shown as percentages in barplots with the median and inter-quartile range, were compared between patients with and without CMV viral-load (**D**) and with and without EBV viral-load (**E**).
Statistical differences were assessed with the Mann-Whitney t test (D and E). *P< .05; **P < .01.

Kinetics of virus-specific T cells with TCR-nucleotide sequences from the infused products in patients with and without viral-loads after infusion

For a number of T-cell populations it was impossible to tell on the basis of TCR-sequence detection alone to what extent their presence was explained by infusion of the T-cell product, given that they were already present in the patients prior to infusion. However, we reasoned that we might still be able to assess the contribution of transfused T cells to viral control by examining their expansion kinetics after infusion of the T-cell

product. If T cells derived from the product would significantly contribute to controlling the virus, we hypothesized that irrespective of their presence or absence in the patient prior to infusion, similar kinetics of the progeny of the virus-specific T-cell populations from the products should be found. Virus-specific T cells would show expansion in case of reactivation and only persistence in the absence of viral-load after infusion. We therefore investigated the kinetics of virus-specific T-cell populations that appeared after infusion of T-cell products in patients with and without viral reactivations (colored) and compared these with the kinetics of virus-specific T-cell populations that were present prior to product infusion (grey). In figures 3, 4 and 5 we separately depicted the kinetics of CMV, EBV and AdV-specific T-cell populations (with TCR-nucleotide sequences that were found in the products) in the presence and absence of viral-loads after infusion.

CMV

As illustrated in figures 3A and 3B, positive CMV viral-loads were detected in 5 out of 13 patients before and after infusion of the T-cell products. In 4 of these 5 patients, CMV viral-loads were also detected at the moment of infusion of the T-cell products. In all 5 patients, CMV-specific TCR-nucleotide sequences that only appeared after infusion and those that were present already before infusion of the T-cell products exhibited similar expansion and contraction that correlated with the increase and decrease of viral-loads (**Figure 3A and 3B**). The responding individual CMV-specific T-cell clones all showed similar expansion kinetics, including T-cell clones that contained public TCRs (TCR-amino-acid sequences that are identical in different individuals) as well as T-cell clones containing private TCRs (**Supplementary Figure 5A and 5B**). During follow-up of the other 8 patients, no CMV viral-loads were detected after infusion. Two patients (patients 7 and 16) had positive CMV viral-loads, which had been cleared before infusion of the products (data not shown). In all 8 patients we detected T cells with CMV-specific TCR-nucleotide sequences from the products that only appeared after infusion and persisted without clear expansions in 6 out of 8 patients (**Figure 3C**). In 4 of these patients, we also detected CMV-specific TCR-nucleotide sequences that were present before and after infusion of the T-cell products showing similar kinetics (**Figure 3D**).

EBV

As shown in figures 4A and 4B, reactivations, as reflected by positive EBV viral-loads, were detected in 8 out of 20 patients after infusion of the T-cell products. Detectable EBV viral-loads were absent in all 8 patients at the time of T-cell product infusions, but EBV viral-loads had been detected before infusion in 3 out of 8 patients (patients 11, 13 and 26: data not shown). In all 8 patients, EBV-specific TCR-nucleotide sequences that only appeared after infusion and EBV-specific TCR-nucleotide sequences that were present before and after infusion of the T-cell products showed similar expansion and contraction that correlated with the increase and decrease of viral-loads (**Figure**

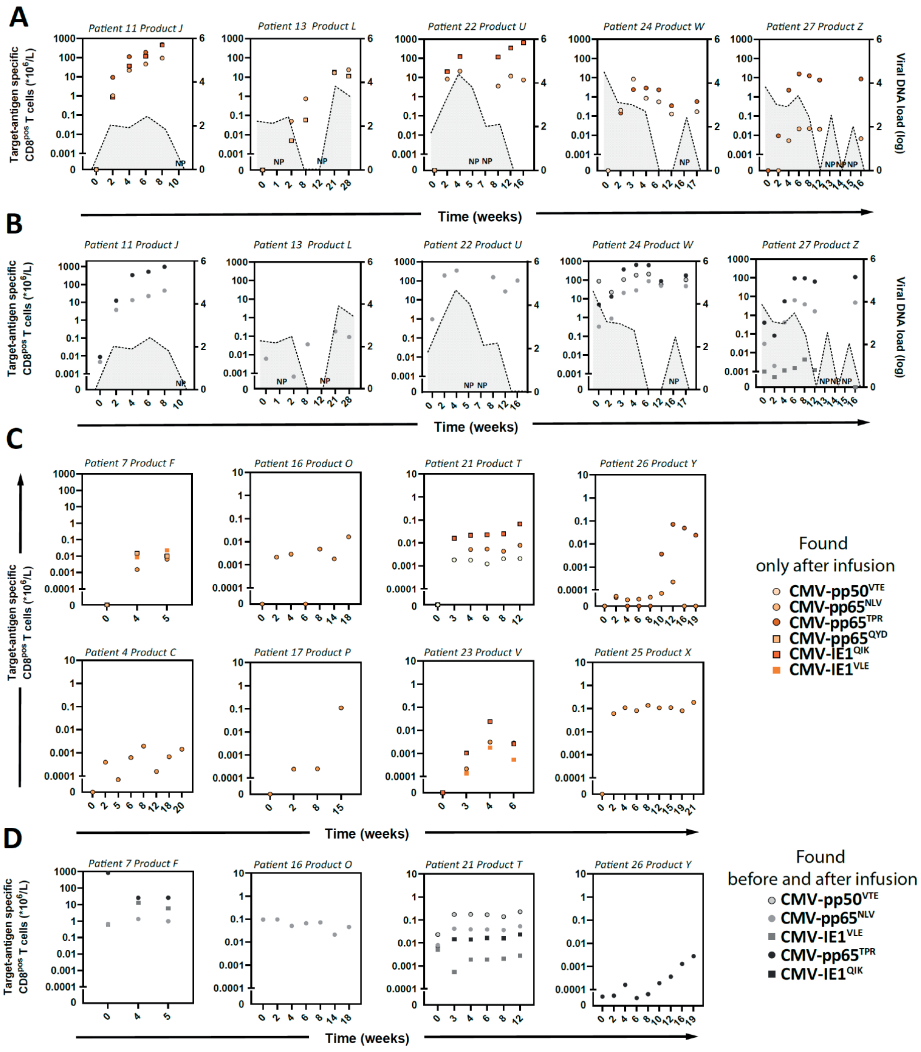

Figure 3. Kinetics of CMV-specific T cells with TCR-nucleotide sequences also present in the infused T-cell products in patients with and without viral-loads after infusion. Positive CMV viral-loads were detected in 5 out of 13 patients that received a T-cell product containing CMV-specific T cells. PB samples that were obtained before and after infusion of the products were MACSorted for $CD8^{pos}$ T cells followed by mRNA isolation and sequencing of the TCRs. The numbers of target-antigen specific $CD8^{pos}$ T cells/L blood were calculated by multiplying the frequencies of CMV-specific TCR-nucleotide sequences with the absolute numbers of $CD3^{pos}/CD8^{pos}$ T cells per liter. CMV viral-loads (dashed lines with grey area under the curve) and absolute numbers of CMV target-antigen-specific T cells in PB samples are illustrated from the moment just before product infusion (day 0) until the end of follow-up. **A and B**) Shown are the kinetics of CMV target-antigen-specific T cells in patients with CMV-reactivations where TCR-nucleotide sequences were found that only appeared after infusion of the products (**A**) and appeared before and after infusion of the products (**B**). **C and D)** Shown are the kinetics of CMV target-antigen-specific T cells in patients without detectable CMV viral-loads where TCR-nucleotide sequences that were identical to the products were found that only appeared after infusion of the products (**C**) and appeared before and after infusion of the products (**D**).
Abbreviations: NP, (TCR-sequencing) Not Performed.

4A and 4B). Individual EBV-specific T-cell clones all showed similar expansion kinetics, including T-cell clones that contained public TCRs and T-cell clones containing private TCRs (**Supplementary Figure 5C and 5D**). During follow-up of the other 12 patients, no EBV viral-loads were detected after infusion, but EBV viral-loads had been detected before infusion in 2 out of 12 patients (patients 3 and 23: data not shown). In 8 out of 12 patients we detected EBV-specific TCR-nucleotide sequences from the products that only appeared after infusion, showing persistence without clear expansions in 5 out of 8 patients (**Figure 4C**). In 5 patients, we also detected T cells with EBV-specific TCR-nucleotide sequences that were present before and after infusion of the T-cell products, showing similar kinetics in all patients, except for patient 25 (**Figure 4D**).

AdV

One out of 17 patients (patient 16) who was prophylactically infused with a product that contained AdV-specific T cells, showed a detectable AdV viral-load at week 16 post infusion. AdV-E1A^{LLD} and AdV-HEXON^{TYF}-specific T cells were present in T-cell product P, but no AdV-specific TCR-nucleotide sequences could be detected in the PB samples before or after infusion of the product. During follow-up of the other 16 patients, AdV viral-loads were undetectable after infusion of the products. In 8 out of 16 patients, we detected T cells with AdV-specific TCR-nucleotide sequences from the products that only appeared after infusion, showing persistence without clear expansions in 7 out of 8 patients (**Figure 5A**). In 2 of these patients, we also detected T cells with AdV-specific TCR-nucleotide sequences that were present before and after infusion of the T-cell products showing similar kinetics, except for patient 26 (**Figure 5B**).

Based on these results, we conclude that in 5/5 patients with a detectable CMV viral-load and in 8/8 patients with a detectable EBV viral-load, T cells with CMV and EBV-specific TCR-nucleotide sequences that were only found after infusion of the products displayed similar kinetics as those that were found before and after infusion of the products. One patient had a detectable AdV viral-load during follow-up, but no T cells with AdV-specific TCR-nucleotide sequences could be detected. In 8/8 patients without detectable CMV viral-loads, 8/12 patients without detectable EBV viral-loads and in 8/16 patients without AdV viral-loads, persistence of CMV, EBV and AdV-specific T cells with TCR-nucleotide sequences that were found in the products were observed, which were not detected before infusion.

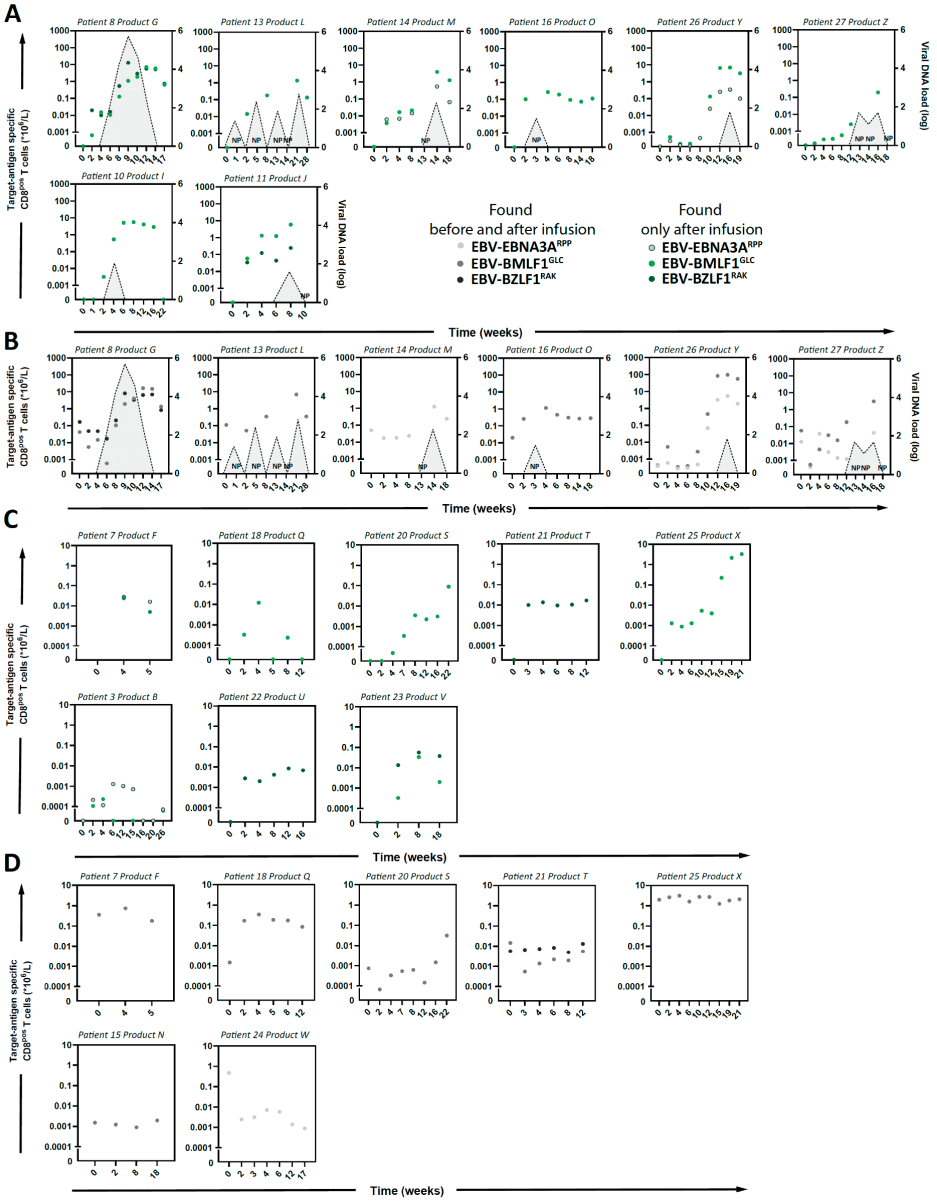

Figure 4. Kinetics of EBV-specific T cells with TCR-nucleotide sequences present in the infused T-cell products in patients with and without viral-loads after infusion. Positive EBV viral-loads were detected in 8 out of 20 patients that received a T-cell product containing EBV-specific T cells. PB samples that were obtained before and after infusion of the product were MACSorted for $CD8^{pos}$ T cells followed by mRNA isolation and sequencing of the TCRs. The numbers of target-antigen specific $CD8^{pos}$ T cells/L blood were calculated by multiplying the frequencies of EBV-specific TCR-nucleotide sequences with the absolute numbers of $CD3^{pos}CD8^{pos}$ T cells per liter blood. EBV viral-loads (dashed lines with grey area under the curve) and absolute numbers of EBV target-antigen-specific T cells in PB samples are illustrated from the moment just before product infusion (day 0) until the end of follow-up. **A and B**) Shown are the kinetics of EBV target-antigen-specific T cells in patients with EBV-reactivations where TCR-nucleotide sequences were found that only appeared after infusion of the products (**A**) and appeared before and

after infusion of the products (**B**). **C and D)** Shown are the kinetics of EBV target-antigen-specific T cells in patients without detectable EBV viral-loads where TCR-nucleotide sequences that were identical to the products were found that only appeared after infusion of the products (**C**) and appeared before and after infusion of the products (**D**).
Abbreviations: NP, (TCR-sequencing) Not Performed.

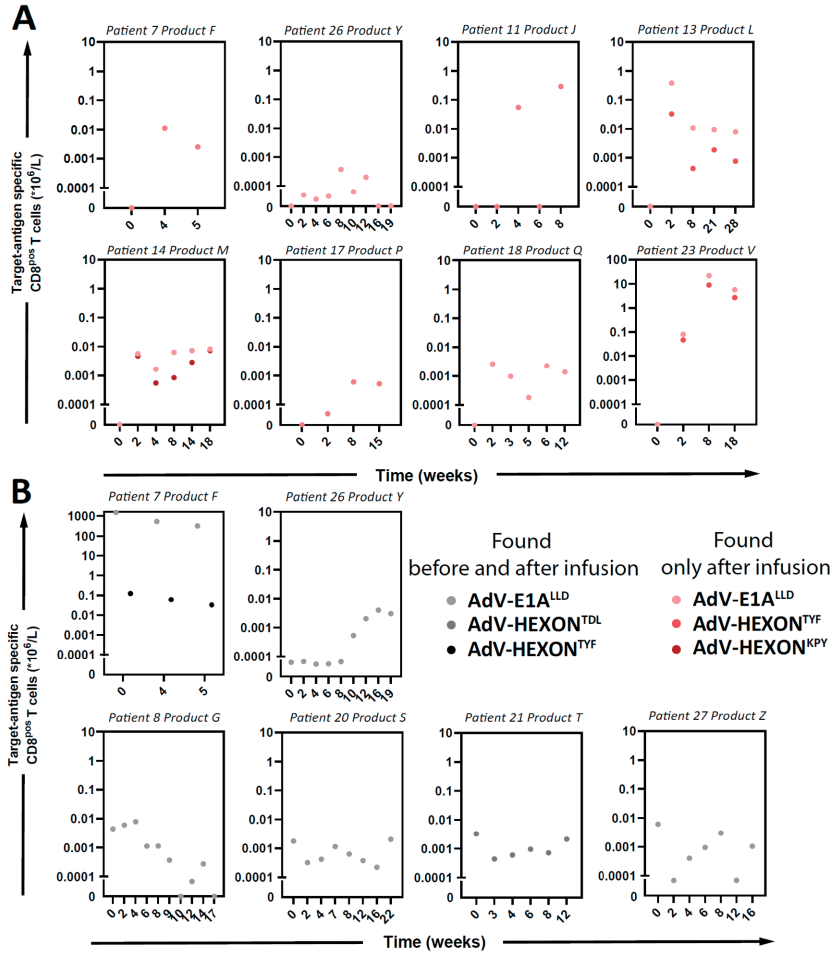

Figure 5. Kinetics of AdV-specific T cells with TCR-nucleotide sequences present in the infused T-cell products in patients without viral-loads after infusion. In 16 out of 17 patients that received a product that contained AdV-specific T cells no AdV viral-load was detected after infusion of the product. Peripheral blood samples of these 16 patients that were obtained before and after infusion of the product were MACSorted for $CD8^{pos}$ T cells followed by mRNA isolation and sequencing of the TCRs. The numbers of target-antigen specific $CD8^{pos}$ T cells/L were calculated by multiplying the frequencies of AdV-specific TCR-nucleotide sequences with the absolute numbers of $CD3^{pos}CD8^{pos}$ T cells per liter blood. Absolute numbers of AdV target-antigen-specific T cells in peripheral blood samples are illustrated from the moment just before product infusion (day 0) until the end of follow-up. **A and B)** Shown are the kinetics of AdV target-antigen-specific T cells in patients without detectable AdV viral-loads where TCR-nucleotide sequences that were identical to the products were found that only appeared after infusion of the products (**A**) and appeared before and after infusion of the products (**B**).
Abbreviations: NP, (TCR-sequencing) Not Performed.

Longitudinal analysis of total numbers of product-derived CMV and EBV-specific T cells

To study the association between viremia and the kinetics of expansion/persistence of adoptively transferred CMV- and EBV-specific T cells, we performed statistical modeling. First, we compared the smoothened Loess curves of the kinetics of all product-derived CMV- and EBV-specific T cells for patients with viral-loads and patients who never had detectable viral-loads during follow-up after product infusion. As expected, product-derived CMV-specific T cells that were only detected after product infusion showed more vigorous expansion in patients with CMV viral-loads during follow-up (Orange solid line) compared to patients who never had detectable CMV viral-loads (Orange dashed line; **Figure 6A**). Product-derived CMV-specific T cells that were detected before and after infusion followed a similar pattern, with more expansion in patients with CMV viral-loads (Grey solid line) than patients without CMV viral-loads (Grey dashed line; **Figure 6B**). Product-derived EBV-specific T cells that were only detected after product infusion showed the same pattern as CMV-specific T cells, with more expansion in patients with EBV viral-loads (Green solid line) compared to patients without EBV viral-loads (Green dashed line; **Figure 6C**). Similar trends were found for EBV-specific T cells that were detected before and after infusion (**Figure 6D**). To investigate whether the increases in numbers of CMV and EBV-specific T cells were indeed significantly associated with viremia, we constructed 4 linear mixed models with the presence of viral-load as a time-dependent covariate. The models contained two fixed effects, being time since infusion in weeks and whether or not the measurement was taken after the first appearance of viral-load (per patient). For the modeling of product-derived T cells that were found only after infusion, a patient-specific random slope effect for time was included to account for the heterogeneity in the trajectories between patients. For the modeling of product-derived T cells that were found before and after infusion, also a random intercept effect was added, since the T cells were already detectable at time of infusion. For CMV, the start of detectable viral-loads was significantly associated with higher T-cell numbers ($p=<0.0001$ and $p=0.0001$ for T cells found only after infusion and T cells found before and after infusion, respectively). Similarly, the appearance of EBV viral-loads was significantly associated with higher numbers of EBV-specific T cells that were found only after infusion ($p=<0.0001$), but a nonsignificant association ($p=0.1771$) with viral-load was observed for EBV-specific T cells that were found before and after infusion. These data show that the in-vivo expansion/persistence kinetics of adoptively transferred CMV- and EBV-specific T cells that were only detected after infusion were significantly different for patients with viral-loads (strongly expanding/proliferating) compared to patients that did not develop detectable viral-loads in the follow-up period after T-cell product infusion (persisting/maintenance).

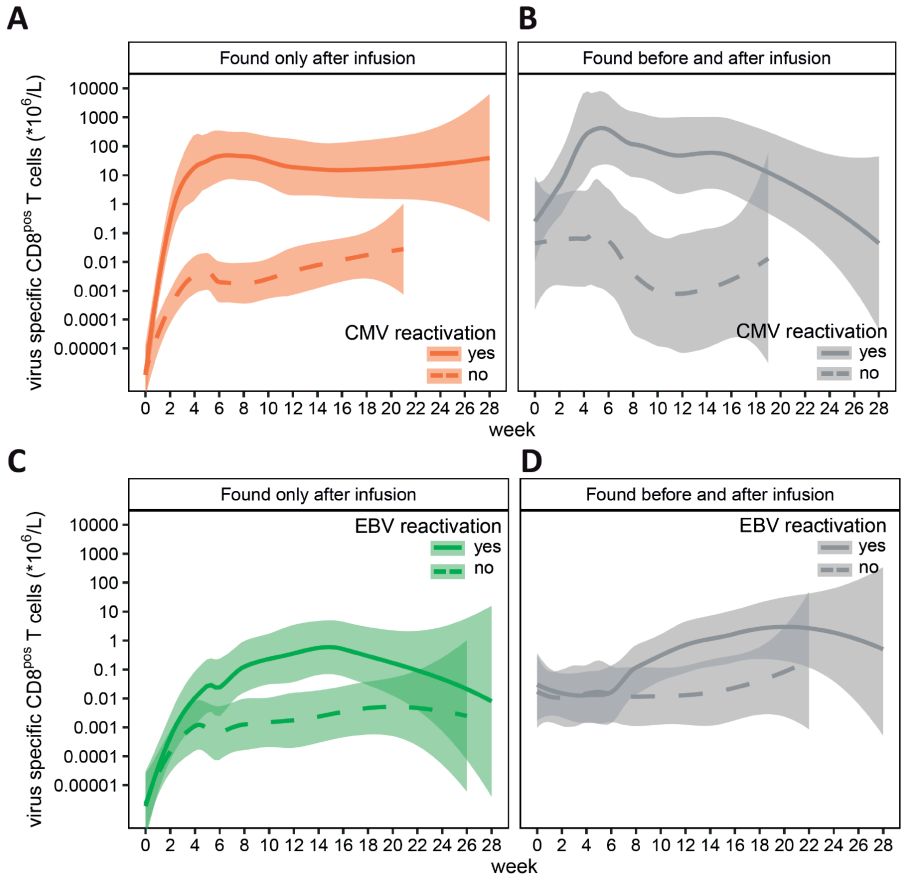

Figure 6. Statistical modeling of the expansion/persistence kinetics of adoptively transferred CMV and EBV-specific T cells and the presence of viral-loads in the follow-up period after T-cell product infusion. Positive CMV or EBV viral-loads were detected after infusion of T-cell products in 5 out of 13 patients and 8 out of 20 patients that received a T-cell product containing CMV-or EBV-specific T cells, respectively. Smoothed Loess curves were plotted to study the association between viremia and expansion/persistence kinetics of adoptively transferred CMV- and EBV-specific T cells. **A and B**) Kinetics of the numbers of product-derived CMV-specific T cells that were only detected after T-cell product infusion (A) or both before and after infusion (B) are shown for patients with a positive CMV viral-load during follow-up (solid line) and without detectable viral-loads in the follow-up period (dashed line). **C and D**) Product-derived EBV-specific T cells that were only detected after infusion (**C**) or both before and after infusion (**D**) are shown for patients with a positive EBV viral-load during follow-up (solid line) and without detectable viral-loads (dashed line).

DISCUSSION

In this study, we investigated the persistence and expansion in patients of *in vitro* isolated and prophylactically infused multi-antigen virus-specific T-cell products in the presence or absence of viral reactivations by *in vivo* tracking of individual T-cell populations. In contrast to the pMHC-tetramer technology that we previously used(20), TCR sequencing of purified viral antigen-specific T-cell populations allowed us to identify multiple different clonal T-cell populations within the antigen-specific T-cell compartments. This permitted their tracking with high sensitivity and specificity in PB of patients after infusion of the virus-specific T-cell products. TCR-mapping of the donor-derived CMV-, EBV- and AdV-specific T cells in the products revealed the presence of medians of 30, 34, and 20 different TCR-sequences per product, respectively. This technology allowed us to follow the presence and kinetics of the virus-specific T cells after infusion into patients after alloSCT. It also made it possible to distinguish donor-derived virus-specific T cells that were already present in the patient before infusion from those exclusively derived from the infused T-cell products. TCR-sequences from the products that were exclusively found in PB after infusion of the products were documented in all patients infused with CMV-specific T cells, in 80% of patients infused with EBV-specific T cells and in 47% of patients infused with AdV-specific T cells. As expected, higher frequencies of TCRs identical to the T-cell products could be tracked in PB of patients with CMV or EBV reactivations, compared to patients without reactivations. Since only one patient experienced AdV reactivation, no conclusions could be drawn about the T-cell kinetics in the presence of this virus. All patients with CMV or EBV reactivations showed expansion of virus-specific TCRs with similar kinetics irrespective of the presence of some of these T cells in the patients prior to infusion, suggesting that the virus-specific T cells from the T-cell products indeed contributed to the antiviral immune response. Statistical modeling of the expansion/persistence kinetics of the adoptively transferred virus-specific T cells showed a significant correlation between the vigorous increase in the numbers of circulating product-derived CMV and EBV-specific T cells and viral-loads. In 100%, 67% and 50% of the patients infused with CMV-, EBV- or AdV- specific T cells, respectively, T cells with TCRs identical to those in infusion products could be tracked for extended periods, even in the absence of viral reactivations, indicating T-cell persistence and a potential role of the adoptively transferred T-cell products in long-term protection against viral reactivations.

Several phase I and phase II clinical studies have been performed exploring safety, feasibility and potential efficacy of adoptive transfer of *in vitro* selected virus-specific T-cell products derived from the original stem-cell donors to control refractory viral reactivations in patients following alloSCT(20, 30-34). Presence of virus-specific T cells in these patients was in some of these studies demonstrated using pMHC-tetramer-

based flow cytometry assays or ELIspot analysis. However, the origin of the detected virus-specific T cells could not be demonstrated using these assays. Similar studies have been performed in patients after solid organ transplantation that received autologous derived virus-specific T-cell products derived from the naïve T-cell repertoire (35). In this case, the origin of the virus-specific T cells that were observed in the patients was clear, but also in this study it remained difficult to unequivocally link the clinical effects to the infused T-cell product. In many cases, control of reactivation or even disappearance of clinical symptoms was documented after infusion of the virus-specific T cells(36, 37). However, even in case of (partial) T-cell depletion of the stem-cell grafts, virus-specific T cells that survive the T-cell depletion are co-administered with the graft and already present at the time of infusion of the T-cell product(28, 29). This makes it difficult to address whether the infused T-cell products were actually responsible for the clinical effects, despite a clear correlation between infusion of the cells and clinical benefit(32). With next-generation TCR sequencing techniques, adoptively transferred donor-derived T cells (e.g. virus-specific, tumor-specific or regulatory T cells) could be more efficiently tracked in peripheral blood samples of patients with virus-reactivations(38), organ transplantations(39), cancer(40) or auto-immune diseases(41). However, so far donor-derived virus-specific T cells were not yet tracked using TCR-sequences in patients that prophylactically received virus-specific T cells. By tracking many individual clonal virus-specific T-cell populations from the T-cell products and tracking them in patients clearing viral reactivations, we could demonstrate that the majority of T cells expanding in response to *in vivo* appearance of the virus were derived from the infused T-cell products. However, *de novo* generation of public TCRs in the recipient that originate from the donor-derived stem cells/naïve precursor T cells cannot be unequivocally excluded. Appearance of T cells expressing public TCRs are therefore difficult to link to the product. However, from previous studies we know that memory $CD8^{pos}$ T cells dominate the immune reconstitution at week 6 post alloSCT compared to naïve T cells. Also, naïve T cells are more efficiently depleted following alemtuzumab based T-cell depletion(28), making it less likely that such T cells expressing public TCRs emerging early after alloSCT would be derived from the naïve donor T-cell repertoire. Additionally, patient-derived virus-specific T cells can also contribute to the control of viral reactivations after alloSCT, especially after NMA TCD alloSCT as described previously (42). Since the focus within the current study was on the *in vivo* fate of donor-derived virus-specific T cells upon adoptive transfer rather than on the entire virus-specific immunity post-transplant, we did not quantify and annotate patient-derived virus-specific T cells and can therefore not elaborate on the role of the host immunity in these specific patients. Gene-edited donor-derived T-cells could circumvent part of this problem by tracking T cells with the inserted gene *in vivo*, thereby limiting the influence of autologous derived TCR-sequences(15, 43, 44). Long-term persistence was shown for adoptively transferred gene-marked EBV-specific T cells using this approach(15). It has been suggested that

co-infusion of virus-specific CD4^{pos} and CD8^{pos} T cells may be beneficial(45-47), our study also strongly supports previous indications that *in vitro* selection based on purification of virus-specific CD8^{pos} T cells by peptide/MHC complexes using the Streptamer technology does not hamper the *in vivo* functionality of these cells after infusion(20), resulting in expansion and long-term persistence without the co-infusion of virus-specific CD4^{pos} T cells. It was reported that the diversity of TCRs of CMV-specific T-cell populations reduces during viral-reactivations(48). However, in our analyses we did not observe any reduction in diversity of the TCR-repertoire, even in those patients where clonal expansion of virus-specific T-cell populations was overt. The high sequencing depth of our strategy allowed us to also detect persisting virus-specific T-cell clones that were not expanding, but that contributed to the diversity of the TCR-repertoire. Since our clinical phase I/II study(20) did not include a control arm without T-cell product infusion or placebo, we can not demonstrate the reconstitution and expansion kinetics in patients who did not receive adoptively transferred virus-specific T cells.

The strategy we applied to perform bulk TCR-beta sequencing of thousands of cells in parallel is a powerful tool to dissect the diversity of the TCR-repertoire of multiple T-cell populations. However, the main limit relies in the impossibility to pair the information regarding TCR-alpha and TCR-beta sequences of individual T cells. Paired single-cell sequencing of TCR-alpha and TCR-beta chains would have allowed us to provide information on the TCR-alpha usage of product-derived virus-specific T cells. However, single-cell sequencing is more limited in the number of cells that can be sequenced simultaneously, thereby losing resolution required for the detection of T-cell populations that are present at low frequencies in the peripheral blood samples of patients. This can potentially result in undetected donor-derived virus-specific T cells before infusion or undetected persisting virus-specific T cells when viral-loads are absent.

Although TCR-beta sequencing of product-derived virus-specific T-cell populations allowed for tracking of individual T-cell clones upon adoptive transfer to patients, it remains possible that TCRs of T cells from the patient contain exactly the same nucleotide sequence as the donor/product—derived TCRs. We recently demonstrated that the magnitude, defined as frequency and occurrence, of such public TCRs is high, but that this was only on the amino-acid level(26), illustrating that the majority of TCRs contained different nucleotide sequences as a results of convergent recombination and random nucleotide inserts between V-D-J regions. Therefore, the chance of shared TCR nucleotide sequences between patient and donor has been estimated to be relatively low.

The majority of studies exploring the potential benefit of *in vitro* selected virus-specific T cells has been performed in a preemptive or therapeutic setting. In these cases,

the cells are infused when viral reactivation already occurs in the patient. This does not allow evaluation of survival/persistence of the T-cell products when they do not immediately encounter their antigen *in vivo*. It has been suggested that under those circumstances survival of the adoptively transferred T cells may be poor. Obviously, in the absence of antigen and expansion, the contribution of the infused T cells to the total peripheral T-cell repertoire in the patient is generally too low to allow detection using the pMHC-tetramer technology(49). However, our approach allowed us to determine the *in vivo* persistence of prophylactically infused T-cell products even in the absence of viral reactivations. In the absence of viral reactivations, we found evidence that infused virus specific T cells persisted at very low frequencies without clear expansion. In a few patients we could also detect late expansion of these infused T cells, supporting the persistent functionality following *in vitro* selection and infusion of the virus-specific T-cell products even in the absence of direct *in vivo* antigen encounter. Statistical modeling of the expansion/persistence kinetics of the adoptively transferred virus-specific T cells showed a significant correlation between the vigorous increase in the numbers of circulating product-derived CMV and EBV-specific T cells and viral-loads. It could be assumed that different virus-specific T-cell populations targeting different antigens can have different efficacies, but modeling of the different CMV/EBV-specificities separately was not possible in our study due to the limited numbers and resulting insufficient power. However, as shown in figures 3 and 4, different virus-specific T-cell populations showed very similar expansion kinetics within each patient, assuming no large differences in efficacies between specificities.

In conclusion, our study shows that TCR sequencing allow highly sensitive and specific tracking and tracing of multiple clonal T-cell populations from *in vitro* selected donor-derived virus-specific $CD8^{pos}$ T-cell products, after infusion into patients after alloSCT. Using this methodology, we were able to distinguish expansion and persistence of virus-specific T-cell populations selectively derived from the infused T-cell products from those T cells that were already present in the patients prior to infusion. We demonstrated after viral reactivation *in vivo* expansion of multiple clonal T-cell populations from multi-virus-specific $CD8^{pos}$ T-cell products *in vitro* purified by the Streptamer technology. We showed persistence of prophylactically infused virus-specific T cells derived from the infused T-cell products in the absence of viral reactivation, illustrating long-term persistence. These results suggest that infusion of donor-derived multi-virus-specific T cells in a prophylactic setting early after T-cell depleted alloSCT may be a viable option to prevent viral complications.

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Supplementary Table 1. Patient and transplantation characteristics.

Patient	Diagnosis	Graft type	Condit-ioning regimen	Anti-viral medication	TCD *in vivo*	TCD *in vitro*	CMV serostatus patient/donor	EBV serostatus patient/donor	HLA-matching
3	MM	unrelated	NMA	No	ALM+ATG	ALM	neg/neg	pos/pos	10/10
4	MM	related	NMA	No	ALM	ALM	neg/pos	neg/pos	12/12
7	AML	unrelated	MA	No	ALM	ALM	pos/pos	pos/pos	10/10
8	AML	unrelated	NMA	CMV: VAL T=70 days EBV: RIT+PRED T=62 days	ALM+ATG	ALM	pos/neg	pos/pos	10/10
10	MM	related	NMA	No	ALM	ALM	pos/neg	pos/pos	12/12
11	MM	unrelated	NMA	CMV: VAL T=0	ALM+ATG	ALM	pos/pos	pos/pos	10/10
13	MM	unrelated	NMA	No	ALM+ATG	ALM	pos/pos	pos/pos	10/10
14	MM	related	NMA	No	ALM	ALM	pos/neg	pos/pos	12/12
15	MCL	related	NMA	No	ALM	ALM	pos/neg	pos/pos	12/12
16	AML	unrelated	NMA	No	ALM+ATG	ALM	pos/pos	pos/pos	10/10
17	MM	unrelated	NMA	No	ALM+ATG	ALM	neg/pos	pos/pos	9/10
18	AML	unrelated	NMA	No	ALM+ATG	ALM	neg/neg	pos/pos	10/10
20	AML	unrelated	MA	No	ALM	ALM	neg/neg	pos/pos	10/10
21	AML	related	MA	No	*No TCD*	ALM	neg/pos	pos/pos	12/12
22	AML	unrelated	MA	CMV: GAN T=14 days	ALM	ALM	pos/pos	pos/pos	10/10
23	AML	related	MA	No	*No TCD*	ALM	neg/pos	pos/pos	12/12
24	B-ALL	unrelated	MA	CMV: VAL T=0	ALM	ALM	pos/pos	pos/pos	9/10
25	Myelo-fibrosis	related	NMA	No	ALM	ALM	neg/pos	pos/pos	12/12
26	LPL	unrelated	NMA	No	ALM+ATG	ALM	pos/pos	pos/pos	10/10
27	MDS	unrelated	NMA	CMV: VAL T=0 and T=30 days	ALM+ATG	ALM	pos/pos	pos/pos	10/10

Table adapted from Supplementary Table 3 from the publication by Roex et al, describing the results of the clinical phase I/II study[4]. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; LPL, lymphoplasmacytic lymphoma; MA, myeloablative; MCL, mantle cell lymphoma; MDS, myelodysplastic syndrome; neg, negative; pos, positive; NMA, non-myeloablative; TCD, T-cell depletion; ALM, Alemtuzumab; ATG, Anti-Thymocyte Globulin.; VAL, valganciclavir; GAN, ganciclovir; RIT+PRED, Rituximab + prednisolone; T=0, anti-viral medication before and at moment of infusion of T-cell product; T=>0, day start of medication after T-cell product infusion

Supplementary Table 2. Primer sequences.

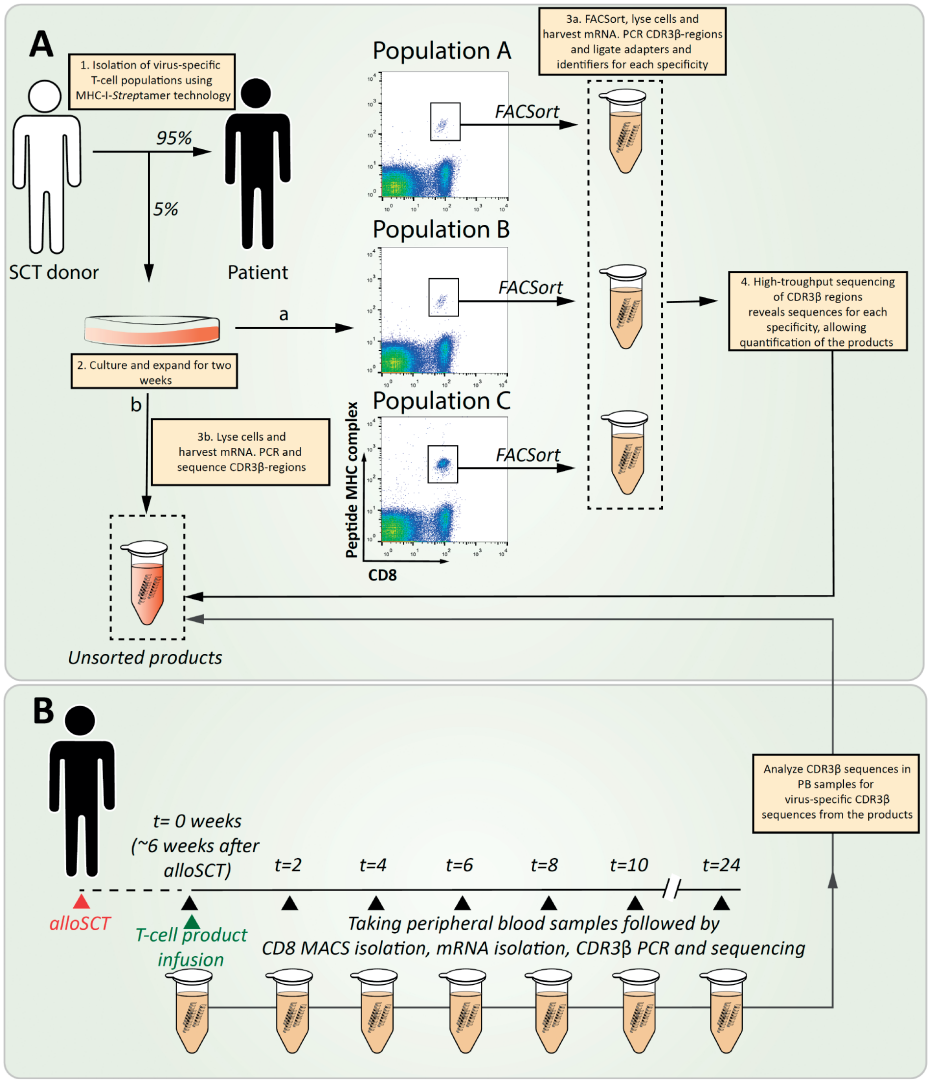
Description	Name	Nucleotide sequence 5'►3'
cDNA primer TRB constant region reverse transcription	TRB_RT	CACGTGGTCGGGGWAGAAGC
cDNA primer SmartSeq2modified template switching oligo	SS2m_TSO	AAGCAGTGGTATCAACGCAGAGTACAT(G)(G){G}
PCR primer SmartSeq2modified forward	SS2m_For	GAGTTCAGACGTGTGCTCTTCCGATCTAAGCAGTGGTATCAACGCAGAGTACAT*G
PCR primer TRBC1 reverse	TRBC1_rev	CCTACACGACGCTCTTCCGATCTGTGGGAACACCTTGTTCAGGTCCT*C
PCR primer TRBC1 reverse	TRBC2_rev	CCTACACGACGCTCTTCCGATCTGTGGGAACACGTTTTTCAGGTCCT*C
Barcode primer SS2m region, forward, backbone	BC_R7xx_For	CAAGCAGAAGACGGCATACGAGAT$_{nnnnnn}$GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C
Barcode primer TRBC region reverse, backbone	BC_R7xx_Rev	AATGATACGGCGACCACCGAGATCTACAC$_{nnnnnn}$ACACTCTTTCCCTACACGACGCTCTTCCGATC*T

Abbreviations: TRB: T-cell Receptor Beta, SS2m: SmartSeq2Modified, TSO: Template Switching Oligo, TRBC: T-cell Receptor Beta Constant, For: Forward, Rev: Reverse, BC: Beta chain, nnnnnn: Identifier sequence
()=RNA, {}=LNA: Locked Nucleic Acid, *:phosphonothioate-binding

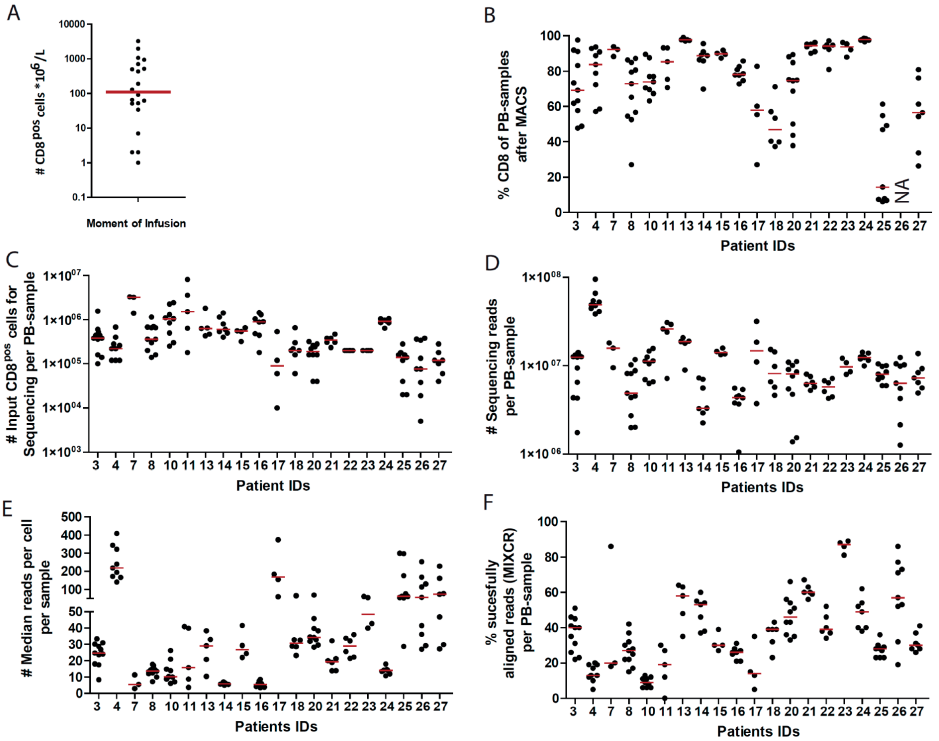
Supplementary Table 3. Identifier sequences.

Identifiers (For) Name	Identifiers (For) Seq	Identifiers (Rev) Name	Identifiers (Rev) Seq
BC_R701	ATCACG	BC_R725	ACTGAT
BC_R702	CGATGT	BC_R726	ATGAGC
BC_R703	TTAGGC	BC_R727	ATTCCT
BC_R704	TGACCA	BC_R728	CAAAAG
BC_R705	ACAGTG	BC_R729	CAACTA
BC_R706	GCCAAT	BC_R730	CACCGG
BC_R707	CAGATC	BC_R731	CACGAT
BC_R708	ACTTGA	BC_R732	CACTCA
BC_R709	GATCAG	BC_R733	CAGGCG
BC_R710	TAGCTT	BC_R734	CATGGC
BC_R711	GGCTAC	BC_R735	CATTTT
BC_R712	CTTGTA	BC_R736	CCAACA
BC_R713	AGTCAA	BC_R737	CGGAAT
BC_R714	AGTTCC	BC_R738	CTAGCT
BC_R715	ATGTCA	BC_R739	CTATAC
BC_R716	CCGTCC	BC_R740	CTCAGA
BC_R717	GTAGAG	BC_R741	GACGAC
BC_R718	GTCCGC	BC_R742	TAATCG
BC_R719	GTGAAA	BC_R743	TACAGC
BC_R720	GTGGCC	BC_R744	TATAAT
BC_R721	GTTTCG	BC_R745	TCATTC
BC_R722	CGTACG	BC_R746	TCCCGA
BC_R723	GAGTGG	BC_R747	TCGAAG
BC_R724	GGTAGC	BC_R748	TCGGCA

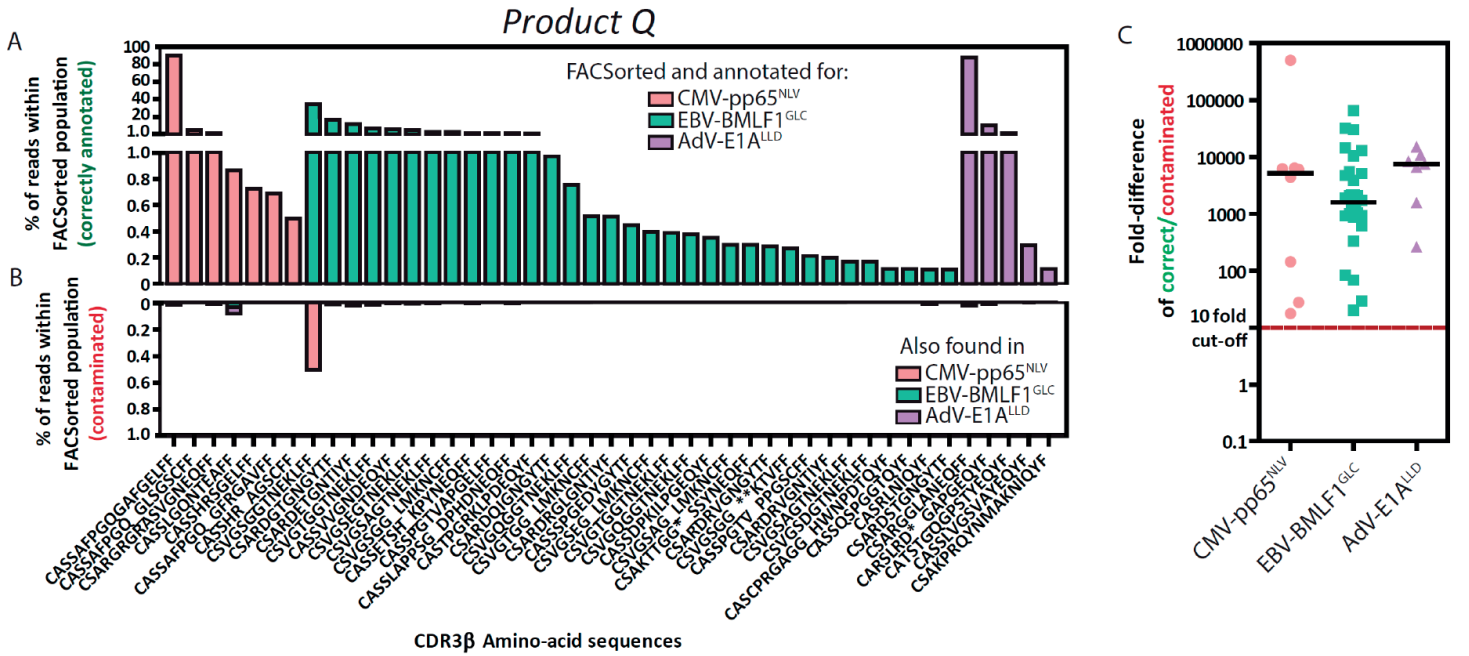
Unique molecular identifiers were used per sample from one patient. Sequencing was performed per patient allowing up to 24 different samples that could be barcoded with unique identifiers.



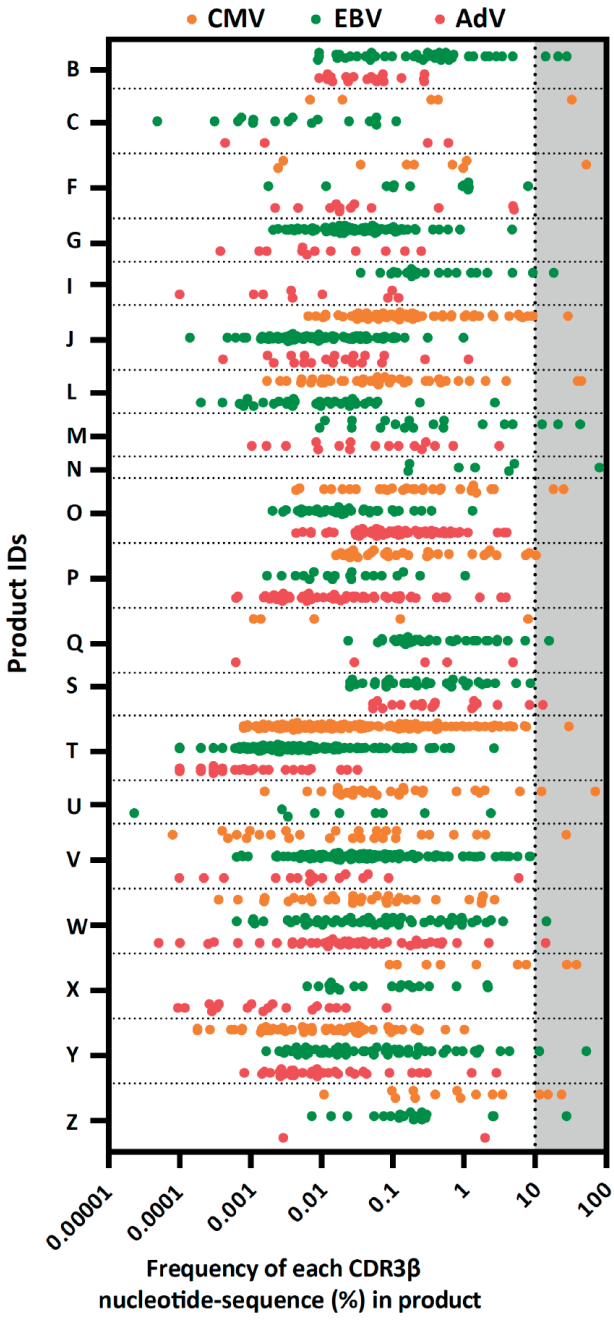
Supplementary Figure 1. Schematic overview of high-throughput sequencing of the T-cell receptors of T cells from virus-specific T-cell products. A) T cells from 20 products were successfully expanded *in vitro*. Virus-specific T-cell populations that target a single antigen were then isolated from the cultured products and mRNA was harvested directly after FACSort. Unique identifiers were ligated to the PCR products for each T-cell population. The remainders of the unsorted products were additionally sequenced to quantify the different TCR-nucleotide sequences (CDR3β-sequences) present within the T-cell products. **B)** PBMCs were isolated from PB of patients drawn before (day 0) and after infusion of the T-cell products. PB samples were collected every ~2 weeks followed by PBMC selection, CD8 MACS isolation, mRNA isolation and sequencing of the TCRs. CDR3β-sequences from these monitoring samples were compared with the CDR3β-sequences from the products.
Abbreviations: alloSCT, allogeneic stem cell transplantation. MACS, magnetic activated cell sorting. SCT, stem cell transplantation. FACS, Fluorescence activated cell sorting. CDR3, complementary determining region 3. MHC, major histocompatibility complex.



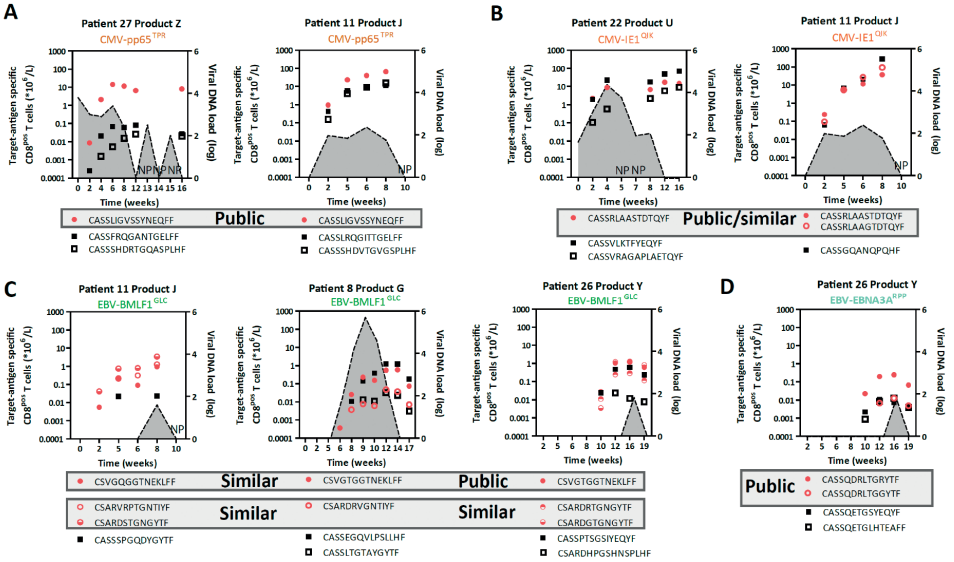
Supplementary Figure 2. Sample quality assessment of peripheral blood samples of patients. A) The absolute numbers of $CD8^{pos}$ T cells are shown for each patient at moment of product infusion **B**) The CD8 purity after CD8 Magnetic Activated Cell Sorted (MACS) per peripheral blood sample is shown for each patient. **C**) The number of CD8 MACSorted T cells used as input for TCR-beta sequencing are shown per peripheral blood sample for each patient. **D**) The total number of TCR sequencing reads for each CD8 MACSorted peripheral blood sample are shown for each patient. (**E**) The median number of TCR sequencing reads per cell are shown per sample for each patient. **F**) The percentage of reads that could successfully be aligned using MIXCR are shown per sample for each patient.
Red lines indicate medians.NA, not available



Supplementary Figure 3. Overlap of TCR-CDR3β sequences between FACSorted specificities. A representative example is shown of FACSorted virus-specific T-cell populations from product Q. A) The percentages of TCR-CDR3β sequences that were present in each of the FACSorted virus-specific T-cell populations are shown on the y-axis. B) The percentages are shown of TCR-CDR3β sequences that were also detected in another FACSorted virus-specific T-cell population (contamination). C) A cut-off fold-difference of 10-fold between the % of sequences annotated to the correct specificity and the % of contamination in another sample was used to determine correct annotation of TCR-CDR3β sequences to the corresponding specificity.



Supplementary Figure 4. Frequencies of CMV, EBV and AdV-specific TCR-nucleotide sequences within each product. Shown are the frequencies (number of specific CDR3β-sequence reads as a proportion of the total number of CDR3β-sequence reads) of different CMV, EBV or AdV-specific TCR-nucleotide sequences (CDR3β-sequences) within each product. Each dot represents the frequency of a single CDR3β-sequence)



Supplementary Figure 5. Tracking of public and private virus-specific T-cell clones that appeared only after infusion of the T-cell product. Representative examples are shown of T-cell products that contained virus-specific T-cell populations with public TCRs or TCRs that were highly-similar to public TCRs. The virus-specific T-cell clones from these examples were only detected in the patient after infusion of the product. Viral DNA loads are shown as filled grey areas. **A** and **B**). Two different CMV-specific T-cell populations were tracked in three patients that contained a CMV-pp65^{TPR}-specific public TCR (red, A) and a CMV-IE1^{QIK}-specific public TCR (red, B). Expansion kinetics of virus-specific T-cell clones that expressed private TCRs is shown in black rectangles. **C** and **D**) Two different EBV-specific T-cell populations were tracked in three patients that contained an EBV-BMLF1^{GLC}-specific public TCR (red, C) and an EBV-EBNA3A^{RPP}-specific public TCR (red, D). Expansion kinetics of T-cell clones with TCRs that are highly-similar to the published public TCR is shown as open red circles. Expansion kinetics of T-cell clones that express private TCRs is shown as black rectangles.
NP; Sequencing not performed.


RISKS

CHAPTER 3

Magnitude of off-target allo-HLA reactivity by third-party donor-derived virus-specific T cells is dictated by HLA-Restriction

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ABSTRACT

T-cell products derived from third-party donors are clinically applied, but harbor the risk of off-target toxicity via induction of allo-HLA cross-reactivity directed against mismatched alleles. We used third-party donor-derived virus-specific T cells as model to investigate whether virus-specificity, HLA restriction and/or HLA background can predict the risk of allo-HLA cross-reactivity. Virus-specific $CD8^{pos}$ T cells were isolated from HLA-A*01:01/B*08:01 or HLA-A*02:01/B*07:02 positive donors. Allo-HLA cross-reactivity was tested using an EBV-LCL panel covering 116 allogeneic HLA molecules and confirmed using K562 cells retrovirally transduced with single HLA-class-I alleles of interest. HLA-B*08:01-restricted T cells showed the highest frequency and diversity of allo-HLA cross-reactivity, regardless of virus-specificity, which was skewed towards multiple recurrent allogeneic HLA-B molecules. Thymic selection for other HLA-B alleles significantly influenced the level of allo-HLA cross-reactivity mediated by HLA-B*08:01-restricted T cells. These results suggest that the degree and specificity of allo-HLA cross-reactivity by T cells follow rules. The risk of off-target toxicity after infusion of incompletely matched third-party donor-derived virus-specific T cells may be reduced by selection of T cells with a specific HLA restriction and background.

INTRODUCTION

Adoptive transfer of autologous or human leukocyte antigen (HLA)-matched patient-specific T-cell products, including antigen-specific T cells, Chimeric Antigen Receptor (CAR) T cells and T-cell receptor (TCR) modified T cells are clinically applied and show feasibility and safety(1-5). Nevertheless, the complex logistics and delays associated with the generation of these products for adoptive immunotherapy strategies are hampering easy broad application. Off-the shelf T-cell products, generated from cells of healthy third-party donors and suitable for treatment of multiple patients, may be an elegant solution, but such products are often only partially HLA-matched with the recipient.

In our study we focused on virus-specific T-cell products derived from healthy third-party donors that can be used for the treatment of uncontrolled viral reactivations and/or viral disease in patients without easy access to autologous or donor-derived virus-specific T cells. Reactivations of cytomegalovirus (CMV), Epstein-Barr virus (EBV) and adenovirus (AdV) are frequently seen and associated with high morbidity and mortality in immune-compromised patients(6, 7), like patients after allogeneic stem cell transplantation (AlloSCT), but also patients after solid organ transplantation. For patients transplanted with stem cells from a virus-non experienced donor or in general for solid-organ donors there is no easy access to (HLA-matched) memory virus-specific T cells. Adoptive transfer of partially HLA-matched virus-specific T cells from healthy third-party donors is a potential strategy to temporarily provide anti-viral immunity to these patients. However, these third party donor-derived virus-specific T cells have not been tolerized by thymic negative selection to the non-matched HLA molecules that are present within the patient(8, 9), thereby implying the risk of off-target toxicity due to allo-HLA cross-reactivity directed against the mismatched HLA alleles(10).

It was demonstrated that third-party derived virus-specific T cells can exert allo-HLA cross-reactivity directed against mismatched HLA alleles *in vitro(11-14).* The viral specificity as well as the allo-HLA cross-reactivity was shown to be mediated by the same T-cell receptor (TCR) complex(11, 14). Additionally, TCR cross-reactivity could be a major trigger of graft rejection, as shown by the association between viral reactivation and graft rejection in recipients of solid organs(15, 16). Despite the clearly documented allo-HLA cross-reactivity of virus-specific T-cell populations documented *in vitro*, only low rates (~5%) of off-target toxicity/*de novo* Graft Versus Host Disease (GVHD) were observed in stem cell recipients that were treated with partially HLA-matched virus-specific T cells(17-21). There are several potential reasons for this discrepancy: 1) the specific allogeneic peptide/HLA complex recognized by the cross-reactive virus-specific T cells is not present in the patient, 2) removal of the *in vitro* off-target (>10% cytotoxic) virus-specific T cells from the product prior to administration to the patient

and/or selection of T-cell products that do not show *in vitro* allo-HLA reactivity(18), 3) low T-cell numbers of cross-reactive virus-specific T cells administered and/or limited *in vivo* proliferation, 4) Rejection of the partly HLA-matched third party virus-specific T cells by the recipient(22). In the last example, such rejection prevents toxicity, but it also diminishes the short-term protection afforded by the third-party derived T cells. In a recent phase I/II clinical study by Neuenhahn et al, survival/persistence was only demonstrated for adoptively transferred virus-specific T cells of the original stem cell donor (8/8 HLA-matched), but not for virus-specific T cells derived from third-party donors with a higher degree of HLA-mismatch(22).

It would be useful if we could predict which non-matched HLA molecules are recognized by third-party derived T cells so that specific donors and/or specific T-cell populations can be selected with a low likelihood of exerting off-target reactivity. Thus far, recurrent off-target reactivity towards the same non-matched HLA molecule was only found for T-cell populations isolated from different individuals using the exact same TCR (public TCR)(14, 23, 24). A classic example of such public cross-reactivity is the HLA-B*08:01-restricted EBV-EBNA3A^{FLR}-specific T-cell population that contains a dominant public TCR showing cross-reactivity against non-self HLA-B*44:02(23, 24). Importantly, this public TCR is not found in the T-cell repertoire of HLA-B*08:01/HLA-B*44:02 positive individuals, demonstrating the deletion of this otherwise potentially auto-reactive public TCR during *in vivo* thymic selection. Many antiviral T-cell responses are, however, not so clearly dominated by a single dominant public TCR, making predictions of cross-reactivity more difficult.

The aim of this study was to investigate whether we could identify and predict allo-HLA cross-reactivity patterns by third-party donor-derived T cells, using virus-specific T cells as a model. We investigated whether the allo-HLA cross-reactivity by third-party donor-derived virus-specific T cells was influenced by virus-specificity, HLA-restriction and/or HLA background of the donors. Our data show that the level of allo-HLA cross-reactivity is not affected by viral-specificity, but surprisingly strongly associated with HLA restriction and influenced by the HLA background of the donors.

MATERIALS AND METHODS

Collection of donor material

After informed consent according to the Declaration of Helsinki, healthy donors (homozygously) expressing HLA-A*01:01 and HLA-B*08:01 or HLA-A*02:01 and HLA-B*07:02 were selected from the Sanquin database and the biobank of the department of Hematology, Leiden University Medical Center (LUMC). Two donors expressing HLA-A*02:01/HLA-B*07:02 were not homozygous. Peripheral blood mononuclear cells

(PBMCs) were isolated by standard Ficoll-Isopaque separation and used directly or thawed after cryopreservation in the vapor phase of liquid nitrogen. Donor characteristics (HLA typing, CMV and EBV serostatus) are provided in **table 1** (Donors 1-24). Healthy donors expressing HLA-B*08:01 and HLA-B*13:02 or HLA-B*35:01 (**Table 1;** donors 25-30) were selected from the biobank of the department of Hematology (LUMC).

Generation of peptide-MHC complexes to isolate virus-specific T cells

All viral peptides were synthesized in-house using standard Fmoc chemistry. Recombinant HLA-A*01:01, HLA-A*02:01, HLA-B*07:02 and HLA-B*08:01 heavy chain and human β2m light chain were in-house produced in Escherichia coli. MHC-class-I refolding was performed as previously described with minor modifications(25). Major histocompatibility complex (MHC)-class-I molecules were purified by gel-filtration using HPLC. Peptide-MHC(pMHC) tetramers were generated by labeling biotinylated pMHC-monomers with streptavidin-coupled phycoerythrin (PE; Invitrogen, Carlsbad, USA), allophycocyanin (APC, Invitrogen), brilliant violet 421 (BV421, Becton Dickinson (BD), Franklin Lakes, USA), brilliant violet 510 (BV510, BD) or peridinin-chlorophyll-protein complex (PerCP, Invitrogen). Complexes were stored at 4 °C. Formation of stable pMHC-monomers was performed using UVexchange technology(26) and according to a previously described protocol(27).

Isolation and expansion of virus-specific T cells

Phycoerythrin (PE), allophycocyanin (APC), BV421, BV510 and/or peridinin-chlorophyll-protein (PerCP)-labeled pMHC-tetramer complexes were used for fluorescence-activated cell sorting (FACSorting). The pMHC-tetramers used are shown in **supplementary table 1**. PeptideMHC-tetramer positive, $CD8^{pos}/CD4^{neg}$ T cells were sorted and seeded at 10,000 cells per well in U-bottom microtiter plates for the generation of bulk T-cell populations. Single pMHC-$tetramer^{pos}$ virus-specific T cells were first specifically expanded, in the presence of 10^{-7} M of the specific peptide in T-cell medium: Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Verviers, Belgium) containing 5% heat-inactivated fetal bovine serum (FBS; Invitrogen), 5% heat-inactivated human serum (ABOS; Sanquin Reagents, Amsterdam, The Netherlands), 100 U/mL penicillin (Lonza), 100 µg/mL streptavidin (Lonza) , 2.7mM L-glutamine (Lonza) 100 IU IL-2/ml (Chiron, Emeryville, USA) and with 5-fold 35 Gy irradiated autologous PBMCs as feeder cells. After two weeks of culture, pMHC-$tetramer^{pos}$ T-cell populations were considered pure if they contained ≥97% pMHC-$tetramer^{pos}$ cells. Polyclonality of the sorted virus-specific T cells was assessed by T-cell receptor-variable β (TCR-Vβ) family analysis using the TCR-Vβ kit (Beckman Coulter, Fullerton, USA). Sub-populations expressing a single TCR-Vβ family were sorted from the bulk using monoclonal antibodies from the TCR-Vβ kit. Sub-populations were then non-specifically expanded using the aforementioned feeder mixture with 0.8µg/ml phytohemagglutinin (PHA; Oxoid Limited, Basingstoke, UK) instead of specific peptide.

Sub-populations using one specific TCR-Vβ family were considered pure if ≥95% of the population was positive for that TCR-Vβ family. Sorting was performed on a FACS ARIA (BD) and analyzed using Diva software (BD). All analyses were performed on a FACS Calibur (BD), and analyzed using Flowjo Software (TreeStar, Ashland, USA).

Selection and generation of stimulator cells for functional analyses

EBV-transformed lymphoblastoid cell-lines (EBV-LCLs) were generated according to standard protocols(28). EBV-LCLs were selected to cover a total of 116 frequently occurring HLA molecules, as listed in **table 2**. HLA-deficient K562 cells were transduced with 40 different single HLA-class-I molecules (**Supplementary Table 2**) including common and rare HLA-class-I molecules. Constructs encoding different HLA-class-I sequences were coupled to an IRES sequence with a truncated form of the nerve growth factor receptor (tNGFR) serving as marker gene and were cloned into LZRS plasmids. Constructs were verified using reverse transcriptase polymerase chain reactions (RT-PCR) and Sanger sequencing. As a control, tNFGR only was cloned into an LZRS plasmid (mock). Retroviral transduction was performed as previously described(29). K562 wildtype cell-lines were transferred to wells containing stable retroviral particles, generated using a puromycin selected stable ϕ-NX-A packaging cell line, and incubated for 24 hrs at 37°C(12). For FACSorting, cells were stained with PE-labelled NGFR antibodies (NGFR(CD271); BD/Pharmingen) for 30 min at 4°C. After sorting, single HLA-class-I transduced K562 cells were analyzed for HLA-class-I expression using FITC-labelled HLA-ABC antibodies (Serotec, Hercules, USA) and PE-labelled NGFR(BD). Single HLA-class-I transduced K562 cell-lines were verified using RT-PCR and Sanger sequencing. EBV-LCLs and K562 cell-lines were cultured in stimulator medium consisting of Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Invitrogen, Carlsbad, USA), 100 U/mL penicillin (Lonza), 100 µg/mL streptavidin (Lonza) and 2.7mM L-glutamine (Lonza).

Cytokine production assays to determine T-cell reactivity

Interferon-γ (IFN-γ) production by virus-specific T cells was quantified using standard enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (Sanquin Reagents, The Netherlands). Responder T cells were co-cultured with stimulator cells at a ratio of 1:10 (responder: stimulator) for 16 hours at 37°C in T-cell medium used for expansion of T-cell populations as described in supplementary material using 25 IU/ml Interleukin-2 (IL-2) instead of 100IU/ml IL-2. Recognition of HLA-mismatched EBV-LCLs, HLA-matched peptide-pulsed EBV-LCLs and K562 cells transduced with specific HLA molecules was defined as production of ≥200 pg/ml of IFN-γ.

Cytotoxicity assay

Cytotoxicity was determined by 51-chromium (^{51}Cr)-release assay. Virus-specific T-cell

populations were added to (10:1, E:T ratio) ^{51}Cr-labeled EBV-LCLs and single HLA-class-I transduced K562 cells for 4 hrs at 37°C in the same IMDM medium used for cytokine production assays. ^{51}Cr release was measured on a γ-counter. Spontaneous ^{51}Cr release of the target cells was determined in medium alone, and maximum ^{51}Cr release was determined by adding Triton (1%; Sigma, Saint louis, USA). Percentages of specific lysis were determined by the following calculation: ((experimental ^{51}Cr release – averaged spontaneous ^{51}Cr release) / (averaged maximal ^{51}Cr release – averaged spontaneous ^{51}Cr release)) x 100. Values for specific ^{51}Cr lysis represent the mean plus and minus standard deviation of triplicate wells. Spontaneous and maximum release represents the mean of sextuplicate wells.

Statistical analysis

Statistical analyses were only performed on quantitative data and were performed using non-parametric tests. The Fisher's-Exact test was used to assess the differences in cross-reactivity (present or absent) of HLA-mismatched EBV-LCLs between groups (i.e. HLA-A*01:01- and HLA-B*08:01-restricted virus-specific T cells). Differences in the numbers of recognized HLA-mismatched EBV-LCLs was first assessed by the Kruskal-Wallis test. Differences between two groups were then further assessed with the non-parametric Mann-Whitney U test. *p*-values were adjusted by the Bonferroni correction for multiple testing. Statistical analyses were conducted using GraphPad Prism (GraphPad Software, version 8).

RESULTS

Virus-specific T-cell populations show profound and diverse cross-reactivity against a panel of HLA-mismatched EBV-LCLs

To study the influence of HLA restriction and antigen specificity on the level of allo-HLA cross-reactivity mediated by virus-specific T cells, bulk virus-specific T-cell populations targeting single epitopes were isolated from total PBMCs of (homozygous) HLA-A*01:01/HLA-B*08:01^{pos} or HLA-A*02:01/HLA-B*07:02^{pos} healthy donors. Two donors did not homozygously express HLA-A*02:01/HLA-B*07:02. All donors were EBV seropositive and 5 out of 12 HLA-A*01:01/HLA-B*08:01^{pos} donors and 9 out of 12 HLA-A*02:01/HLA-B*07:02^{pos} donors were CMV seropositive (Table 1). The serostatus for AdV was unknown for all donors. Virus-specific T cells were isolated by FACS using pMHC-tetramers for various peptides (n=21) from CMV, EBV and AdV (**Supplementary Table 1**). In total, 45 CMV, 95 EBV and 24 AdV-specific T-cell populations were isolated (**Table 3**). CMV and EBV-specific T-cell populations could be isolated from all CMV^{pos} and EBV^{pos} donors, respectively. Although no AdV serostatus was known, AdV-specific T-cell populations could be isolated from 18 out of 24 donors. We isolated 22 different

Table 1. HLA typing and CMV/EBV serostatus of healthy donors.

#	CMV	EBV	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ		HLA-DP	
1	Pos	Pos	01:01		08:01		07:01		03:01	15:01	02:01	06:02	N.D	
2	Pos	Pos	01:01		08:01		07:01		03:01	01:02	02:01	05:01	01:01	04:01
3	Neg	Pos	01:01		08:01		07:01		03:XX		02:XX		N.D	
4	Pos	Pos	01:01		08:01		07:01		03:01		02:01		04:01	
5	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	
6	Neg	Pos	01:01		08:01		07:01		03:01		02:01		01:01	09:01
7	Pos	Pos	01:01		08:01		07:01		03:XX		02:XX		N.D	
8	Neg	Pos	01:01		08:01		07:01		03:XX		02:XX		N.D	
9	Pos	Pos	01:01		08:01		07:01		03:01		02:01		01:01	04:01
10	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	04:02/01
11	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	05:01
12	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	05:01
13	Pos	Pos	02:01		07:02		07:02		15:01		06:02		04:01	
14	Pos	Pos	02:01		07:02	44:02	07:02	05:01	15:01	04:01	06:02	03:01	04:XX	02:01
15	Pos	Pos	02:01	03:01	07:02		07:02		15:01		06:02		04:01	03:01
16	Pos	Pos	02:01	03:01	07:02	44:02	07:02	05:01	15:01	01:01	06:02	05:01	04:01	14:01
17	Pos	Pos	02:01		07:02		07:02		15:01		06:02		04:01	05:01
18	Pos	Pos	02:01		07:02		07:02		15:01		06:02		04:01	
19	Neg	Pos	02:01		07:02		07:02		15:01		06:02		04:01	
20	Pos	Pos	02:01		07:02		07:02		15:01		06:02		02:01	04:01
21	Neg	Pos	02:01		07:02		07:02		15:01		06:02		04:01	13:01
22	Pos	Pos	02:01		07:02		07:02		07:01	15:01	03:03	06:02	04:01	13:01
23	Neg	Pos	02:01		07:02		07:02		15:XX		06:XX		N.D	
24	Pos	Pos	02:01		07:02		07:02		15:XX		06:XX		N.D	
25	Pos	Pos	01:01	68:01	08:01	35:01	04:01	07:01	01:01	03:01	02	05:01	04:01	04:02
26	Neg	pos	01:01	24:02	08:01	35:01	04:01	07:01	03:01	08:01	02:01	04:02	04:01	
27	Pos	pos	02:01	24:02	08:01	35:01	07:01	11:01	02:02	03:01	02:02	03:01	02:01	13:01
28	Pos	pos	01:01	30:01	08:01	13:02	07:01	06:02	03:01	04:01	02:01	03:01	04:01	
29	Pos	pos	01:01	30:01	08:01	13:02	07:01	06:02	04:07	15:01	03:01	06:02	04:01	
30	Pos	pos	01:01	30:01	08:01	13:02	07:01	06:02	03:01	07:01	02:01	02:02	04:01	09:01

Virus-specific T cells restricted to HLA-A*01:01/HLA-B*08:01 or HLA-A*02:01/HLA-B*07:02 were isolated from donors #1-12 and donors #13-24, respectively. CMV and EBV serostatus are indicated for each donor. HLA typing was determined either by serology, where the second digits could not be determined (XX) or with high resolution HLA typing, unless indicated by N.D. HLA-B*08:01-restricted EBV-EBNA3A^{QAK} and EBV-BZLF1^{RAK}-specific T cells were additionally isolated from donors #25-30 for specific experiments to investigate the role of HLA-backgrounds. Blanks indicate homozygosity for the given allele.

HLA-A*01:01-restricted virus-specific T-cell populations, 69 HLA-A*02:01-restricted virus-specific T-cell populations, 34 HLA-B*07:02-restricted virus-specific T-cell populations and 39 HLA-B*08:01-restricted virus-specific T-cell populations (**Table 3**). These T-cell populations were analyzed for allo-HLA cross-reactivity using a panel of HLA-mismatched EBV-LCLs, expressing the most frequent (>2%) HLA-class-I and class-II

antigens in the Caucasian population (**Table 2**). EBV-specific T-cell populations were only tested against HLA-mismatched EBV-LCLs that did not express the specific restriction molecules to avoid recognition of EBV-derived peptides in self-HLA. In total, 65 out of 164 (39%) virus-specific T-cell populations produced interferon-γ in response to stimulation with at least one HLA-mismatched EBV-LCL (**Supplementary Figure 1A**).

Table 2. HLA typing of the HLA-mismatched EBV-LCL panel.

EBV-LCL	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ		HLA-DP	
UBX	01:01	03:01	08:01	18:01	01:02	07:01	03:01	10:XX	02:01	05:01	01:01	02:01
ACD	01:01	24:02	39:06	51:01	07:02	14:02	01:02	11:01	03:01	05:01	04:01	04:02
GME	26:01	02:06	38:01	35:01	12:03	04:01	04:04	01:01	05:01	03:02	04:01	04:02
ABF	30:04	68:02	38:01	55:01	03:03	12:03	03:01	13:01	02:01	06:03	02:01	04:01
LSR	32:01	68:01	35:03	52:01	12:02	12:03	15:02	16:02	05:02	06:01	04:01	14:01
GML	23:01		41:01	51:01	15:02	17:01	07:01	15:01	02:01	06:02	02:01	04:01
UCE	03:01	11:01	07:02	27:05	02:02	07:02	11:01	14:54	03:01	05:03	02:01	16:01
GMS	01:01	11:01	51:01	50:01	15:02	06:02	07:01	04:07	02:01	03:01	03:01	02:01
WKD	11:01	24:02	15:02	40:01	07:02	08:01	08:03	09:XX	03:03	06:01	05:01	
UVN	03:01	11:01	14:02	35:01	04:01	08:02	01:01	13:02	05:01	06:09	05:01	10:01
MWX	01:01	34:01	15:21	35:03	04:03	12:03	01:01	15:02	05:01	06:01	06:01	13:01
GMK	01:01		07:02	57:01	06:02	07:02	04:04	13:01	03:02	06:03	04:02	15:01
MSV	03:01	33:01	07:02	14:02	07:02	08:02	01:02	04:05	03:03	05:01	02:01	04:01
CBF	02:01	11:01	35:01	44:02	04:01	05:01	03:01	09:01	02:01	03:03	01:01	04:01
AVZ	02:20	24:02	08:01	14:01	07:01	08:02	03:01	07:01	02:01	02:02	02:01	04:01
BSR	02:01	68:01	35:03	37:01	04:01	06:02	04:03	10:01	03:01	05:01	02;01	04:01
RHP	03:01	31:01	07:02	40:01	03:04	07:02	13:02	15:01	06:02	06:04	04:01	13:01
SOM	02:60	23:01	15:10	57:03	03:04	18:02	11:01	13:01	03:01	05:01	04:02	40:01
UBM	03:01	24:02	15:01	44:03	03:04	16:01	04:01	07:01	02:02	03:02	03:01	
UBG	02:01	30:02	15:01	39:01	03:03	12:03	01:01	13:01	05:04	06:03	02:01	04:01
LMB	29:02		44:03	51:01	14:02	16:01	07:01	08:01	02:02	04:02	04:01	11:01
FAQ	23:01	68:02	14:02	38:01	08:02	12:03	13:01	13:03	03:01	06:03	02:01	
OBB	01:01	02:01	07:02	08:01	07:01	07:02	03:01	15:01	02:01	06:02	01:01	05:01

The panel of HLA-mismatched EBV-LCLs was composed of high resolution HLA-typed EBV-LCLs that together covered all HLA-class-I and almost all frequently HLA-class-II molecules that are frequently (2%) occurring in the Caucasian population. The HLA typing was determined molecularly. XX indicates that only the allele group could be determined (2 digit resolution). Blanks indicate homozygosity for the given allele.

Next, we investigated whether the T-cell populations that did not recognize any HLA-mismatched EBV-LCL contained smaller sub-population(s) of T cells that could recognize HLA-mismatched EBV-LCLs, but were missed in the initial bulk analysis. Sub-populations were sorted based on expression of single TCR-Vβ families. Twenty-four different TCR-Vβ families can be identified with the provided monoclonal antibodies in the kit that was used for flow cytometry, covering around 70% of the human TCR-Vβ repertoire(30). Sub-

populations that could not be stained with the antibody kit could not be separated from the bulk populations using this strategy and were not analyzed for recognition of HLA-mismatched EBV-LCLs. In total, 165 sub-populations expressing a single TCR-Vβ family were isolated from the 99 bulk T-cell populations that initially did not show reactivity against the EBV-LCL panel. These sub-populations were subsequently analyzed for their capacity to recognize HLA-mismatched EBV-LCLs. We observed that 31 of these isolated sub-populations contained T cells that were capable of exerting allo-HLA cross-reactivity (**Supplementary Figure 1B**). Additionally, 193 sub-populations were sorted from bulk T-cell populations that did already demonstrate HLA-mismatched EBV-LCL recognition in the initial analysis (derived from 65 initial bulk populations). Eighty-six of these isolated sub-populations contained T cells that recognized HLA-mismatched EBV-LCLs. Recognition of additional HLA-mismatched EBV-LCLs could be observed that were not detected in the initial analysis of 25 different bulk T-cell populations (**Supplementary Figure 2**). In summary, a total of 83 bulk T-cell populations contained T cells that showed detectable cross-reactivity against one or more HLA-mismatched EBV-LCL(s).

These 83 T-cell populations were used to investigate whether the virus specificity (CMV, EBV or AdV) or HLA restriction (HLA-A*01:01, HLA-A*02:01, HLA-B*07:02 or HLA-B*08:01) of the virus-specific reactivity influences the occurrence and frequency of HLA-mismatched EBV-LCL recognition. A similar proportion of the virus-specific T-cell populations targeting CMV, EBV or AdV exerted reactivity against at least one HLA-mismatched EBV-LCL (**Figure 1A**). in contrast, a significantly larger fraction of the HLA-B*08:01-restricted virus-specific T-cell populations showed recognition of HLA-mismatched EBV-LCLs, as compared to the HLA-A*01:01, HLA-A*02:01 and HLA-B*07:02-restricted virus-specific T-cell populations (**Figure 1B**). To assess the broadness of the cross-reactivity patterns, we counted how many different HLA-mismatched EBV-LCLs were recognized by the individual T-cell populations (65 bulk T-cell populations including 86 additional sub-populations derived from these T-cell populations, and 31 sub-populations derived from the 18 bulk T-cell populations that did not recognize any HLA-mismatched EBV-LCLs in the initial analysis). In these analyses, HLA-B*08:01-restricted virus-specific T cells exhibited a significantly broader cross-reactivity pattern, illustrated by reactivity against a median of 6 different HLA-mismatched EBV-LCLs, whereas HLA-A*01:01, HLA-A*02:01 and HLA-B*07:02-restricted virus-specific T-cell populations showed reactivity against a median of only 2, 2 and 3 HLA-mismatched EBV-LCLs, respectively (**Figure 1C**). Within the different HLA-B*08:01-restricted T-cell populations, similar high frequencies of T cells capable of exerting cross-reactivity against HLA-mismatched EBV-LCLs were observed, regardless of viral antigen-specificity (**Figure 1D**). These results show that the occurrence and frequency of cross-reactivity against HLA-mismatched EBV-LCLs is highly affected by HLA-restriction and not by virus-specificity (CMV, EBV or AdV).

Table 3. Isolated virus-specific T-cell populations.

				Number of isolated T-cell populations / maximum number of isolations (%)		
Virus	**Antigen**	**HLA**	**Peptide**	**Per specificity**	**Per virus**	**Per HLA**
CMV	pp50	HLA-A*01:01	VTEHDTLLY	4/5 (80%)	**CMV:** 45/56 (80.3%)	**HLA-A*01:01:** 22/28 (78.6%)
	pp65	HLA-A*01:01	YSEHPTFTSQY	4/5 (80%)		
	pp65	HLA-A*02:01	NLVPMVATV	7/9 (78%)		
	IE-1	HLA-A*02:01	VLEETSVML	5/9 (56%)		
	pp65	HLA-B*07:02	TPRVTGGGAM	8/9 (89%)		
	pp65	HLA-B*07:02	RPHERNGFTVL	8/9 (89%)		**HLA-A*02:01:** 69/90 (76.7%)
	IE-1	HLA-B*08:01	ELRRKMMYM	5/5 (100%)		
	IE-1	HLA-B*08:01	QIKVRVDMV	4/5 (80%)		
EBV	LMP2	HLA-A*01:01	ESEERPPTPY	5/6 (83%)	**EBV:** 95/114 (83.3%)	
	LMP2	HLA-A*02:01	FLYALALLL	11/12 (92%)		
	LMP2	HLA-A*02:01	CLGGLLTMV	9/12 (75%)		
	EBNA3C	HLA-A*02:01	LLDFVRFMGV	7/12 (58%)		**HLA-B*07:02**: 34/42 (81%)
	BMLF1	HLA-A*02:01	GLCTLVAML	10/12 (83%)		
	BRLF1	HLA-A*02:01	YVLDHLIVV	12/12 (100%)		
	EBNA3A	HLA-B*07:02	RPPIFIRRL	11/12 (92%)		
	BZLF1	HLA-B*08:01	RAKFKQLL	9/12 (75%)		
	EBNA3A	HLA-B*08:01	FLRGRAYGL	10/12 (83%)		**HLA-B*08:01:** 39/46 (84.8%)
	EBNA3A	HLA-B*08:01	QAKWRLQTL	11/12 (92%)		
AdV	HEXON	HLA-A*01:01	TDLGQNLLY	9/12 (75%)	**AdV:** 24/36 (66.7%)	
	E1A	HLA-A*02:01	LLDQLIEEV	8/12 (67%)		
	HEXON	HLA-B*07:02	KPYSGTAYNAL	7/12 (58%)		

Twelve donors were used to isolate HLA-A*01:01/B*08:01-restricted virus-specific T-cell populations and 12 donors were used to isolate HLA-A*02:01/B*07:02-restricted virus-specific T-cell populations. Five out of 12 HLA-A*01:01/B*08:01^{pos} donors were seropositive for CMV and 9 out of 12 HLA-A*02:01/B*07:02^{pos} donors were seropositive for CMV.
CMV, Cytomegalovirus; EBV, Epstein-Barr virus; AdV, Adenovirus

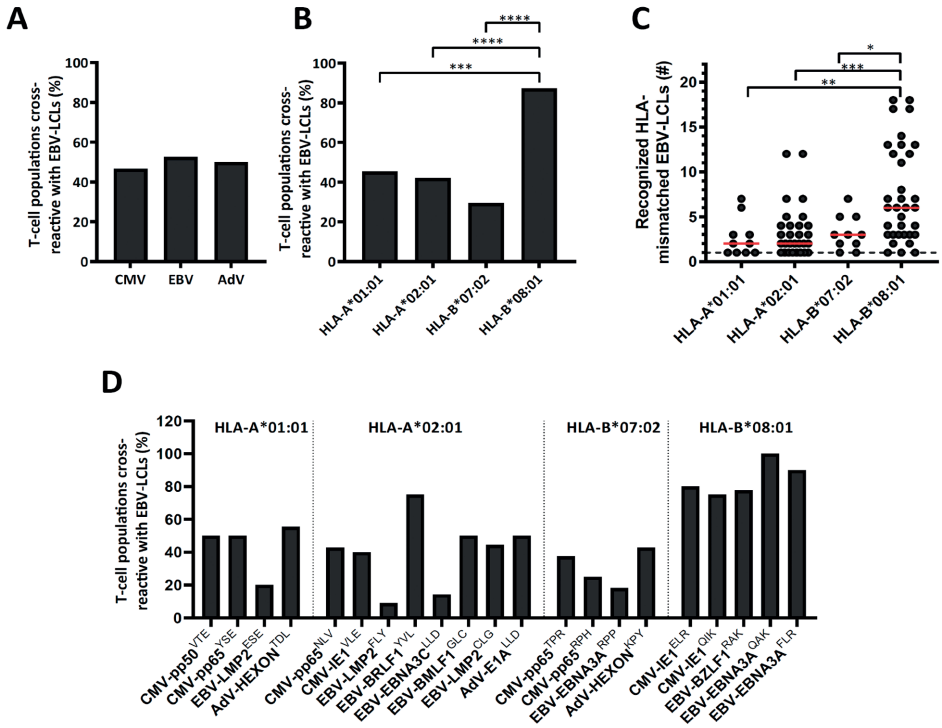

Figure 1. The scope of HLA-mismatched EBV-LCL recognition by virus-specific T-cell populations. Virus-specific T-cell populations (n=164) were stimulated with a panel of HLA-mismatched EBV-LCLs for 16hrs and IFNγ production was measured by ELISA. EBV-specific T-cell populations were tested only against those HLA-mismatched EBV-LCLs that did not express the specific restriction molecule of the viral specificity of those T cells. In total, 83 virus-specific T-cell populations contained T cells that showed recognition of HLA-mismatched EBV-LCLs, defined as production of >200pg IFNγ/ml. **A and B)** Shown are the frequencies of virus-specific T-cell populations that recognized HLA-mismatched EBV-LCLs per virus-specificity **(A)** and per HLA-restriction of the viral specificity **(B). C)** The number of recognized HLA-mismatched EBV-LCLs is shown for the 83 virus-specific T-cell populations that contained T cells that showed cross-reactivity against one or more HLA-mismatched EBV-LCLs (117 sub-populations were included). Recognition of the same HLA-mismatched EBV-LCL by the bulk T-cell populations and sub-population(s) derived from those initial populations was counted once. **D)** Shown are frequencies of virus-specific T-cell populations recognizing HLA-mismatched EBV-LCLs for each viral specificity.
Statistical differences were assessed with the Chi-Squared Fishers Exact Test (A/B) or the Mann-Whitney *t* test (C). **P* <.05; ***P* <.01; ****P* <.001; *****P* <.0001. Red lines represent medians. AdV, Adenovirus. EBV, Epstein-Barr virus. CMV, Cytomegalovirus.

Cross-reactivity against HLA-mismatched EBV-LCLs is mediated by recognition of allogeneic HLA molecules

To investigate if the recognition of HLA-mismatched EBV-LCLs was indeed caused by recognition of allogeneic HLA molecules, HLA-deficient EBV^{neg} K562 cell-lines transduced with single HLA-class-I molecules were used as stimulator cells(12, 31). T-cell populations exhibiting a clear pattern of EBV-LCL recognition, corresponding with the expression of a single HLA allele, were tested against K562 cells transduced with the respective HLA-molecule. For example, a population of EBV-EBNA3A^{QAK}-specific T cells recognized HLA-mismatched EBV-LCL ABF, which uniquely expressed HLA-A*30:04 and HLA-B*55:01 (**Figure 2A**). Recognition of K562 cells that were transduced with HLA-B*55:01 confirmed part of this respective cross-reactivity pattern (**Figure 2B**). In another example, a population of EBV-BRLF1^{YVL}-specific T cells recognized HLA-mismatched EBV-LCLs ACD, WKD, AVZ and UBM that all expressed HLA-A*24:02 (**Figure 2A**) and this was confirmed by recognition of K562 cells transduced with HLA-A*24:02 (**Figure 2B**). Some virus-specific T-cell populations (especially HLA-B*08:01-restricted T cells) showed more complex reactivity patterns when tested against the EBV-LCL panel, that could not be (fully) attributed to recognition of a single allogeneic HLA-molecule. The first representative example shows CMV-pp65^{RPH}-specific T cells that recognized multiple different EBV-LCLs, not allowing direct complete elucidation of the HLA allele(s) being recognized (**Figure 2C**). Only part of the reactivity could be explained by the unique shared expression of HLA-B*40:01 in EBV-LCLs WKD and RHP, that were both recognized. EBV-LCL UCE was the only EBV-LCL expressing HLA-B*27:05. However, the HLA alleles underlying the recognition of EBV-LCLs GML, GMS and MWX could not be deduced. Similarly, EBV-LMP2^{CLG}-specific T cells recognized 4 EBV-LCLs with unique shared expression of HLA-B*35:01 or HLA-B*35:03, while the recognition of EBV-LCL GMK could not be traced back to a specific HLA allele (**Figure 2C; Supplementary Figure 3**). Recognition of the HLA molecules that were anticipated to partly underlie the cross-reactivity patterns was confirmed using K562 cells transduced with the respective HLA molecules (**Figure 2D**). No recognition was observed of K562 cells transduced with irrelevant HLA molecules, whereas recognition of K562 cells transduced with the HLA restriction molecule of the respective virus-specific T-cell population only occurred upon exogenous peptide loading (**Figure 2B and 2D**). Allo-HLA cross-reactive virus-specific T cells also showed to be able to lyse HLA-mismatched target cells (Supplementary Figure 4), in line with previous studies(11, 14).These results show that recognition of HLA-mismatched EBV-LCLs can be mediated by recognition of single or multiple allogeneic HLA-molecules.

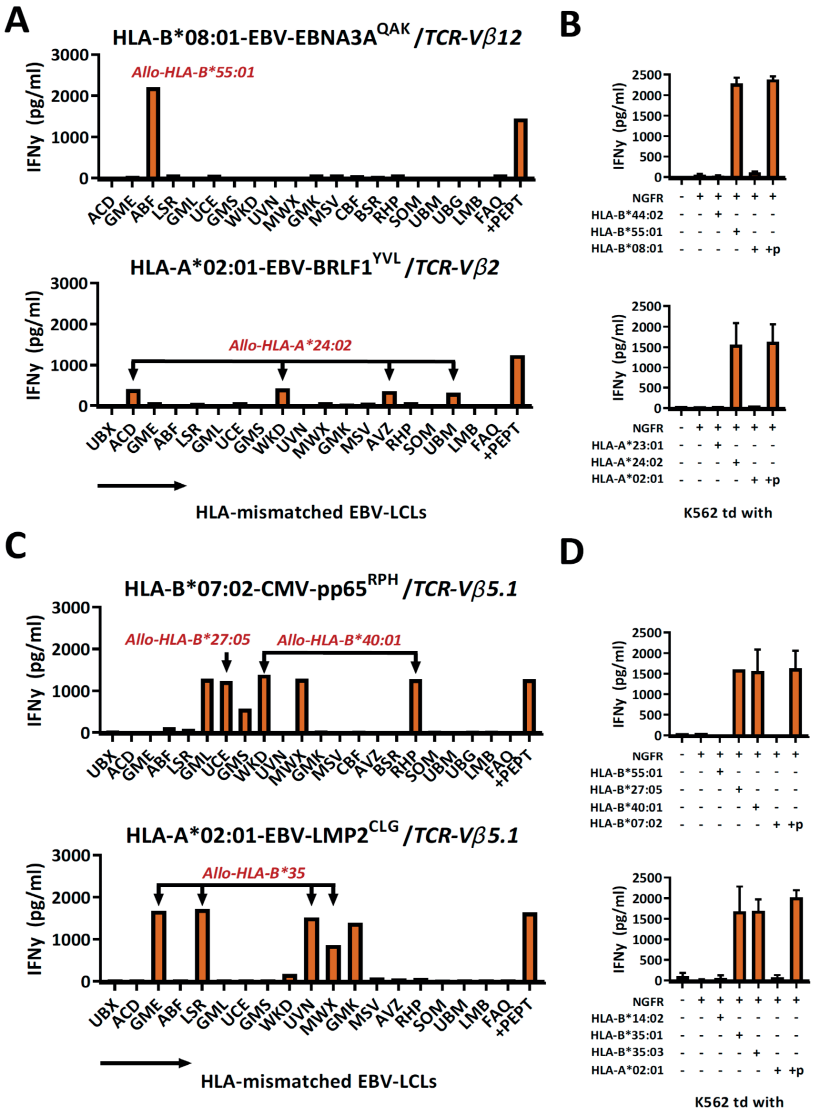

Figure 2. Cross-reactivity against HLA-mismatched EBV-LCLs is mediated by recognition of allogeneic HLA molecules. Sub-populations expressing a single TCR-Vβ family were sorted from the bulk virus-specific T-cell populations targeting a single epitope and were stimulated with a panel of HLA-mismatched EBV-LCLs for 16hrs and IFNγ production was measured by ELISA. EBV-specific T-cell populations were tested only against those HLA-mismatched EBV-LCLs that did not express the specific restriction molecule of the viral reactivity of those T cells. Reactivity was defined as production of >200pg/ml IFNγ. **A)** Shown are two representative examples of virus-specific T-cell sub-populations that showed production of IFNγ (y-axis) in response to stimulation with HLA-mismatched EBV-LCLs (x-axis). **B)** EBV-EBNA3A^{QAK} and EBV-BRLF1^{YVL}-specific T cells were stimulated with K562 cells transduced with the HLA molecules that were expected to be recognized based on the patterns of reactivity against the EBV-LCL panel. K562 cells were also transduced with HLA molecules that were not expected to be recognized as negative control. K562 cells transduced with HLA-A*02:01 or HLA-B*08:01 exogenously loaded with 10^{-6}M of the respective viral peptide were used as positive control. **C)** Shown are two representative examples of virus-specific T-cell sub-populations that showed production of IFNγ (y-axis) in response to stimulation with HLA-mismatched EBV-LCLs (x-axis) that shared multiple different allogeneic HLA

molecules. **D)** CMV-pp65^{RPH} and EBVLMP2^{CLG}-specific T-cell populations were stimulated with K562 cells transduced with HLA molecules that were expected to be recognized based on the reactivities seen against the EBV-LCL panel. K562 cells transduced with HLA-B*07:02 or HLA-B*08:01 exogenously loaded with 10^{-6}M of the respective viral peptide were used as positive control.
TCR, T-cell Receptor. Vβ, Variable Beta Chain. +p, peptide pulsed. NGFR, Nerve Growth Factor Receptor; td, transduced.

HLA-B*08:01-restricted virus-specific T cells recognize multiple allogeneic HLA molecules, skewed towards recognition of HLA-B alleles

The cross-reactivity patterns against the EBV-LCL panel of more than half of the HLA-B*08:01-restricted T-cell populations were rather complex and extensive (observed in 11 out of the 12 HLA-B*08:01^{pos} donors), even when the complexity of the T-cell populations was reduced by selecting for cells expressing a single TCR-Vβ family (Representative examples; **Figure 3A**). No correlation could be observed for recognition of EBV-LCLs that show shared expression of specific HLA-class-II molecules. To investigate whether the reactivity patterns of these HLA-B*08:01-restricted T-cell populations could be (fully) attributed to recognition of a limited number of allogeneic HLA-class-I alleles, a panel of 40 different single HLA-class-I-transduced K562 cell-lines was used as stimulator cells (**Supplementary Table 2**). With this panel we covered 63% of the HLA-A, 73% of the HLA-B and 37% of the HLA-C alleles present in our EBV-LCL panel. Testing the HLA-B*08:01-restricted T-cell populations against this K562 panel revealed recognition of multiple specific groups of allogeneic HLA alleles by single T-cell populations, which could in part explain the cross-reactivity patterns observed when tested against the EBV-LCL panel (**Figure 3B and 3C**).

Next, we determined if the cross-reactivity of HLA-B*08:01-restricted T-cell populations was skewed towards HLA-A, B, or C molecules. In total, 22 HLA-B*08:01-restricted bulk or sub-populations (derived from the 11 HLA-B*08:01^{pos} donors that contained complex and extensive cross-reactive virus-specific T-cell populations) were tested against the K562 panel expressing a selection of HLA-A, B, and C alleles. Twenty-one out of 22 HLA-B*08:01-restricted T-cell populations recognized at least one allogeneic HLA-B molecule and 1 HLA-B*08:01-restricted T-cell population (CMV-IE1^{QIK} from donor 7) only recognized multiple HLA-A molecules in this panel (**Figure 4A; Supplementary Figure 5**). Twelve out of 21 allo-HLA-B-reactive HLA-B*08:01-restricted T-cell populations recognized only allogeneic HLA-B molecules and 9 T-cell populations additionally recognized allogeneic HLA-A and/or HLA-C molecules (**Supplementary Figure 5**). The number of allogeneic HLA-class-I molecules in the K562 panel recognized by the 22 HLA-B*08:01-restricted virus-specific T-cell populations ranged from 1-10 per T-cell population (median of 3; **Figure 4B**). HLA-B*35:01, B*44:02 and B*44:03 were most frequently recognized, whereas HLA-B*13:02, HLA-B*14:02 and HLA-B*41:01 were never recognized by HLA-B*08:01-restricted T cells (**Figure 4C**).

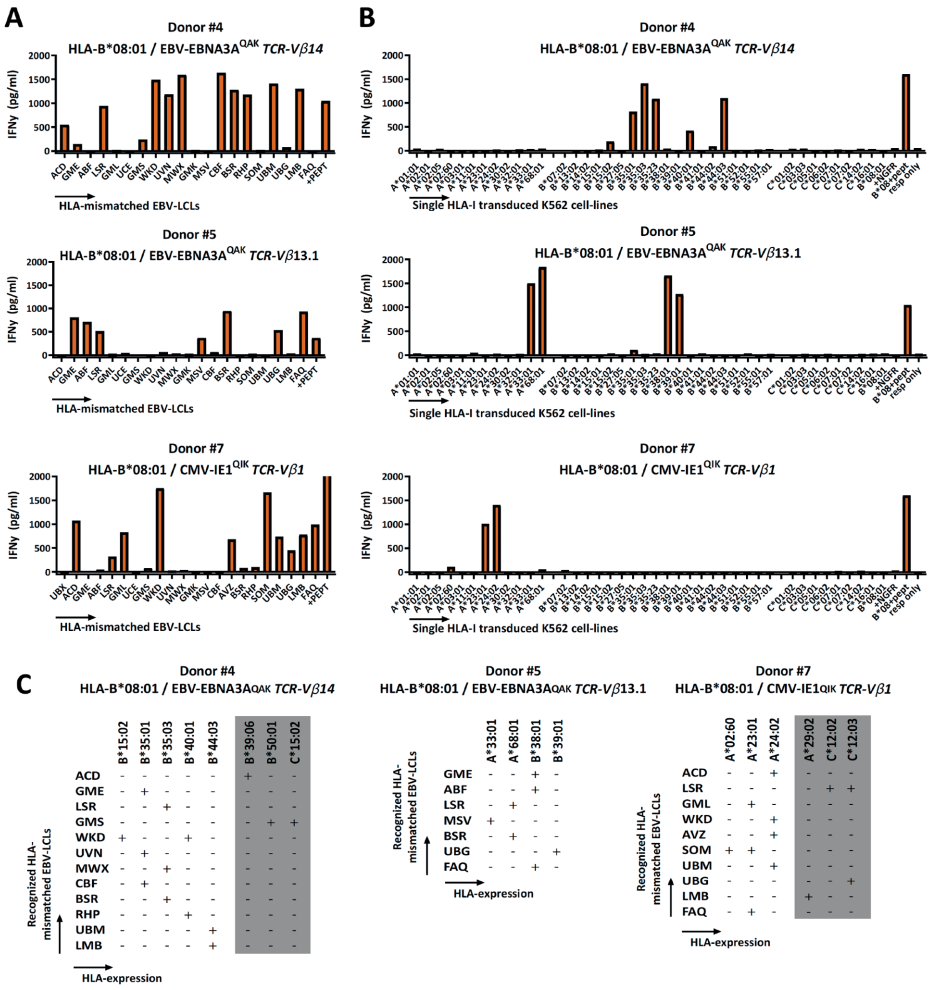

Figure 3. Recognition of multiple HLA-mismatched EBV-LCLs and multiple allogeneic HLA molecules by virus-specific T-cell populations

HLA-B*08:01-restricted T-cell populations that showed reactivity against multiple HLA-mismatched EBV-LCLs were subsequently tested against a panel of single HLA-class-I-transduced K562 cell-lines (n=40) and IFNγ production was measured by ELISA after 16 hours to analyze which specific HLA molecules were being recognized. Reactivity was defined as production of >200pg/ml IFNγ. The K562 panel covered 63% of HLA-A, 73% of HLA-B and 37% of the HLA-C molecules that were present in the EBV-LCL panel. **A)** Shown are three representative examples of HLA-B*08:01-restricted virus-specific T-cell populations, sorted for expression of a single TCR-Vβ family tested for production of IFNγ (γ-axis) in response to stimulation with a panel of HLA mismatched EBV-LCLs (x-axis) **B)** A panel of single HLA-class-I transduced K562 cell-lines allowed partial deduction of the recognized allogeneic HLA molecules. **C)** Listed are the EBV-LCLs that were recognized by the respective virus-specific T-cell populations. The reactivity pattern observed with the EBV-LCL panel could partly be explained by recognition of specific HLA-class-I alleles confirmed with the K562 panel. However, some EBV-LCLs did not express any of the HLA molecules present in the K562 panel. Their recognition might be explained by recognition of HLA molecules that were not present in our K562 panel (grey).

TCR, T-cell Receptor. Vß, Variable Beta Chain, NGFR Nerve growth factor receptor, Resp; responder

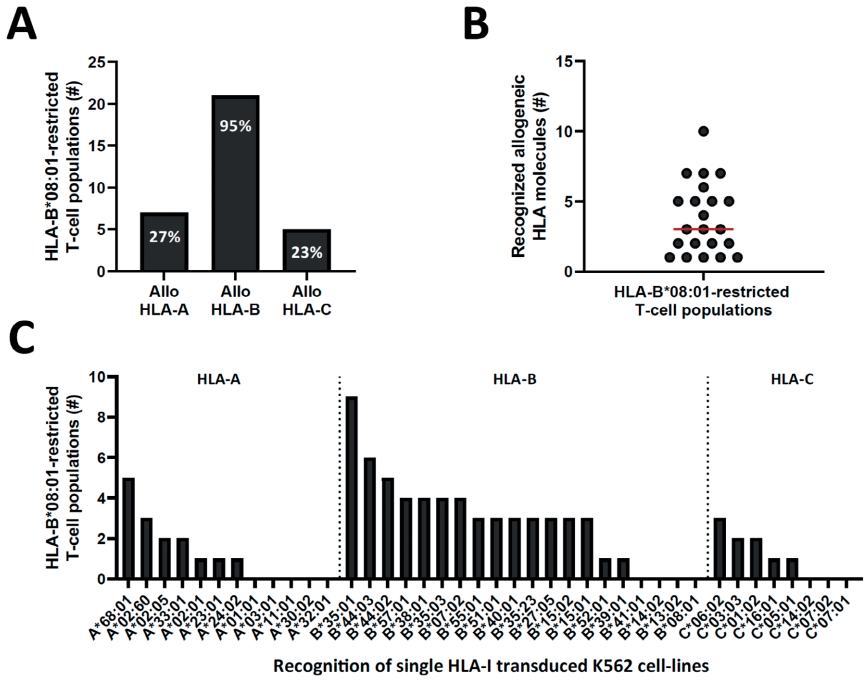

Figure 4. Cross-reactivity by HLA-B*08:01-restricted virus-specific T cells is skewed towards recognition of certain allogeneic HLA-B alleles. Eleven out of 12 HLA-B*08:01^{pos} donors contained HLA-B*08:01-restricted T-cell populations (n=22) with no clear recognition pattern when tested against the HLA-mismatched EBV-LCL panel. To analyze which HLA molecules were being recognized, virus-specific T-cell populations ;were stimulated with a panel of single HLA-class-I-transduced K562 cell-lines (n=40) for 16hrs and IFNγ production was measured by ELISA. Reactivity was defined as production of >200pg/ml IFNγ. **A)** Shown are the number of HLA-B*08:01-restricted T-cell populations that contained T cells that recognized allogeneic HLA-A, B or C alleles. Some populations were allocated to multiple groups. **B)** Shown are the number of recognized allogeneic HLA molecules for each HLA-B*08:01-restricted T-cell population. Red line represents median **C)** Shown are the numbers of HLA-B*08:01-restricted T-cell populations (y-axis) that show recognition of specific allogeneic HLA-A, B or C alleles (x-axis).

To investigate whether the complex and extensive recognition of allogeneic HLA molecules could be mediated by one T-cell clone, we generated T-cell clones from three cross-reactive EBV-EBNA3A^{QAK}-specific T-cell populations (donor #4; donor #8 and #12; **Supplementary Figure 5**). Indeed, all T-cell clones recognized multiple HLA-B alleles in the K562 panel, in the same pattern as the initial EBV-EBNA3A^{QAK}-specific T-cell populations (1 representative example per donor; **Figure 5**)**,** demonstrating that single T-cell clones can exert complex and extensive cross-reactivity against allogeneic HLA molecules. Although these T-cell clones expressed different TCR-Vβ families, all T-cell clones showed a recurrent pattern of recognition of both HLA-B*15:01, HLA-B*35:01, HLA-B*35:03, HLA-B*40:01 and HLA-B*44:03.

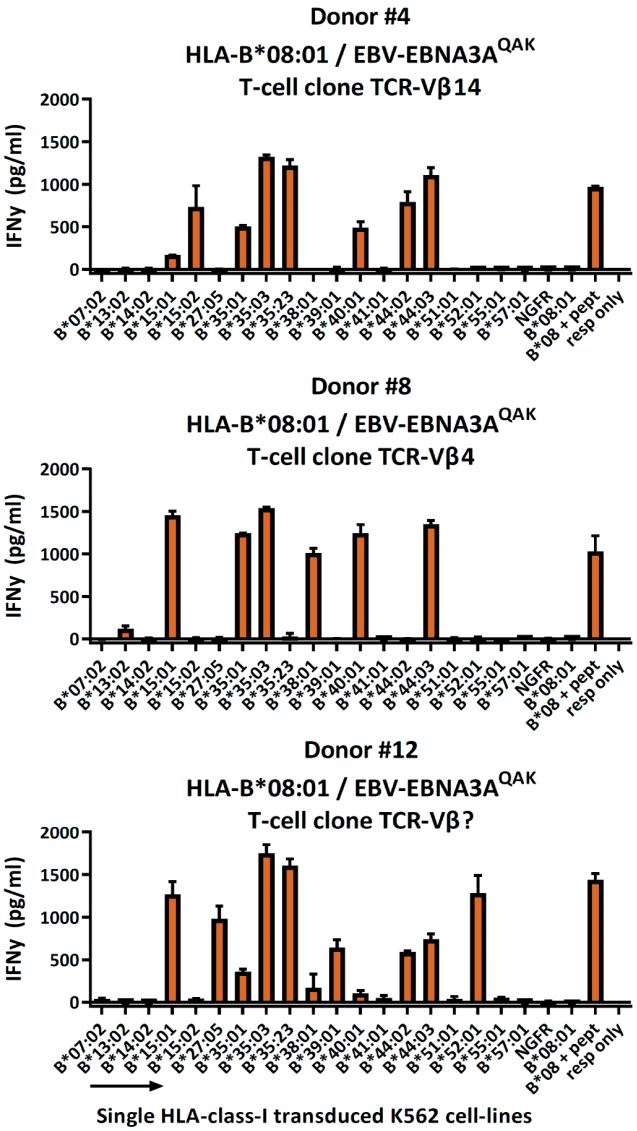

Figure 5. HLA-B*08:01-restricted EBV-EBNA3A^{QAK}-specific T-cell clones recognize multiple allogeneic HLA molecules. HLA-B*08:01-restricted EBV-EBNA3A^{QAK}-specific T-cell clones showing cross-reactivity against allogeneic HLA-B*35:01 were sorted from EBV-EBNA3A^{QAK}-tetramer positive bulk T-cell populations based on expression of activation marker CD137 after stimulation with K562 cells transduced with HLA-B*35:01. All virus-specific T-cell clones showed 100% positive EBV-EBNA3A^{QAK}-tetramer staining. Six T-cell clones per donor were stimulated with a panel of single HLA-B molecule-transduced K562-cell lines (x-axis) for 16hrs and IFNγ production (y-axis) was measured by ELISA to analyze which HLA molecules were being recognized. Reactivity was defined as production of >200pg/ml IFNγ. One representative T-cell clone is shown for each donor. T-cell clones from donor #4 expressed TCR-Vβ14, T-cell clones from donor #8 expressed TCR-Vβ4 and T-cell clones from donor #12 expressed a TCR-Vβ family that could not be determined by the TCR-Vβ flow cytometry kit.
Shown are means with standard deviations of 1 experiment carried out in triplicate.
TCR, T-cell Receptor. Vß, Variable Beta Chain, NGFR Nerve growth factor receptor, Resp; responder

The HLA background of donors shapes the allo-HLA cross-reactivity of HLA-B*08:01-restricted T cells

Virus-specific T cells from HLA-B*08:01 homozygous donors frequently recognized HLA-B*35:01. We therefore reasoned that heterozygosity for HLA-B*35:01 would purge much of the cross-reactivity from the HLA-B*08:01-restricted TCR-repertoire through thymic negative selection. For these reasons, HLA-B*08:01-restricted EBV-EBNA3A^{QAK} and EBV-BZLF1^{RAK}-specific T-cell populations (n=35) were isolated from 3 HLA-B*08:01/HLA-B*35:01^{pos} heterozygous donors (**Table 1**). Contrary, HLA-B*13:02 was never recognized by T cells from HLA-B*08:01^{pos} donors. Therefore, we also isolated T-cell populations (n=10) with the same specificities from 3 HLA-B*08:01^{pos} donors, heterozygous for HLA-B*13:02 (**Table 1**). Strikingly, only 33% of the T-cell populations isolated from HLA-B*08:01/B*35:01^{pos} heterozygous donors recognized one or more HLA-mismatched EBV-LCLs, while 90% of the corresponding T-cell populations from HLA-B*08:01^{pos} homozygous donors demonstrated recognition of HLA-mismatched EBV-LCLs (**Figure 6A**). In contrast, 80% of the T-cell populations isolated from HLA-B*08:01/HLA-B*13:02 heterozygous donors recognized one or more HLA-mismatched EBV-LCLs (**Figure 6A**). HLA-B*08:01-restricted T cells isolated from HLA-B*08:01/HLA-B*35:01 donors also recognized significantly fewer HLA-mismatched EBV-LCLs than the corresponding T-cell populations isolated from HLA-B*08:01/HLA-B*13:02 heterozygous or HLA-B*08:02 homozygous donors (**Figure 6B**). These results show that the occurrence and broadness of allo-HLA cross-reactivity by virus-specific-specific T cells is influenced by the HLA background of the donors.

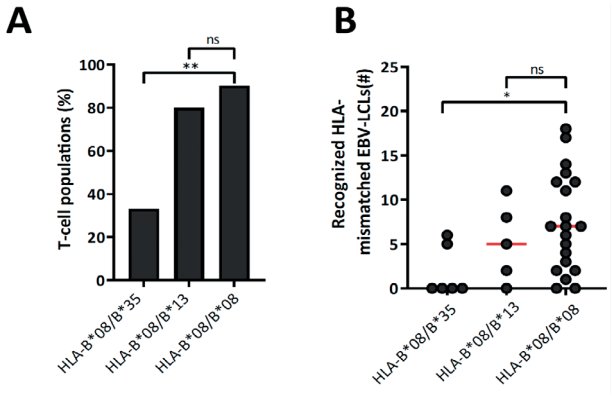

Figure 6. HLA-background of donors shapes the allo-HLA cross-reactivity of HLA-B*08:01-restricted T cells. HLA-B*08:01-restricted EBV-EBNA3A^{QAK} and EBV-BZLF1^{RAK}-specific T-cell populations isolated from HLA-B*08:01/B*35:01^{pos} donors, HLA-B*08:01/B*13:02^{pos} donors or HLA-B*08:01 homozygous donors were stimulated with a panel of HLA-mismatched EBV-LCLs for 16hrs and IFNγ production was measured by ELISA. Reactivity was defined as production of >200pg/ml IFNγ. EBV-LCLs expressing the HLA restriction molecules of the viral specificity of the respective T-cell populations were excluded. Sub-populations of T cells expressing single TCR-Vβ families derived from the initial HLA-B*08:01-restricted EBV-EBNA3A^{QAK} and EBV-BZLF1^{RAK}-specific T-cell populations were included in the analysis of the level of reactivity against the HLA-mismatched EBV-LCL panel. **A)** The percentages of the HLA-B*08:01-restricted EBV-BZLF1^{RAK} and EBV-EBNA3A^{QAK}-specific T-cell populations isolated from HLA-B*08:01/B*35:01^{pos} donors, HLA-B*08:01/B*13:02^{pos} donors or HLA-B*08:01 homozygous donors that recognize 1 or more HLA-mismatched EBV-LCLs were compared. **B)** Shown are the number of HLA-mismatched EBV-LCLs (y-axis) that are recognized by HLA-B*08:01-restricted EBV-EBNA3A^{QAK} and EBV-BZLF1^{RAK}-specific T-cell populations isolated from HLA-B*08:01/B*35:01^{pos}, HLA-B*08:01/B*13:02^{pos} or HLA-B*08:01^{pos} homozygous donors.
Statistical differences were assessed with a Chi-Squared Fishers Exact Test (A) and the Mann-Whitney *t* test (B). *P< .05; **P < .01; ***P <.001; ****P <.0001. Red lines represent medians.

DISCUSSION

In this study, we demonstrated that 50% (83/164) of virus-specific T-cell populations contained T cells that cross-reacted against HLA-mismatched EBV-LCLs, in line with previous findings(11). We showed that the level of allo-HLA cross-reactivity is highly influenced by HLA restriction and not by the viral specificity of the virus-specific T-cell populations. HLA-B*08:01-restricted virus-specific T cells showed the highest frequencies and diversities of allo-HLA cross-reactivity compared to the HLA-A*01:01, HLA-A*02:01 or HLA-B*07:02-restricted virus-specific T-cell populations. Cross-reactivity against HLA-mismatched EBV-LCLs was shown to be mediated by recognition of allogeneic HLA molecules, which was confirmed by recognition of EBV^{neg} K562 cells transduced with specific HLA-class-I molecules, illustrating that the peptides presented by these allogeneic HLA molecules were not EBV or B-cell-derived. HLA-B*08:01-restricted virus-specific T cells showed a skewed pattern of recognition of a group of allogeneic

HLA-B alleles, with HLA-B*35:01 being recognized most often. We demonstrated that cross-reactivities against multiple allogeneic HLA-class-I molecules by HLA-B*08:01-restricted EBV-EBNA3^{QAK}-specific T cells could be mediated by single T-cell clones. Finally, heterozygosity for HLA-B*35:01, but not HLA-B*13:02 significantly reduced the degree of HLA cross-reactivity by HLA-B*08:01-restricted T cells, demonstrating that the HLA background of donors influences the off-target reactivity of virus-specific T cells.

Several groups have investigated whether the allo-HLA cross-reactive risk of virus-specific T cells could be predicted. In most of these studies, allo-HLA cross-reactive patterns could only be predicted when a T-cell population used a public TCR(14, 23, 24). Public T-cell populations could often be found by analysis of sub-populations of T cells expressing a single TCR-Vβ family. However, no pattern of allo-HLA cross-reactivity could be observed in our study, except for HLA-A*02:01-restricted EBV-LMP2^{CLG}-specific T cells sorted for expression of TCR-Vβ5.1 (**Figure 2C**). Although virus-specific T cells often expressed the same TCR-Vβ family, differences in the Complementary Determining Region 3 (CDR3) or a different TCR-alpha chain could result in variation in the allo-HLA cross-reactivity patterns. Allo-HLA cross-reactivity can therefore not be predicted based on TCR-Vβ-family usage alone and may only result in clear patterns if the TCR-Vβ family consist of a public TCR(31).

Similar to other studies we observed that part of the allo-HLA cross-reactive T-cell populations showed recognition of HLA-mismatched EBV-LCLs, but no recognition of our panel of single HLA-class-I transduced K562 cells expressing 58% (n=37/64) of the HLA-class-I molecules present in the EBV-LCL panel(11). The scope our current study was not to fully unravel the recognized allogeneic peptide in allo-HLA molecules. However, this may demonstrate that allo-HLA cross-reactive T cells do not solely recognize an household peptide in the context of allogeneic HLA, but potentially also lineage-specific peptide-allo-HLA cross-reactivity exists(32). Also recognition of HLA-class-II molecules by HLA-class-I-restricted CD8^{pos} virus-specific T cells has previously been described(11). However, in our study we did not see a correlation with the pattern of recognition against the EBV-LCL panel and the expression of specific HLA-class-II molecules. Therefore, HLA-class-II-restricted cross-reactivity was not further analyzed in depth in our current study. Finding third-party donors with anti-viral T cells that are fully (HLA-class-I) matched to the recipient patients is probably difficult. When allo-HLA cross-reactive T cells targeting HLA alleles expressed on cells of the patient or (organ) donor are present in the virus-specific T-cell product, acute graft versus host disease (GVHD) or graft rejection could occur. Strikingly, only a very low incidence of *de novo* acute GVHD or graft rejection has been observed in clinical trials analyzing the effect of adoptive T-cell therapy with third-party donor-derived products, either in the setting of HLA-mismatched stem cell transplantation or of solid organ transplantation(18, 33). It has therefore been assumed

that third-party virus-specific T cells do not mediate GVHD or graft rejection(18). It is not clear whether, the observed absence of GVHD or graft rejection in these cases was the result of: 1) no expression of the particular mismatched HLA allele recognized by the transferred virus-specific T cells, 2) removal of the *in vitro* off-target (>10% cytotoxic) virus-specific T cells from the product prior to administration to the patient and/or selection of T-cell products that do not show *in vitro* allo-HLA reactivity(18), 3) extensive culturing of the virus-specific T cells prior to adoptive transfer, leading to senescence and impaired cytokine production(34), 4) weak adhesion molecule expression (i.e. ICAM-1) by the target organ(35), 5) biased production and administration of HLA-A*02:01-restricted virus-specific T cells with an intrinsic low risk of off-target toxicity, as shown in this study, 6) low T-cell numbers of cross-reactive virus-specific T cells administered and/or limited *in vivo* proliferation, or 7) rapid rejection of the virus-specific T cells(22)

Here, we demonstrated that around 40% of HLA-A*01:01, HLA-A*02:01 or HLA-B*07:02-restricted T-cell populations recognized one or more HLA-mismatched EBV-LCLs. For each T-cell population this recognition was found to be limited to only a few HLA-mismatched EBV-LCLs and could be attributed to recognition of one or a couple of allogeneic HLA alleles. The risk for accidentally mismatching for the particular allogeneic HLA allele(s) cross-recognized by the virus-specific T cells would be low, but studies do report cases of GVHD after infusion of virus-specific T cells derived from the SCT donor(36, 37) or derived from a third-party donor(37-39). Importantly, we found that HLA-B*08:01-restricted T cells isolated from donors that were homozygous for HLA-B*08:01 or heterozygous for HLA-B*08:01 and a specific HLA-B allele (e.g. HLA-B*13:02) showed abundant allo-HLA cross-reactivity *in vitro* and are therefore likely to cause graft rejection or GVHD *in vivo*. Since in the majority of studies so far, the adoptive transfer of third-party donor-derived virus-specific T cells was focused on HLA-A*02:01- and/or HLA-B*07:02-restricted virus-specific T cells(40), the effect of HLA-B*08:01-restricted virus-specific T cells has not been extensively studied(41). Our results on the higher incidence of HLA-cross-reactivity by HLA-B*08:01-restricted compared to HLA-A*01:01, HLA-A*02:01 or HLA-B*07:02-restricted virus-specific T cells may have important value for the design of future clinical trials. Since the specificity did not contribute to the allo-HLA cross-reactivity, these results have also important value for third-party derived CAR-T cell therapies or in the field of organ transplantations. Intriguingly, studies in the field of organ transplantations show a significant increase of acute graft rejections in recipients that express HLA-B*08:01, HLA-C*07:01 and HLA-DRB1*03:01(42, 43). These three HLA molecules are part of a common haplotype(44) and the homozygous donors used in our study have the same haplotype, suggesting that these rejections are mediated by HLA-B*08:01-restricted T cells. Altogether, these results imply that the HLA background of the donor is important for the broadness of the allo-HLA cross-reactivity. Therefore, the most compatible HLA background of the donor should be aimed for and homozygous donors should not be used despite the lower chance of rejection.

Since we only analyzed virus-specific T cells restricted to four different HLA molecules, it remains unclear whether T cells with another HLA restriction could show similar reactivity patterns as HLA-B*08:01-restricted T cells. However, we hypothesize that these findings might only be restricted to a few HLA molecules since the peptidome of HLA-B*08:01 shows an unique pattern, that is specific for only HLA-B*08:01 and HLA-B*08:02. Based on binding data and sequence information, Sidney J. et al classified the majority of HLA-B molecules into 9 super families(45). We hypothesized that super families with only a few HLA-B alleles, have unique peptidomes and T cells with this specific HLA background are likely to be cross-reactive against HLA molecules from other HLA super families, since negative thymic selection for these peptide-HLA complexes has not taken place. In the present study, virus-specific T cells isolated from donors that expressed HLA-B*08:01 and HLA-B*35:01 proved to be less allo-HLA cross-reactive than those from donors that were homozygous for HLA-B*08:01 or heterozygous for HLA-B*08:01 and HLA-B*13:02. We hypothesize that HLA-B*35:01 may elicit thymic negative selection for all HLA molecules present in the B07 superfamily to which it belongs (e.g. HLA-B*07:02, HLA-B*35:03, HLA-B*42:01). Being heterozygous for any of the HLA molecules from this B07 superfamily would then presumably result in the same outcome as heterozygosity for HLA-B*35:01. HLA-B*13:02 could not be assigned to a particular HLA superfamily(45), possibly explaining why it did little to the level of allo-HLA cross-reactivity of the HLA-B*08:01-restricted repertoire in our study. Therefore, if full matching for HLA-B is not possible, we propose that donors should be used that express HLA-B molecules that are part of different superfamilies to reduce the chance for a broad off-target toxicity in clinical application of third-party donor-derived T-cell products.

Altogether, our results indicate that selection of virus-specific T-cells with specific HLA restrictions and donors with specific HLA backgrounds may decrease the risk of developing GvHD or (organ) graft rejection after infusion of third-party donor-derived virus-specific T cells into patients with uncontrolled viral reactivation. Ideally, if complete HLA-class-I matching is not feasible, donor and recipient should at least be fully matched for HLA-B or matched for HLA-B alleles from the same HLA-B superfamily. Mismatching of HLA-B alleles that are unclassified should be avoided, because the peptides presented by these HLA-molecules are unique and could mediate allo-HLA cross-reactivity.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Peptide-MHC-tetramers used for the isolation of virus-specific T-cell populations.

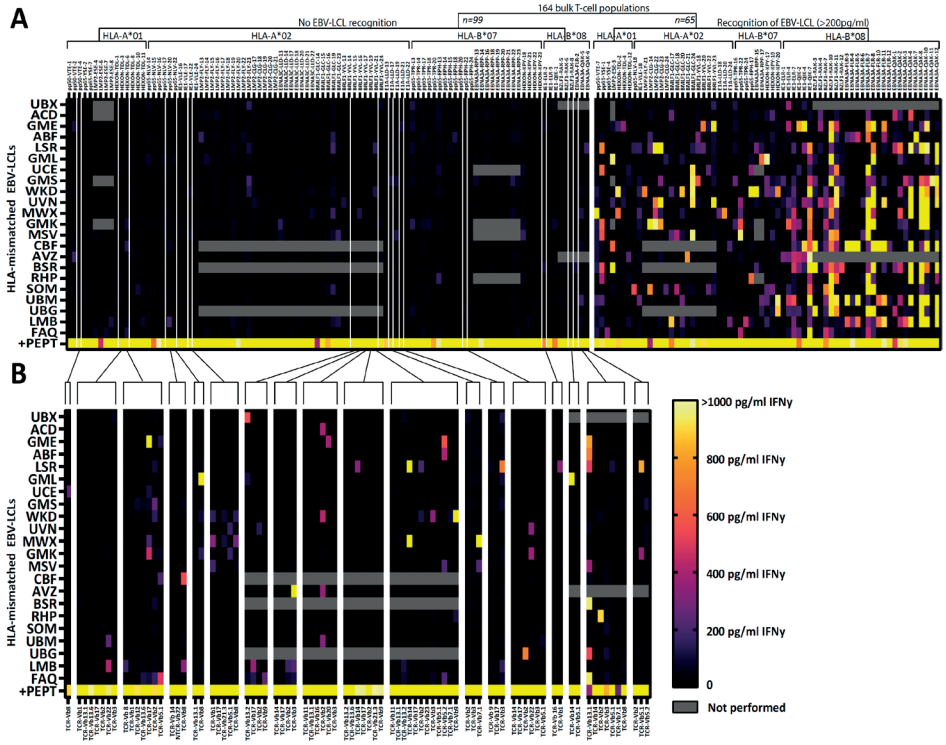
Virus	Antigen	HLA	Peptide
CMV	pp50	HLA-A*01:01	VTEHDTLLY
	pp65	HLA-A*01:01	YSEHPTFTSQY
	pp65	HLA-A*02:01	NLVPMVATV
	IE-1	HLA-A*02:01	VLEETSVML
	pp65	HLA-B*07:02	TPRVTGGGAM
	pp65	HLA-B*07:02	RPHERNGFTVL
	IE-1	HLA-B*08:01	ELRRKMMYM
	IE-1	HLA-B*08:01	QIKVRVDMV
EBV	LMP2	HLA-A*01:01	ESEERPPTPY
	LMP2	HLA-A*02:01	FLYALALLL
	LMP2	HLA-A*02:01	CLGGLLTMV
	EBNA3C	HLA-A*02:01	LLDFVRFMGV
	BMLF1	HLA-A*02:01	GLCTLVAML
	BRLF1	HLA-A*02:01	YVLDHLIVV
	EBNA3A	HLA-B*07:02	RPPIFIRRL
	BZLF1	HLA-B*08:01	RAKFKQLL
	EBNA3A	HLA-B*08:01	FLRGRAYGL
	EBNA3A	HLA-B*08:01	QAKWRLQTL
AdV	HEXON	HLA-A*01:01	TDLGQNLLY
	E1A	HLA-A*02:01	LLDQLIEEV
	HEXON	HLA-B*07:02	KPYSGTAYNAL

CMV, Cytomegalovirus; EBV, Epstein-Barr virus; AdV, Adenovirus

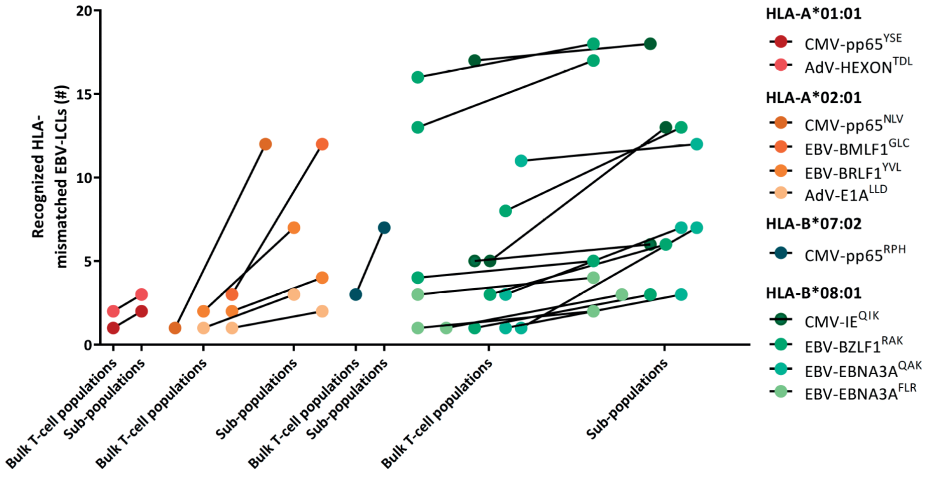
Supplementary Table 2. Single HLA-class-I transduced K562 cell-lines.

HLA-A	HLA-B	HLA-C
01:01	07:02	01:02
02:01	08:01	03:03
02:05	13:02	05:01
02:60	14:02	06:02
03:01	15:01	07:01
11:01	15:02	07:02
23:01	27:05	14:02
24:02	35:01	16:01
30:02	35:03	
32:01	35:231	
33:01	38:01	
68:01	39:01	
	40:01	
	41:01	
	44:02	
	44:03	
	51:01	
	52:01	
	55:01	
	57:01	
n=12	*n=20*	*n=8*

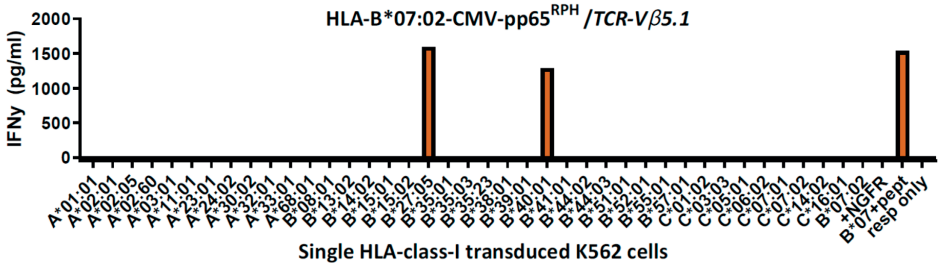
The K562 panel was composed of HLA-deficient K562 cells retrovirally transduced with constructs encoding specific HLA-class-I alleles. All HLA-constructs and transduced K562 cell-lines were checked by Sanger sequencing.



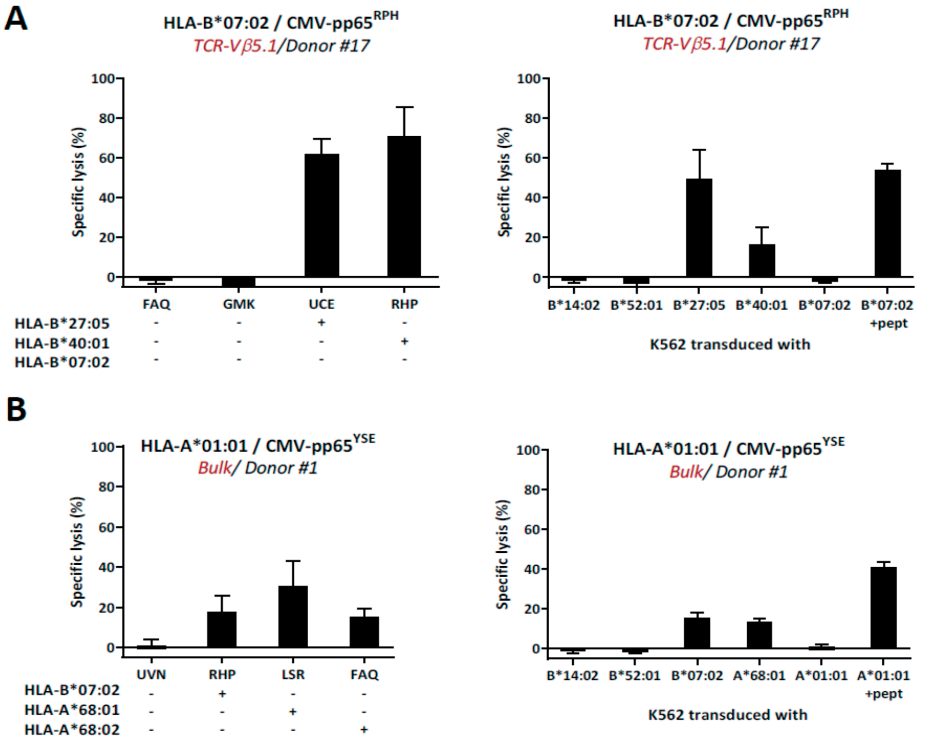
Supplementary Figure 1. Virus-specific T-cell populations show profound and diverse cross-reactivity against a panel of HLA-mismatched EBV-LCLs. Virus-specific T-cell populations were stimulated with a panel of HLA-mismatched EBV-LCLs for 16hrs and IFNγ production was measured by ELISA. Reactivity was defined as production of >200pg/ml IFNγ. EBV-specific T-cell populations were tested only against those HLA-mismatched EBV-LCLs that did not express the specific restriction molecule of the viral specificity of those T cells. EBV-LCLs from donor OBB exogenously loaded with 10^{-6}M of the respective viral peptide were used as positive control. **A)** The results of cross-reactivities against HLA-mismatched EBV-LCLs mediated by bulk virus-specific T cells is shown and divided in bulk virus-specific T-cell populations that did not show any cross-reactivity (<200pg/ml IFNγ, left) and bulk virus-specific T-cell populations that showed profound and diverse cross-reactivity against the HLA-mismatched EBV-LCLs (right). The different bulk virus-specific T-cell populations are shown on the x-axis clustered per HLA-restriction and their specificity and origin are shown as protein-peptide-donorID. **B)** Shown are virus-specific T cells that were sorted for expression of a single TCR-Vβ family and that were stimulated with HLA-mismatched EBV-LCLs and demonstrated recognition of HLA-mismatched EBV-LCLs, while the original bulk virus-specific T-cell population did not show any recognition. The x-axis shows the different TCR-Vβ family expression of the sorted T-cell populations.
TCR, T-cell Receptor. Vβ, Variable Beta Chain.



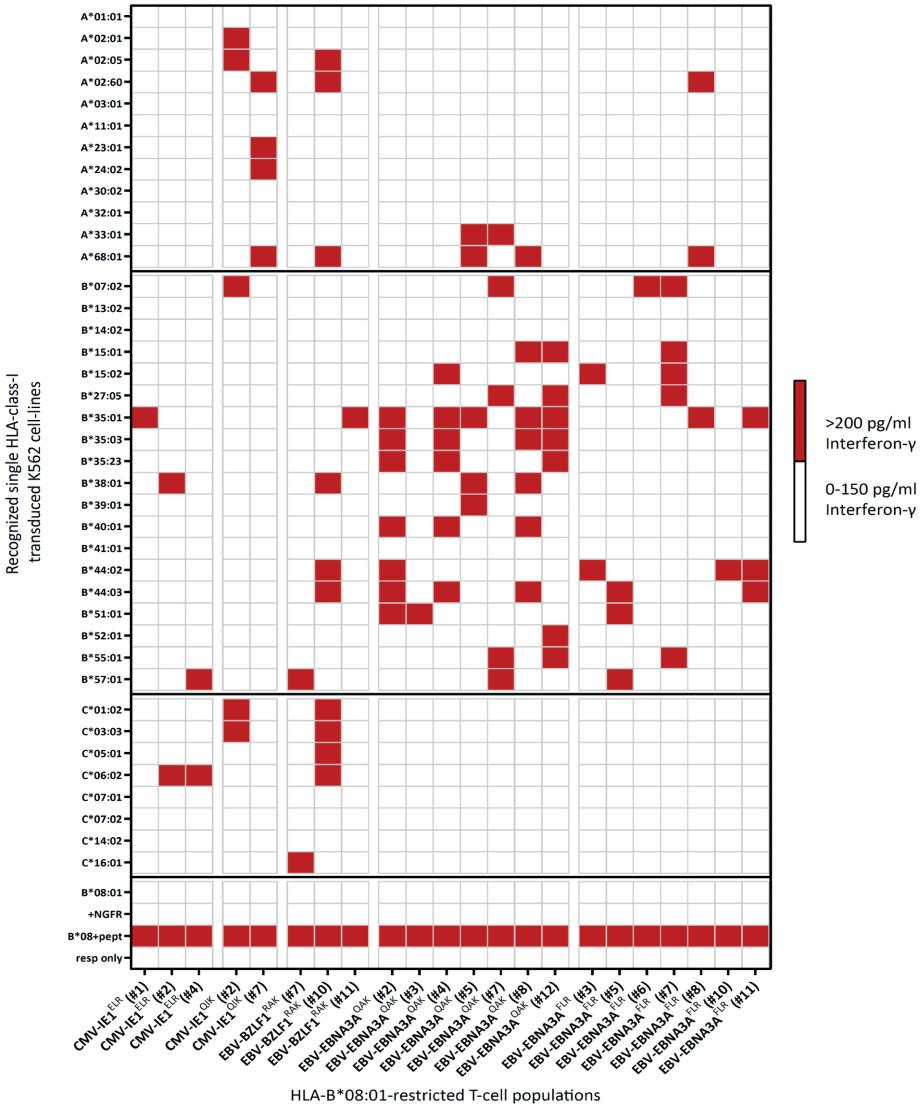
Supplementary Figure 2. Recognition of additional HLA-mismatched EBV-LCLs when sub-populations expressing single TCR-Vβ families were included in the analysis. T-cell populations expressing a single TCR-Vβ family were sorted from the bulk virus-specific T-cell populations and stimulated with a panel of HLA-mismatched EBV-LCLs for 16hrs and IFNγ production was measured by ELISA. Recognition of HLA-mismatched EBV-LCLs was defined as production of >200pg/ml IFNγ by the virus-specific T cells. Shown are 25 bulk T-cell populations from which sub-populations expressing single TCR-Vβ families were sorted that demonstrated additional recognition of HLA-mismatched EBV-LCLs.



Supplementary Figure 3. Cross-reactivity of HLA-B*07:02-restricted CMV-pp65^{RPH} T cells. The CMV-pp65^{RPH}-specific T-cell population was stimulated with a panel of K562 cells transduced with HLA molecules to investigate which other HLA-molecules were recognized based on the reactivities seen against the EBV-LCL panel. K562 cells transduced with HLA-B*07:02 exogenously loaded with 10^{-6}M of the respective viral peptide was used as positive control.



Supplementary Figure 4. Allo-HLA cross-reactive virus-specific T cells lyse HLA mismatched target cells. To investigate whether the allo-HLA cross-reactivity of virus-specific T cells also results in cytotoxicity, virus-specific T-cell lines were tested in cytotoxicity assays against HLA-mismatched EBV-LCLs and single HLA-class-I transduced K562 cell-lines. Two representative examples are shown. **A)** The allo-HLA-B*27:05/HLA-B*40:01 cross-reactive HLA-B*07:02-restricted TCR-Vβ5.1 sorted CMV-pp65^{RPH}-specific T-cell population derived from donor #17 was tested against 4 HLA-mismatched EBV-LCLs and 5 single HLA-class-I transduced K562 cell-lines **B)** The allo-HLA-A*68/HLA-B*07:02 cross-reactive HLA-A*01:01-restricted CMV-pp65^{YSE}-specific T-cell population derived from donor #1 was tested against 4 HLA-mismatched EBV-LCLs and 5 single HLA transduced K562 cell lines (HLA-B*14:02, HLA-B*52:01, HLA-A*68:01, HLA-B*07:02 and peptide "YSE" pulsed HLA-A*01:01).
Shown are means with standard deviations of 1 experiment carried out in triplicate, with an effector: target (E:T) ratio of 10:1.
TCR, T-cell Receptor. Vβ, Variable Beta Chain



Supplementary Figure 5. Skewed cross-reactivity against certain allogeneic HLA-B alleles by HLA-B*08:01-restricted virus-specific T cells. HLA-B*08:01-restricted T-cell populations (n=22) with no clear recognition pattern when tested against the HLA-mismatched EBV-LCL panel, were stimulated with a panel of single HLA-class-I transduced K562 cell-lines (n=40) for 16hrs and IFNγ production was measured by ELISA to analyze which HLA molecules were being recognized. Reactivity was defined as production of >200pg/ml IFNγ. Shown are the specific allogeneic HLA-A, B or C molecules (y-axis) recognized by the HLA-B*08:01-restricted T-cell populations directed against different CMV or EBV epitopes (x-axis). The numbers between brackets represent the identification numbers of the donors from which the respective T-cell population originate.


BENEFITS

CHAPTER 4

Public T-cell receptors (TCRs) revisited by analysis of the magnitude of identical and highly-similar TCRs in virus-specific T-cell repertoires of healthy individuals

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ABSTRACT

Since multiple different T-cell receptor (TCR) sequences can bind to the same peptide-MHC combination and the number of TCR-sequences that can theoretically be generated even exceeds the number of T cells in a human body, the likelihood that many public identical (PUB-I) TCR-sequences frequently contribute to immune responses has been estimated to be low. Here, we quantitatively analyzed the TCR-repertoires of 190 purified virus-specific memory T-cell populations, directed against 21 epitopes of Cytomegalovirus, Epstein-Barr virus and Adenovirus isolated from 29 healthy individuals, and determined the magnitude, defined as prevalence within the population and frequencies within individuals, of PUB-I TCR and of TCR-sequences that are highly-similar (PUB-HS) to these PUB-I TCR-sequences. We found that almost one third of all TCR nucleotide-sequences represented PUB-I TCR amino-acid (AA) sequences and found an additional 12% of PUB-HS TCRs differing by maximally 3 AAs. We illustrate that these PUB-I and PUB-HS TCRs were structurally related and contained shared core-sequences in their TCR-sequences. We found a prevalence of PUB-I and PUB-HS TCRs of up to 50% among individuals and showed frequencies of virus-specific PUB-I and PUB-HS TCRs making up more than 10% of each virus-specific T-cell population. These findings were confirmed by using an independent TCR-database of virus-specific TCRs. We therefore conclude that the magnitude of the contribution of PUB-I and PUB-HS TCRs to these virus-specific T-cell responses is high. Because the T cells from these virus-specific memory TCR-repertoires were the result of successful control of the virus in these healthy individuals, these PUB-HS TCRs and PUB-I TCRs may be attractive candidates for immunotherapy in immunocompromised patients that lack virus-specific T cells to control viral reactivation.

INTRODUCTION

Human virus-specific $CD8^{pos}$ T cells express heterodimeric alpha(α)/beta(β) TCRs that can specifically recognize viral peptides presented by HLA-class-I molecules(1). The TCRα- and the TCRβ-chain repertoires are highly variable due to the genetic recombination process involved in their generation. For the TCRβ-chains, recombination of 1 of 48 functional T-cell Receptor Beta Variable (TRBV), 1 of 2 functional T-cell receptor Beta Diversity (TRBD) and 1 of 12 functional T-cell Receptor Beta Joining (TRBJ) gene segments leads to a V-D-J reading frame(2). The TCRα-chains are generated by a similar recombination process with the exception of a diversity gene, resulting in a V-J reading frame(3). Insertion of template-independent nucleotides between the recombined segments (junctional region) results in a significant further increase in variability(4). The sequence around these junctions encodes for the Complementary Determining Region 3 (CDR3), a loop that reaches out and interacts with a peptide embedded in an HLA molecule, together with the loops of the CDR1 and CDR2 regions, which are fixed within the germline variable gene sequence(5, 6). It has been calculated that these gene rearrangements could potentially generate a repertoire of 10^{15}-10^{20} unique TCRs that may interact with all possible peptide-HLA complexes(7).

Pathogenic viruses like Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Adenovirus (AdV) can infect humans for life by staying latently present in target cells after a primary infection. In healthy individuals these latent viruses are controlled by the virus-specific T cells. As a result, reactivations of these latent viruses are observed frequently, but do not result in severe virus-associated disorders like malignancies and/or organ failure. However, in the absence of a competent immune system, these latent viruses remain uncontrolled and are associated with high-morbidity and mortality in immune-compromised patients, including patients after stem cells or organ tranplantation(8, 9). To control these viruses, antigen-experienced (central-memory and effector-memory) virus-specific T cells have to develop from the naïve T-cell repertoire. Due to the high diversity of the naïve T-cell repertoire(10), T-cell responses against the many potential viral epitopes presented in multiple HLA alleles may be composed of a large variety of different TCRs. Indeed, when naïve umbilical cord blood-derived T cells were stimulated *in vitro* to generate *de novo* responses against proteins from CMV or Human Immunodeficiency Virus (HIV), this resulted in responding virus-specific T-cell populations with a highly diverse repertoire of TCRs, recognizing many different CMV(11) or HIV-derived peptides(12). However, from *ex vivo* analyses in adults it became clear that *in vivo* the virus-specific memory T-cell populations are shaped during control and clearance of the infection and target only a limited number of viral-peptides, as was shown for T-cell populations specific for viruses like CMV(13), EBV(14), AdV(15), Influenza A(16) and also more recently SARS-Cov-2(17, 18). Nevertheless, the multiple viral-peptides that are targeted in the

various HLA alleles make it theoretically unlikely that individuals would frequently share exactly the same virus-specific TCR, unless T cells expressing certain TCRs would favor control of infections and would therefore dominate the responses. Since latent viruses like CMV, EBV and AdV are not fully eradicated, reactivations are frequent and trigger the expansion of antigen-experienced virus-specific memory T cells. This unique biology might contribute to the favor and skewing of specific TCRs expressed by T cells that dominate the response and control the virus.

Evidence for selection of certain virus-specific TCR-expressing T cells in controlling viruses has come from several reports identifying identical TCR amino-acid (AA) sequences in dominant virus-specific memory T-cell populations in different individuals, designated as public TCR-sequences (from here on referred to as public-identical (PUB-I) TCR-sequences). Most studies investigated the presence of PUB-I TCR-sequences in TCRβ-chains, since this is the most diverse TCR chain and the CDR3β sequence of CD8 T cells is positioned to interact with the antigenic peptide presented by HLA-class-I molecules. However, dominant virus-specific memory T-cell populations with PUB-I TCRα chains have also been described previously(19). Such PUB-I TCR sequences are most overtly observed in antigen-experienced memory virus-specific T cells due to the *in vivo* antigen-driven proliferation, but are also present within the naïve T-cell compartment, although at low frequency(20). PUB-I TCRβ sequences have been found in T-cell populations specific for latent viruses like CMV-specific T-cell responses(21-23), EBV-specific T-cell responses(24, 25), but also for non-latent viruses like Influenza-specific T-cell responses(16), respiratory syncytial virus-specific T-cell responses(26) and SARS-Cov-2-specific T-cell responses(17, 27). In addition, some of these virus-specific T-cell populations also contained TCR AA-sequences that were highly-similar to the identical shared TCR AA-sequence (from here on referred to as highly-similar to PUB-I (PUB-HS) TCR-sequences). However, the magnitude, defined as prevalence within the population and frequencies within virus-specific T-cell repertoires, of PUB-I and PUB-HS TCR-sequences is not known. A high probability to be generated during V-D-J recombination may play a role(28), but since virus-specific memory T-cell repertoires in the circulation are shaped based on antigen encounter and subsequent proliferation, the PUB-I and PUB-HS TCR-sequences most likely reflect highly functional T cells capable of antigen-driven proliferation.

We hypothesize that frequent induction of antigen-driven proliferation of virus-specific T cells targeting frequently reactivating latent viruses will increase the prevalence and frequencies of PUB-I and PUB-HS TCR-sequences within the repertoire of antigen-experienced virus-specific T-cells. Molecular analysis of these TCRs will add in the analysis of the development, presence and quality of memory T-cell responses, and tracking of virus-specific T-cell responses. Furthermore, Identification of dominant TCRs

with shared core-sequences may be utilized for the design of future immunotherapy purposes including TCR-gene transfer. Therefore, the aim of our study was to quantitively analyze the magnitude of PUB-I and PUB-HS TCRβ-sequences within the antigen-experienced virus-specific TCR-repertoires of CMV, EBV and AdV-specific $CD8^{pos}$ memory T cells. We confirmed that healthy individuals generate many different virus-specific TCRs, illustrated by the >3000 TCR nucleotide-sequences that were found *ex vivo* in virus-specific memory T-cell populations. However, a significant part of the virus-specific TCR-repertoires contained PUB-I and PUB-HS TCR nucleotide-sequences. The AAs of these PUB-HS TCRs varied on specific positions in the CDR3β-region, while maintaining a conserved core-AA-sequence that was also present in the respective PUB-I TCR.. We identified conserved TCR core-AA-sequences for each specificity that could be used for diagnostic purposes looking at anti-viral immune responses. Additionally, PUB-I or PUB-HS TCRs with the highest frequencies in healthy individuals may be utilized to develop off-the-shelf immunotherapeutics (using TCR-gene transfer) to effectively control CMV, EBV or AdV-infections or reactivations in immunocompromised patients.

MATERIALS AND METHODS

Collection of donor material

After informed consent according to the Declaration of Helsinki, healthy individuals (homozygously) expressing HLA-A*01:01 and HLA-B*08:01 or HLA-A*02:01 and HLA-B*07:02 were selected from the Sanquin database and the biobank of the department of Hematology, Leiden University Medical Center (LUMC). Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Isopaque separation and used directly or thawed after cryopreservation in the vapor phase of liquid nitrogen. Donor characteristics (HLA typing, CMV and EBV serostatus) are provided in **Table 1**.

Generation of peptide-MHC complexes to isolate virus-specific T cells

All viral peptides were synthesized in-house using standard Fmoc chemistry. Recombinant HLA-A*01:01, HLA-A*02:01, HLA-B*07:02 and HLA-B*08:01 heavy chain and human β2m light chain were in-house produced in Escherichia coli. MHC-class-I refolding was performed as previously described with minor modifications(29). Major histocompatibility complex (MHC)-class-I molecules were purified by gel-filtration using HPLC. Peptide-MHC(pMHC) tetramers were generated by labeling biotinylated pMHC-monomers with streptavidin-coupled phycoerythrin (PE; Invitrogen, Carlsbad, USA), allophycocyanin (APC, Invitrogen), brilliant violet 421 (BV421, Becton Dickinson (BD), Franklin Lakes, USA), brilliant violet 510 (BV510, BD) or peridinin-chlorophyll-protein complex (PerCP, Invitrogen). Complexes were stored at 4 °C. Formation of stable pMHC-monomers was performed using UVexchange technology(30) and according to a

previously described protocol(31).

Isolation and expansion of virus-specific T cells

Phycoerythrin (PE), allophycocyanin (APC), BV421, BV510 and/or peridinin-chlorophyll-protein (PerCP)-labeled pMHC-tetramer complexes were used for fluorescence-activated cell sorting (FACSorting). The pMHC-tetramers used are shown in Table 2. Per specificity, $30*10^6$ PBMCs were first incubated with pMHC-tetramers at 4°C for 30 min, followed by labeling with APC-H7 CD8 (BD) and fluorescein isothiocyanate-labeled (FITC) CD4 and CD14 (BD) antibodies at 4°C for 30 min. PeptideMHC-tetramer positive, $CD8^{pos}/CD4^{neg}$ T cells were FACsorted and seeded at 10,000 cells per well in U-bottom microtiter plates for the generation of bulk T-cell populations (**Supplementary Figure 1**). Peptide-MHC-tetramer^{pos} virus-specific T cells, targeting a single antigen, were first specifically expanded in the presence of 10^{-7}M of the specific peptide in T-cell medium: Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Verviers, Belgium) containing 5% heat-inactivated fetal bovine serum (FBS; Invitrogen), 5% heat-inactivated human serum (ABOS; Sanquin Reagents, Amsterdam, The Netherlands), 100U/mL penicillin (Lonza), 100µg/mL streptavidin (Lonza) , 2.7mM L-glutamine (Lonza), and 100IU IL-2/mL (Chiron, Emeryville, USA) and with 5-fold 35 Gy irradiated autologous PBMCs as feeder cells. Initial specific stimulation and expansion with 10^{-6}M peptide was performed to stimulate preferential outgrowth of pMHC-tetramer^{pos} T cells. After two weeks of culture, pMHC-tetramer^{pos} T-cell populations were qualified as pure populations when they contained ≥97% pMHC-tetramer^{pos} cells. Sorting was performed on a FACS ARIA (BD) and analyzed using Diva software (BD). All analyses were performed on a FACS Calibur (BD), and analyzed using Flowjo Software (TreeStar, Ashland, USA).

TCRβ-library preparation

TCRβ-sequences were identified using ARTISAN PCR adapted for TCR PCR(32, 33). Total mRNA was extracted from 190 pMHC-tetramer^{pos} purified (**Supplementary Figure 2A**) virus-specific T-cell populations(34) using magnetic beads (Dynabead mRNA DIRECT kit; Invitrogen, Thermo Fisher Scientific). Ten µl (~1µg) of mRNA per sample was mixed with TCRβ constant region-specific primers (1µM final concentration) and SmartSeq2modified template-switching oligonucleotide (SS2m_TSO; 1µM final concentration) and denatured for 3 minutes at 72°C. After cooling, cDNA was synthesized for 90 minutes at 42°C with 170 U SMARTscribe reverse transcriptase (Takara, Clontech) in a total volume of 20µl containing 1.7U/µl RNasin (Promega), 1.7mM DTT (Invitrogen, Thermo Fisher Scientific), 0.8mM each of high-purity RNAse-free dNTPs (Invitrogen, Thermo Fisher Scientific) and 4µl of 5x first-strand buffer. During cDNA synthesis, a non-templated 3'polycytosine terminus was added (**Supplementary Figure 2B**), which created a template for extension of the cDNA with the TSO(35). PCR (2min at 98°C followed by 40 cycles of [1s at 98°C, 15s at 67°C, 15s at 72°C], 2 min at 72°C) of 5µl of cDNA was then performed using

Phusion Flash (Thermo Fisher Scientific) with anchor-specific primer (SS2m_For; 1µM final concentration) and each (1µM final concentration) of the nested primers specific for the constant regions of TCRβ constant 1 and TCRβ constant 2. Both forward and reverse PCR primers contained overhanging sequences suitable for barcoding. Amplicons were purified and underwent a second PCR (2min at 98°C followed by 10 cycles of [1s at 98°C, 15s at 65°C, 30s at 72°C], 2 min at 72°C) using forward and reverse primers (1µM final concentration) with overhanging sequences containing identifiers (sequences of 6 base-pairs) and adapter sequences appropriate for Illumina HiSeq platforms (or PacBio; Pacific Biosciences). Unique identifiers were used for each T-cell population targeting one epitope. Forward or reverse identifiers were shared between T-cell populations targeting different epitopes. For all primer sequences see **Supplementary Table 1**. For identifier sequences see **Supplementary Table 2**. Amplicons with identifiers were purified, quantified and pooled into one library for paired-end sequencing of 150bp on an Illumina HiSeq4000. Deep sequencing was performed at GenomeScan (Leiden, The Netherlands). Raw data were de-multiplexed and aligned to the matching TRBV, TRBD, TRBJ and constant (TRBC) genes. CDR3β-sequences were built using MIXCR software using a bi-directional approach (5'-3' and 3'-5' read)(36). CDR3β-sequences with a stop-codon were removed from the library. Bi-directional readings using MIXCR could result in out-of-frame CDR3β AA-sequences due to the even number of nucleotides. These sequences (n=392) were manually aligned with the germline TRBV and TRBJ-sequence. CDR3β-sequences were further processed using custom scripts in R to compare specificities and sharing of CDR3β-sequences.

Computational unbiased repertoire analysis

The following R-packages were used in R-software to generate a nodal plot of CDR3β AA-sequences with the levenshtein distance as parameter for similarity: "igraph" to create network objects, obtain the degree of a node and its betweenness(37), "data.table" to organize CDR3β-sequences; "stringdist" to calculate Levenshtein distances(38), "Biostrings" for fast manipulation of large biological sequences or sets of sequences(39), "dplyr" to arrange and filter data(40), "tibble" for providing opinionated data frames, "ggplot2" for generating figures(41) and "RColorBrewer" to create graphics(42). A levenshtein distance of 0.25 was added to visualize multiple identical sequences. Nodes with identical sequences (levenshtein distance of 0.25) were manually replaced by pie-charts using Adobe Illustrator CC 2018.

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

#	Age	CMV	EBV	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ		HLA-DP	
1	62	Pos	Pos	01:01	30:01	08:01	13:02	07:01	06:02	03:01	07:01	02:01	02:02	04:01	09:01
2	48	Pos	Pos	01:01		08:01		07:01		03:01	01:02	02:01	05:01	01:01	04:01
3	53	Pos	Pos	01:01		08:01		07:01		03:01	15:01	02:01	06:02	N.D	
4	44	Pos	Pos	01:01		08:01		07:01		03:01		02:01		04:01	
5	65	Pos	pos	01:01	30:01	08:01	13:02	07:01	06:02	04:07	15:01	03:01	06:02	04:01	
6	26	Pos	Pos	01:01		08:01		07:01		03:XX		02:XX		N.D	
7	42	Pos	pos	01:01	30:01	08:01	13:02	07:01	06:02	03:01	04:01	02:01	03:01	04:01	
8	41	Pos	pos	02:01	24:02	08:01	35:01	07:01	11:01	02:02	03:01	02:02	03:01	02:01	13:01
9	27	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	
10	48	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	05:01
11	35	Neg	Pos	01:01		08:01		07:01		03:01		02:01		01:01	09:01
12	49	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	05:01
13	50	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	04:02/01
14	57	Pos	Pos	01:01	68:01	08:01	35:01	04:01	07:01	01:01	03:01	02	05:01	04:01	04:02
15	32	Neg	Pos	01:01		08:01		07:0		03:XX		02:XX		N.D	
16	25	Neg	pos	01:01	24:02	08:01	35:01	04:01	07:01	03:01	08:01	02:01	04:02	04:01	
17	38	Neg	Pos	01:01		08:01		07:01		03:XX		02:XX		N.D	
18	N.K.	Pos	Pos	02:01		07:02	44:02	07:02	05:01	15:01	04:01	06:02	03:01	04:XX	02:01
19	64	Pos	Pos	02:01		07:02		07:02		15:01		06:02		04:01	
20	33	Pos	Pos	02:01	03:01	07:02		07:02		15:01		06:02		04:01	03:01
21	53	Pos	Pos	02:01		07:02		07:02		15:01		06:02		04:01	05:01
22	62	Pos	Pos	02:01		07:02		07:02		15:01		06:02		02:01	04:01
23	43	Pos	Pos	02:01		07:02		07:02		15:01		06:02		04:01	
24	50	Pos	Pos	02:01	03:01	07:02	44:02	07:02	05:01	15:01	01:01	06:02	05:01	04:01	14:01
25	50	Pos	Pos	02:01		07:02		07:02		07:01	15:01	03:03	06:02	04:01	13:01
26	63	Pos	Pos	02:01		07:02		07:02		15:XX		06:XX		N.D	
27	29	Neg	Pos	02:01		07:02		07:02		15:01		06:02		04:01	

Table 1. Continued.

#	Age	CMV	EBV	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ		HLA-DP	
28	26	Neg	Pos	02:01		07:02		07:02		15:01		06:02		04:01	13:01
29	52	Neg	Pos	02:01		07:02		07:02		15:XX		06:XX		N.D	

Virus-specific T cells restricted to HLA-A*01:01/HLA-B*08:01 or HLA-A*02:01/HLA-B*07:02 were isolated from donors #1-17 and donors #18-29, respectively. CMV and EBV serostatus and age are indicated for each donor. HLA typing was determined either by serology, where the second digits could not be determined (XX), or with high resolution HLA typing unless indicated by N.D.. Blanks indicate homozygosity for the given allele. For one of the unrelated stem cell donors the age was not known (N.K.).

Sequence logo plots

To identify which positions of PUB-I and PUB-HS CDR3β AA-sequences were conserved and which were variable, all CDR3β AA-sequences with the most frequent CDR3 length were included and the AAs were stacked for each position in the sequence. The overall height of the stacks indicates the sequence conservation at that position, while the height of symbols within the stacks indicates the relative frequency of each AA at that position. AAs have colors according to their chemical properties; polar AAs (G, S, T, Y, C, Q, N) show as green, basic (K, R, H) blue, acidic (D, E) red, and hydrophobic (A, V, L, I, P, W, F, M) AAs as black(43).

Generation of independent TCR-database from virus-specific T-cell products generated for a clinical trial

As an independent TCR-database containing TCR-sequences from virus-specific T cells, we used the information obtained from the virus-specific T-cell products generated in the context of the phase I/II safety and feasibility study T Control (EudraCT-number 2014-003171-39) using the MHC-I-Streptamer isolation technology (Juno Therapeutics, Munich, Germany)(20, 44). Sequencing was performed as described above for all virus-specific T-cell populations per donor, resulting in unique identifiers for all virus-specific T-cell populations in the TCRβ-library.

Data deposition

TCR-Sequencing data is deposited to the Sequence Read Archive (SRA); submission: SUB10993301 (access to bioproject: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA803981)

RESULTS

Generation and validation of a library of TCR-sequences derived from FACsorted virus-specific T-cell populations

To examine the composition of the virus-specific TCR-repertoires in different individuals, the CDR3β-regions of purified expanded pMHC-tetramer-binding virus-specific T-cell populations were sequenced (**Supplementary Figure 2**). We analyzed the TCR-repertoires of CMV, EBV and AdV-specific T cells, restricted to four prevalent HLA alleles (HLA-A*01:01, HLA-A*02:01, HLA-B*07:02 and HLA-B*08:01) and specific for 21 different peptides (**Table 2**). Purified CMV, EBV and AdV-specific T-cell populations targeting CMV (n=8), EBV (n=10) or AdV (n=3)-derived peptides were isolated from 17 HLA-A*01:01/B*08:01^{pos} individuals and 12 HLA-A*02:01/B*07:02^{pos} individuals (**Table 1 and Table 2**). In total, 190 virus-specific T-cell populations, each targeting a single viral epitope, were successfully isolated and showed high purity (>97% pMHC-tetramer positive).

The mean precursor frequencies of the different T-cell specificities in the starting PBMC materials are shown in **Supplementary Figure 3**. Sequencing of the CDR3β-regions of these virus-specific T-cell populations resulted in 3346 CDR3β nucleotide-sequences that occurred at frequencies of more than 0.1%. 135 of these nucleotide-sequences were present at high frequencies (>5%) within one specificity, but were also found at low frequencies (around 0.1%) in another specificity, indicating contamination due to FACSorting impurities. These low frequency nucleotide-sequences were discarded from further analysis. 41 nucleotide-sequences were present at low frequencies in two unrelated specificities and could not be correctly annotated and these 41 duplicates (n=82) were also discarded from the analysis. Therefore, a total of 3129 nucleotide-sequences could be annotated. In total, 2224 (71%) of these nucleotide-sequences represented unique CDR3β AA-sequences that were found in only one individual and 905 nucleotide-sequences (29%) resulted in 131 different PUB-I CDR3β AA-sequences that were found in two or more unrelated individuals (**Flowchart; Figure 1**).

Table 2. Number of isolated virus-specific T-cell populations

				# of isolated T-cell populations / # of attempted isolations (%)		
Virus	**Antigen**	**HLA**	**Peptide**	**Per specificity**	**Per virus**	**Per HLA**
CMV	pp50	HLA-A*01:01	VTEHDTLLY	7/8 (88%)	**CMV:** 53/70 *(76%)*	**HLA-A*01:01:** 29/39 *(74%)*
	pp65	HLA-A*01:01	YSEHPTFTSQY	6/8 (75%)		
	pp65	HLA-A*02:01	NLVPMVATV	8/9 (89%)		
	IE-1	HLA-A*02:01	VLEETSVML	5/9 (56%)		
	pp65	HLA-B*07:02	TPRVTGGGAM	8/9 (89%)		
	pp65	HLA-B*07:02	RPHERNGFTVL	8/9 (89%)		**HLA-A*02:01:** 70/90 *(78%)*
	IE-1	HLA-B*08:01	ELRRKMMYM	5/9 (56%)		
	IE-1	HLA-B*08:01	QIKVRVDMV	6/9 (67%)		
EBV	LMP2	HLA-A*01:01	ESEERPPTPY	4/6 (67%)	**EBV:** 111/129 (86%)	
	LMP2	HLA-A*02:01	FLYALALLL	11/12 (92%)		
	LMP2	HLA-A*02:01	CLGGLLTMV	10/12 (83%)		
	EBNA3C	HLA-A*02:01	LLDFVRFMGV	7/12 (58%)		**HLA-B*07:02**: 33/42 *(79%)*
	BMLF1	HLA-A*02:01	GLCTLVAML	10/12 (83%)		
	BRLF1	HLA-A*02:01	YVLDHLIVV	11/12 (92%)		
	EBNA3A	HLA-B*07:02	RPPIFIRRL	11/12 (92%)		
	BZLF1	HLA-B*08:01	RAKFKQLL	17/17 (100%)		
	EBNA3A	HLA-B*08:01	FLRGRAYGL	13/17 (76%)		**HLA-B*08:01:** 58/69 *(84%)*
	EBNA3A	HLA-B*08:01	QAKWRLQTL	17/17 (100%)		
AdV	HEXON	HLA-A*01:01	TDLGQNLLY	12/17 (71%)	**AdV:** 26/41 (63%)	
	E1A	HLA-A*02:01	LLDQLIEEV	8/12 (67%)		
	HEXON	HLA-B*07:02	KPYSGTAYNAL	6/12 (50%)		

Seventeen donors were used to isolate HLA-A*01:01/B*08:01-restricted virus-specific T cells and 12 donors were used to isolate HLA-A*02:01/B*07:02-restricted virus-specific T cells.
CMV, Cytomegalovirus; EBV, Epstein-Barr virus; AdV, Adenovirus

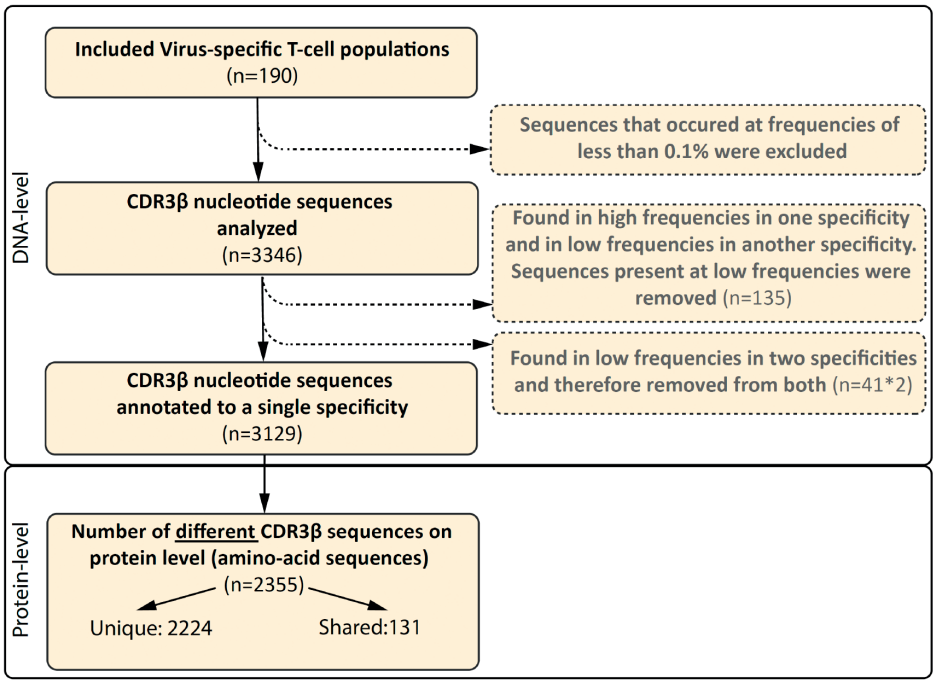

Figure 1. Flowchart of included and excluded CDR3β nucleotide and AA-sequences. In total, 190 different virus-specific T-cell populations were FACsorted using pMHC-tetramers, followed by a short-term *in vitro* stimulation. The CDR3β nucleotide-sequences were determined using next-gen Illumina sequencing. CDR3β nucleotide-sequences that occurred at a frequency of less than 0.1% in each sample were excluded. CDR3β nucleotide-sequences that were identical and present in two different specificities, but present at high frequencies in one specificity, were only removed from the specificity that contained the sequences at very low frequencies (0.1-0.5%; n=135). CDR3β nucleotide-sequences that were identical and present in two different specificities at low frequency were considered contamination and removed from the library (82 sequences, 41 different-sequences). The numbers of different CDR3β AA-sequences that were encoded by the CDR3β nucleotide-sequence are shown at protein level. We then assessed how many CDR3β-AA-sequences were found in multiple individuals (shared) and how many were only found in a single individual (unique).

To investigate the relationship between the numbers of CDR3β nucleotide-sequences and the translated number of CDR3β AA-sequences, we compared the nucleotide-sequences of the 131 PUB-I CDR3β AA-sequences found in different individuals. Different nucleotide-sequences can result in the same CDR3β AA-sequence, a phenomenon known as convergent recombination. We found that PUB-I CDR3β AA-sequences present at high (representative example**; Figure 2A**) or low frequencies (range 0.1-1%) (representative example; **Figure 2B**) could be encoded by different CDR3β nucleotide-sequences in the junctional regions of the CDR3β-regions in TCRs of T cells isolated from different individuals (**Figure 2C and 2D; Supplementary Figure 4**). Because the majority of nucleotide-sequences encoding the same CDR3β AA-sequences were different

between individuals, these data exclude contamination as an explanation for the finding of PUB-I CDR3β AA-sequences.

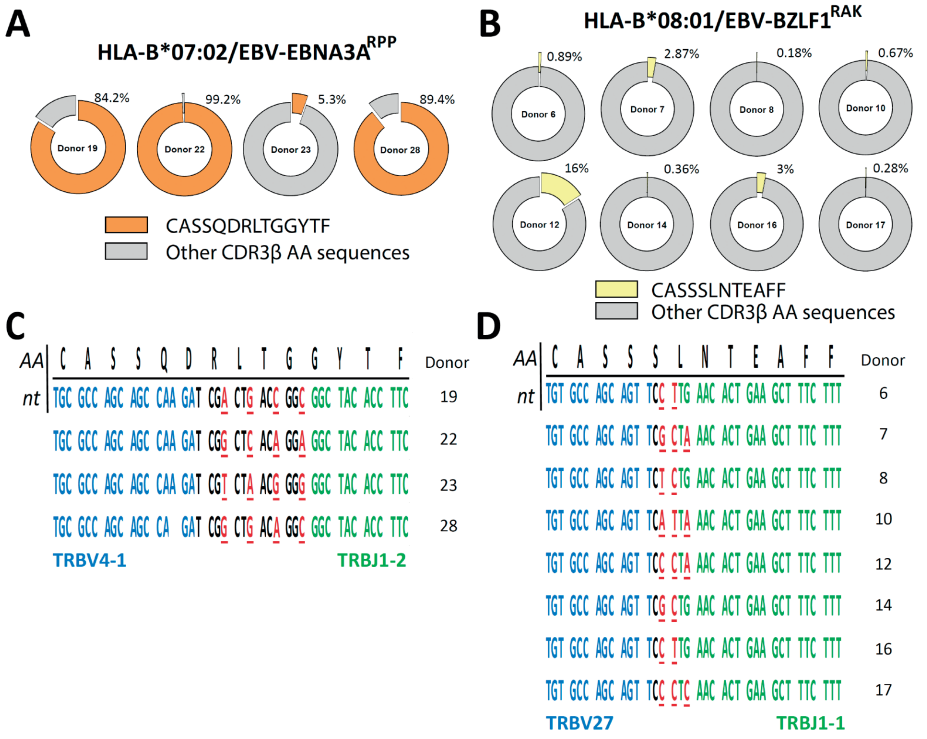

Figure 2. PUB-I CDR3β-sequences can be found in different individuals with small nucleotide-differences as a result of convergent recombination. The library of virus-specific CDR3β AA-sequences contained 131 sequences that were found in multiple different individuals (≥2). The nucleotide-sequences of these CDR3β AA-sequences were analyzed to investigate differences and similarities. **A and B**) Shown are two representative examples of the frequencies of the PUB-I CDR3β AA-sequences CASQDRLTGGYTF and CASSSLNTEAFF, that were specific for HLA-B*07:02-restricted EBV-EBNA3A^{RPP} and HLA-B*08:01-restricted EBV-BZLF1^{RAK}, respectively. **C and D**) Shown are the nucleotide-sequences of the CDR3β AA-sequences CASSQDRLTGGYTF and CASSSLNTEAFF that were shared by different individuals. Underlined nucleotides represent differences between the different individuals. Nucleotides in red represent differences to the consensus sequence. Nucleotide-sequences in blue and green represent perfect alignment with the germline sequences of the TRBV-gene and TRBJ-gene, respectively.
AA; amino-acids, nt; nucleotides

PUB-I and PUB-HS CMV-, EBV- and AdV-specific CDR3β AA-sequences are abundant in virus-specific T-cell populations

We then investigated the distribution of the 131 PUB-I CDR3β AA-sequences within the 21 different specificities and the prevalence among individuals for each of the PUB-I CDR3β AA-sequences per viral-epitope. T cells with PUB-I CDR3β AA-sequences were found

for 19 out of the 21 specificities (**Supplementary Table 3**). PUB-I CDR3β AA-sequences were not observed in AdV-IE1^{LLD} and EBV-LMP2^{ESE}-specific T-cell populations. Some T-cell populations (e.g. EBV-LMP2^{FLY}) contained many different PUB-I CDR3β AA-sequences (n=24) that were all highly-similar. For this reason, we investigated the distribution of PUB-I CDR3β AA-sequences with unique TRBV and TRBJ-gene usage. This resulted in 29 different PUB-I CDR3β AA-sequences, distributed over 19 specificities (**Figure 3A; grey bars**). Six specificities contained two or three different (expressing different TRBV and/or TRBJ-genes) PUB-I CDR3β AA-sequences that were highly prevalent among individuals. To investigate how often these PUB-I CDR3β AA-sequences could be found in our cohort of healthy donors, we quantified the prevalence of each of these 29 PUB-I CDR3β AA-sequence (**Figure 3A; grey bars**). Because we classified a PUB-I CDR3β AA-sequence as being present in at least 2 individuals, the prevalence among donors could not be less than 2 out of 17 (12%; 17 is maximum number of T-cell populations for 1 specificity). Only 4 out of 29 PUB-I CDR3β AA-sequences were found in only 2 individuals (**Figure 3A; grey bars**). Overall, these 29 PUB-I CDR3β AA-sequences had a prevalence of 33% among healthy individuals (median; range 12%-82%). Importantly, most PUB-I CDR3β AA-sequences were found in at least 25% of individuals and 5 were even present in more than half the donors.

We and others hypothesized that the binding/docking of TCRs to HLA-peptide complexes might allow for small changes/flexibility in the CDR3 AA-sequences without significantly changing the conformation or interaction(45). Therefore, we investigated if there were CDR3β AA-sequences present in our data set that were highly-similar (PUB-HS) to PUB-I CDR3β AA-sequences and differed by 1, 2 or 3 AAs. The 2224 unique TCR nucleotide-sequences identified in our previous analysis may contain PUB-HS CDR3β AA-sequences that are in fact part of the same public response as the respective PUB-I TCRs. In total, 379 PUB-HS CDR3β nucleotide-sequences were present that also resulted in 379 PUB-HS CDR3β AA-sequences that differed by 1, 2 or 3 AAs from one of the 131 PUB-I CDR3β AA-sequences. This shows that 41% of the total virus-specific TCR-repertoire contained PUB-I and PUB-HS CDR3β nucleotide-sequences. We investigated if these PUB-HS CDR3β AA-sequences were also present in individuals that did not contain the respective PUB-I CDR3β AA-sequences. PUB-HS CDR3β AA-sequences were present for 21 out of 29 PUB-I CDR3β AA-sequences (**Figure 3A; shaded orange bars**). When we include the PUB-HS CDR3β AA-sequences and quantified the 29 PUB-I and PUB-HS CDR3β AA-sequences, these had a median prevalence of 50% among healthy individuals (range 23%-100%). The AdV-IE1^{LLD} and EBV-LMP2^{ESE}-specific T-cell populations, where PUB-I CDR3β AA-sequences were not found, did contain PUB-HS CDR3β AA-sequences in multiple individuals at high frequencies(46) (**Supplementary Figure 5**). The frequencies of PUB-I combined with PUB-HS CDR3β AA-sequences were relatively high within each virus-specific T-cell population of each individual (**Figure 3B**). The frequencies of all PUB-I and PUB-HS CDR3β AA-sequences ranged from 0.1%-99.4% within the 19 different virus-

specific T-cell populations with a median of 13.1%. When combined, all but one PUB-I plus PUB-HS CDR3β AA-sequences were found in at least 25% of individuals and 3 were even found in over 75% of individuals. These data show that for many PUB-I CDR3β AA-sequences we found sequences that were similar (1, 2 or 3 AA-differences), making up more than 40% of the total virus-specific TCR-repertoire and together these sequences were found in the majority of individuals at high frequencies.

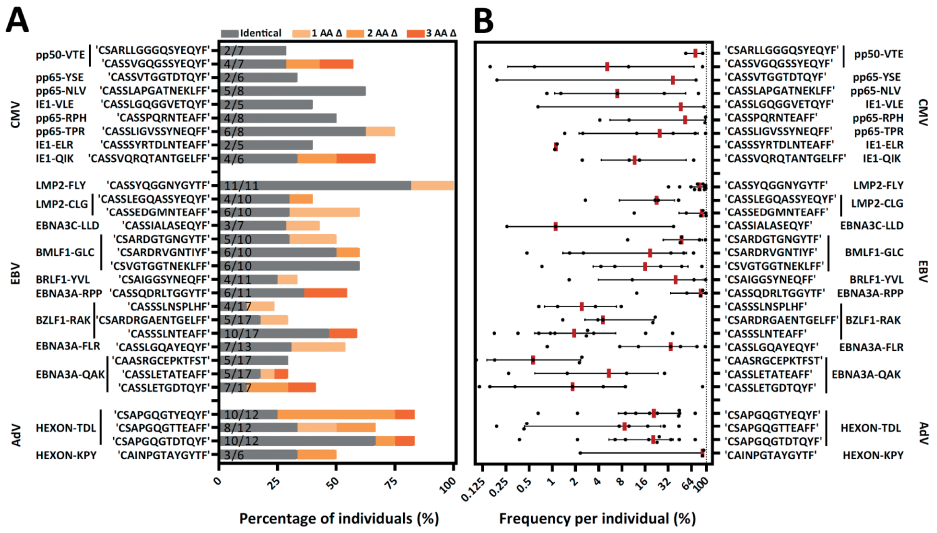

Figure 3. PUB-I and PUB-HS CMV, EBV and AdV-specific CDR3β AA-sequences are common in different individuals and of high frequencies within the T-cell specificities. **A**) Nine-teen different specificities contained PUB-I CDR3β-sequences, shared by at least 2 individuals. Six specificities contained 2 or 3 different (with different TRBV and/or TRBJ-genes) CDR3β AA-sequences that were found frequently in different individuals. The total numbers of different T-cell populations (different donors) that contained the PUB-I or PUB-HS CDR3β AA-sequences are indicated at the inner-side of the y-axis. The occurrence, shown as percentage among healthy individuals, is shown per CDR3β AA-sequence. PUB-I sequences are shown in grey. Individuals containing PUB-HS sequences with 1, 2 or 3 AA-differences (shown in light-dark orange) compared to the PUB-I sequence were stacked on top of the individuals where the PUB-I sequence was already identified. **B**) Shown is the sum of frequencies of the PUB-I and PUB-HS (1, 2 and 3 AA-differences) CDR3β AA-sequences per individual. Each dot is one individual, and the red-lines represent the medians with interquartile ranges.
AA; amino-acids, nt; nucleotides, Δ; difference(s)

Identical and highly-similar CDR3β AA-sequences contain conserved regions in the junctional region

To investigate how the PUB-HS CDR3β AA-sequences related to the PUB-I CDR3β AA-sequences, we analyzed if the PUB-HS (1, 2 or 3 AA-differences) CDR3β AA-sequences showed variations at random positions or at specific positions compared to the PUB-I

CDR3β AA-sequences. We hypothesized that if the binding/docking of PUB-HS TCRs was not significantly different, conserved regions and regions that allow for some variation could be identified in the CDR3β AA-sequences. As Illustrated in **Figure 4A** for the EBV-LMP2^{FLY}-specific PUB-I CDR3β AA-sequence CASSYQGGNYGYTF, two motifs were identified with AA-differences predominantly located at positions 5 and 9/10 of the CDR3β-region. The AAs [QGG] at positions 6-8 were conserved for both motifs. In total, the two motifs consisted of 86 PUB-HS CDR3β AA-sequences with 1 or 2 AA-differences. The majority (n=71) had the same CDR3 length of 14 AAs as the PUB-I CDR3β AA-sequence, implying that variations were caused by AA-substitutions. The remaining 15 PUB-HS CDR3β AA-sequences had a CDR3 length of 15 AAs, due to AA-inserts, compared to the PUB-I CDR3β AA-sequence (**Figure 4B**). Similar rules were found for the other 20 PUB-I CDR3β AA-sequences. Also here, some AA-positions were highly conserved, whereas others were variable. However, the precise locations of the variable AAs differed between specificities (Representative examples; **Figure 4C**). Interestingly, the corresponding CDR3-alpha sequences of a few highly-frequent PUB-I and PUB-HS CDR3β-sequences were also identical or highly-similar between different individuals (**Supplementary table 4**).

As a control, we assessed if these conserved motifs were predictive for the specificity when searching in our database of 2355 unique CDR3β AA-sequences. The requirement was that each motif should not be present in another specificity. We observed that some specificities contained motifs of only 3 or 4 AAs that were exclusive for that specificity and were not observed in any other specificity (**Table 3**). Altogether, these data show that the variations in the CDR3β AA-sequences were not random, but occurred at specific positions that resulted in conserved regions that were predictive for the specificities.

Computational analysis reveals conserved regions in CDR3β AA-sequences despite using different TRBJ-genes

We hypothesized that if the conserved junctional region is a crucial part of the peptide-HLA binding, virus-specific TCR-repertoires could also contain CDR3β AA-sequences with the same conserved region, while allowing different TRBJ-gene usage, as long as the 3-dimensional conformation would allow this. Since the TRBJ-regions often differ by more than 3 AAs, we were not able to include these as PUB-HS CDR3β AA-sequences. Such PUB-HS CDR3β AA-sequences that use different TRBJ-genes might even further increase the prevalence of PUB-I and PUB-HS CDR3β AA-sequences in the virus-specific TCR-repertoire. To investigate this, we performed a computational analysis using the levenshtein-distances (AA-differences) between all different CDR3β AA-sequences. For four different specificities (EBV-LMP2^{FLY}, EBV-EBNA3A^{RPP}, AdV-E1A^{LLD}, and AdV-HEXON^{TDL}) we observed clustering of CDR3β AA-sequences that expressed the same TRBV-genes while using different TRBJ-genes. For example (**Figure 5A**), the HLA-A*02:01-restricted

EBV-LMP2^{FLY}-specific CD8^{pos} T-cell repertoire contained 2 clusters within the cluster of TRBV6-5-expressing T cells (TRBV6-5/TRBJ1-2 and TRBV6-5/TRBJ2-1). The majority of CDR3β AA-sequences within the TRBJ1-2 cluster had a length of 14 AAs, while CDR3β AA-sequences from the TRBJ2-1 cluster had a length of 13 AAs (**Figure 5B**). Analysis of the junctional regions of the TRBV6-5/TRBJ1-2 and TRBV6-5/TRBJ2-1-encoded CDR3β AA-sequences revealed strong conservation of AAs [QGG] on positions 6-8, despite different TRBJ-usage and CDR3 lengths (**Figure 5C**). Similarly, the HLA-A*01:01-restricted AdV-HEXON^{TDL}-specific CD8^{pos} T-cell repertoire contained two large clusters of CDR3β AA-sequences, using TRBV20-1 or TRBV5-1 (**Figure 5D**), all with a CDR3 length of 13 AAs.

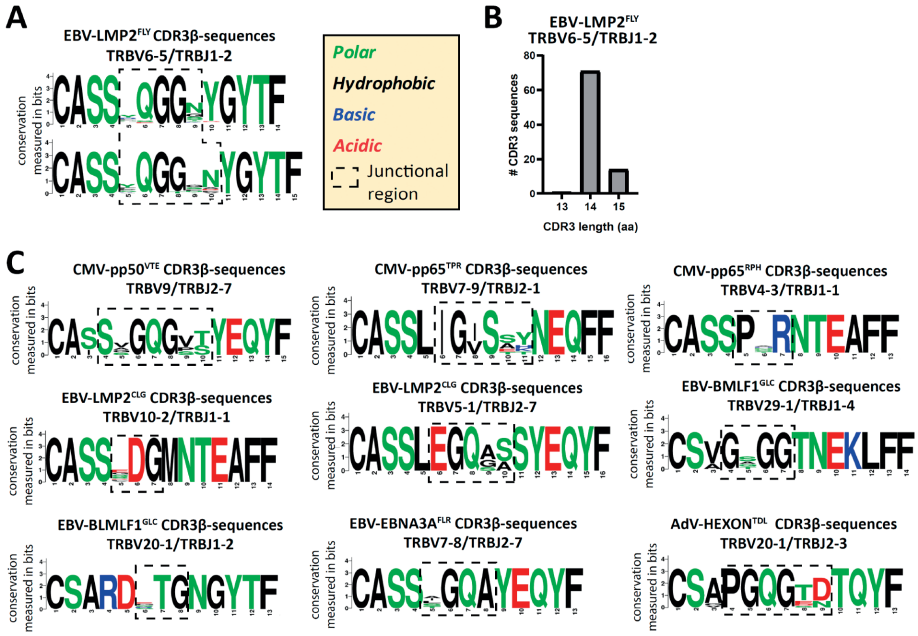

Figure 4. PUB-HS CDR3β AA-sequences show conserved regions and regions with high variability. The levenshtein distance was calculated (i.e. substitution, deletions or insertions of AAs) between CDR3β AA-sequences that express the same TRBV/TRBJ-genes as the PUB-I CDR3β AA-sequence. PUB-HS CDR3β AA-sequences were included with 1, 2 and 3 AA-differences compared to the PUB-I CDR3β AA-sequence. Sequence logos generated using WebLogo (http://weblogo.berkeley.edu/logo.cgi) show the relative frequency of each AA at each given position. The junctional regions (AAs that do not align with the germline TRBV or TRBJ-gene) are shown within the dotted-line box. **A**) The CDR3β AA-sequences specific for HLA-A*02:01-restricted EBV-LMP2^{FLY} with a CDR3 length of 14 and 15 AAs were stacked and show conserved and variable regions in the CDR3β-region. **B**) Shown are the CDR3 length distributions of CDR3β AA-sequences specific for EBV-LMP2^{FLY}-expressing TRBV6-5/TRBJ1-2. **C**) Shown are representative examples of PUB-HS (1, 2 or 3 AA differences) and PUB-I CDR3β AA-sequences that were stacked per specificity and TRBV/TRBJ-usage.

Table 3. Conserved motifs that predict the specificity

Specificity	TRBV	Shared TCR	TRBJ	Motif
CMV-pp50^{VTE}	9	CAS**S**V**GQG**SSYEQYF	2-7	**S**x**GQG**
CMV-pp65^{YSE}	9	CASS**VTGGT**DTQYF	2-3	**VTGGT**
CMV-pp65^{NLV}	7-6	CASS**LAPG**ATNEKLFF	1-4	**LAPG**
CMV-IE1^{VLE}	9	CASSL**GQGGV**ETQYF	2-5	**GQGGV**
CMV-pp65^{TPR}	7-9	CAS**SLIG**V**S**SYNEQFF	2-1	**SLIG**x**S**
CMV-pp65^{RPH}	4-3	CASS**P**Q**RNT**EAFF	1-1	**P**x**RNT**
CMV-IE1^{QIK}	9	CASS**V**Q**R**QT**ANT**GELFF	2-2	**V**x**R**xx**ANT**
CMV-IE1^{ELR}	27	CASSSYR**TDLN**TEAFF	1-1	**TDLN**
EBV-LMP2^{FLY}	6-5	CAS**S**Y**QGG**NYGYTF	1-2	**S**x**QGG**
EBV-LMP2^{CLG}	10-2	CASSE**DGMN**TEAFF	1-1	**DGMN**
	5-1	CASSL**EGQ**AS**S**YEQYF	2-7	**EGQ**xx**S**
EBV-EBNA3C^{LLD}	19	CASS**IAL**ASEQYF	2-7	**IAL**
EBV-BMLF1^{GLC}	20-1	CSA**RDR**V**G**NTIYF	1-3	**RDR**(V/T)**G**
	29-1	CSV**G**T**GGTN**EKLFF	1-4	**G**x**GGTN**
EBV-BRLF1^{YVL}	20-1	CS**AIGGS**YNEQFF	2-1	**AIGGS**
EBV-EBNA3A^{RPP}	4-1	CASSQD**RLTG**GYTF	1-2	**RLTG**
EBV-BZLF1^{RAK}	27	CA**SSSLN**TEAFF	2-1	**SSSLN**
	20-1	CSA**RDR**GA**EN**TGELFF	2-2	**RDR**xx**EN**
EBV-EBNA3A^{FLR}	7-8	CAS**S**L**GQA**YEQYF	2-7	**S**x**GQA**
EBV-EBNA3A^{QAK}	5-1	CASS**LET**GGYGYTF	1-2	**LET**(A/G)
AdV-HEXON^{TDL}	20-1	CSA**PGQ**GTDTQYF	2-3	(A/V)**PGQ**
AdV-E1A^{LLD}	20-1	CS**AR**P**GLA**DEQFF	2-1	**AR**x**GLA**
AdV-HEXON^{KPY}	10-3	CA**INP**GTAYGYTF	1-2	**INP**

We investigated which regions of PUB-I or PUB-HS CDR3β AA-sequences were predictive for the specificity. We searched for each motif in our library of 2355 CDR3β AA-sequences to determine what part of the junctional regions were unique for each specificity, without being present in any other specificity. Underscores with an x represent any of the 20 AAs. Some motifs contain two possible AAs that can be part of the motif which are shown between brackets. The minimum motifs are also shown in bold font in the original CDR3β AA-sequences.

The first cluster (TRBV20-1) contained sub-clusters of CDR3β AA-sequences using TRBJ1-1, TRBJ2-3 or TRBJ2-7 and the second cluster (TRBV5-1) contained CDR3β AA-sequences using TRBJ2-1 or TRBJ2-7. AdV-HEXON^{TDL}-specific CDR3β AA-sequences expressing TRBV20-1 revealed strong conservation of AAs [PGQG] on positions 4-7, which fell outside the region encoded by TRBJ (**Figure 5E**). Additionally, AdV-HEXON^{TDL}-specific CDR3β AA-sequences expressing TRBV5-1 revealed strong conservation of AAs [N__D] on positions 4 and 7, despite different TRBJ-usage. These examples illustrate that virus-specific TCR-repertoires can have conserved CDR3β-regions, while using different TRBJ-genes, allowing substantial variability at specific positions encoded by the TRBJ-region. This will further increase the prevalence of PUB-I and PUB-HS CDR3β AA-sequences in the total virus-specific TCR-repertoire.

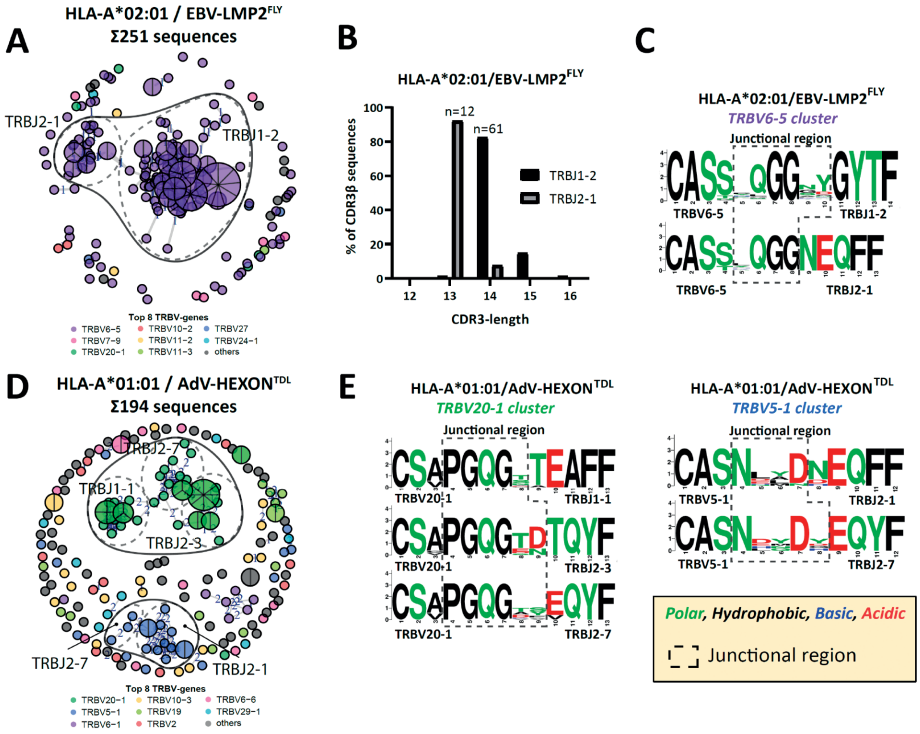

Figure 5. Computation analysis reveals clustering of PUB-HS CDR3β AA-sequences that contained conserved regions in the CDR3β-region. Computational analysis was performed using the levenshtein distance (differences in AAs) between CDR3β AA-sequences of one specificity. CDR3β AA-sequences were plotted and colored according to the top 8 most frequent TRBV-genes and were clustered and linked by a line if they were similar, with a number (levenshtein distance of 1, 2 or 3) representing the differences in AAs. PUB-I CDR3β AA-sequences were plotted as a pie-chart, whereby the size and number of slices indicate in how many individuals this CDR3β AA-sequence was present. Sequence logos generated using WebLogo (http://weblogo.berkeley.edu/logo.cgi) show the relative frequency of each AA at each given position. The junctional region (AAs that do not align with the germline TRBV or TRBJ-gene) are shown within the box with the dotted-line **A**) Shown is a representative example of a virus-specific $CD8^{pos}$ T-cell population, specific for EBV-$LMP2^{FLY}$ with overlapping clusters of sequences that express different TRBJ-genes, while expressing the same TRBV-gene. **B**) The lengths of the CDR3β-regions of the two clusters of EBV-$LMP2^{FLY}$-specific CDR3β-sequences are shown. Varying lengths of the CDR3β-region within a cluster would suggest deletions or insertions, whereby the same length would indicate AA substitutions. **C**) Shown are the sequence motifs of the two EBV-$LMP2^{FLY}$-specific clusters. **D**) Shown is a second representative example of a virus-specific $CD8^{pos}$ T-cell population, specific for AdV-$HEXON^{TDL}$, with overlapping clusters of sequences that express different TRBJ-genes, while expressing the same TRBV-gene. **E**) Shown are the sequence motifs of the TRBV20-1 and TRBV5-1-expressing EBV-$HEXON^{TDL}$-specific clusters.

Individuals with heterozygous HLA backgrounds contain the same shared identical and highly-similar CDR3β AA-sequences

To determine whether the magnitude of PUB-I and PUB-HS CDR3β AA-sequences was particular for our cohort of individuals with a homozygous HLA background, we investigated if the same phenomenon was also present in individuals with a heterogeneous HLA background. We performed the same analyses on virus-specific CD8^{pos} T-cell populations targeting 11 different viral epitopes that were generated and used in the context of a clinical study(20). A total of 1157 CDR3β nucleotide-sequences could be correctly annotated. In total, 695 (61%) nucleotide-sequences resulted in unique CDR3β AA-sequences, that were only found in one individual, and 462 nucleotide-sequences (39%) resulted in 89 different PUB-I CDR3β AA-sequences. From the 695 unique CDR3β AA-sequences, 134 PUB-HS CDR3β nucleotide-sequences were present that differed by 1, 2 or 3 AAs from one of the 89 PUB-I CDR3β AA-sequences.

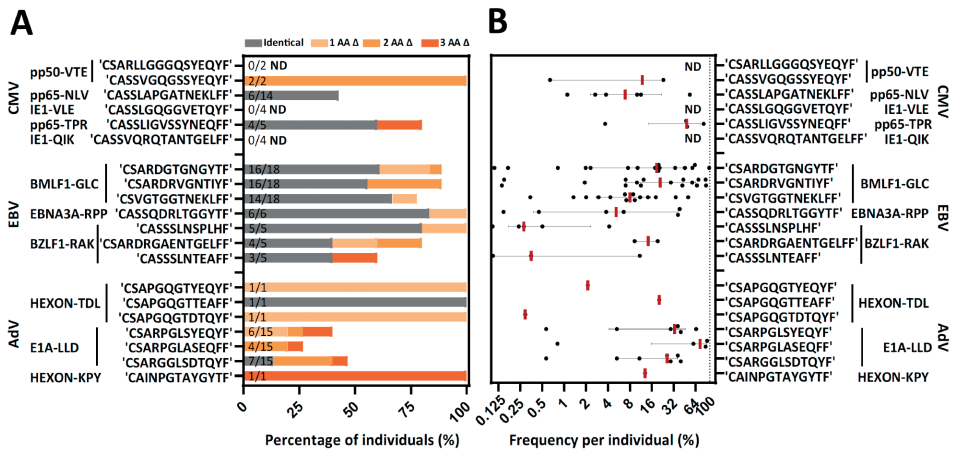

Figure 6. Individuals with a different HLA background from an independent database contain the same PUB-I and PUB-HS CDR3β AA-sequences. Virus-specific T-cell populations targeting 11 different viral-antigens, derived from an independent database, could be evaluated for the occurrence of PUB-I pr PUB-HS CDR3β AA-sequences. **A**) Nine out of the 11 specificities contained the same PUB-I or PUB-HS CDR3β AA-sequences as in our database. The occurrence, shown as percentages among healthy donors, is shown per CDR3β AA-sequence. PUB-I sequences are shown in grey. PUB-HS CDR3β AA-sequences from other individuals with 1, 2 or 3 AA differences, were stacked on top of the already identified public-identical sequences. The total numbers of different T-cell populations (different donors) that contained the PUB-I or PUB-HS CDR3β-sequences are indicated at the inner-side of the y-axis. **B**) Shown are the sum of frequencies of the PUB-I or PUB-HS CDR3β AA-sequences per donor. Each dot is one donor shown and the red-lines represent the medians with interquartile ranges.
AA; amino-acid, Δ; difference(s), ND; Not detected

This shows again that also in this cohort a large part (51%) of the total virus-specific TCR-repertoire contained PUB-I and PUB-HS CDR3β nucleotide-sequences. Because the targeted viral epitopes were not fully identical in both cohorts, we could investigate the prevalence of 20 out of 29 PUB-I CDR3β AA-sequences in this cohort. In total, 17 out of 20 CDR3β AA-sequences that were previously identified, could also be identified in this independent cohort. When we included the PUB-HS CDR3β AA-sequences and quantified the 17 PUB-I and PUB-HS CDR3β AA-sequences, these sequences had a similar high prevalence of a median of 89% among healthy individuals (range 26-100%). (**Figure 6A**). These CDR3β AA-sequences were also present at high frequencies within each virus-specific T-cell population (**Figure 6B**). These data show that the same PUB-I or PUB-HS CDR3β AA-sequences are also present in virus-specific T cells isolated from an independent cohort of individuals with a heterogeneous HLA background with a similar prevalence among donors and frequency within donors.

DISCUSSION

In this study, we quantitatively analyzed the magnitude, defined as prevalence within the population and frequencies within individuals, of public-identical (PUB-I) together with public-highly-similar (PUB-HS) TCRs in TCR-repertoires of CMV, EBV and AdV-specific $CD8^{pos}$ T-cell populations. In total, 2224 (71%) TCR-CDR3β nucleotide-sequences resulted in unique CDR3β AA-sequences, and 905 nucleotide-sequences (29%) resulted in 131 different PUB-I CDR3β AA-sequences that were found in two or more unrelated individuals. These PUB-I CDR3β AA-sequences were distributed over 19 out of 21 virus-specificities and contained 29 different PUB-I CDR3β AA-sequences that were often found in multiple individuals at high frequencies. The virus-specific T-cell populations additionally contained 12% PUB-HS CDR3β AA-sequences, which differed by 1, 2 or 3 AAs compared to the respective PUB-I CDR3β AA-sequences. PUB-HS CDR3β AA-sequences could be found in virus-specific T-cell populations of individuals who did not contain the PUB-I CDR3β AA-sequence as well as of individuals who already contained the PUB-I CDR3β AA-sequence. Analysis of the PUB-I and PUB-HS CDR3β AA-sequences revealed strong conservation of specific AA motifs in the junctional region together with variability of AAs at specific positions at the TRBV/TRBD- and/or TRBD/TRBJ-border regions. Positions with high variability were often adjacent to or even interspersed with the conserved motif. The conserved motifs that we identified were unique for each specificity, and could not be identified in any other specificity in our database. This makes it very likely that these motifs are important for binding of the TCRs to the peptide-HLA complexes. Combined, 41% of the total virus-specific TCR-repertoire consisted of PUB-I and PUB-HS CDR3β nucleotide-sequences. These findings were based on virus-specific T-cell populations derived from two homogeneous donor cohorts that homozygously

expressed HLA-A*01:01/HLA-B*08:01 or HLA-A*02:01/HLA-B*07:02. However, we found similar high percentages (51%) of PUB-I and PUB-HS CDR3β nucleotide-sequences within virus-specific T-cell populations from healthy donors with heterogeneous HLA-backgrounds that were generated for a recent clinical study(20). These dominant PUB-I and PUB-HS TCRs probably are a reflection of the viral-antigen-specific T-cell responses that most optimally encountered the peptide-HLA complexes on the infected target cells and could be utilized for the design of future immunotherapy purposes including TCR-gene transfer strategies.

Various explanations have been suggested to underlie the development of public TCRs in T-cell responses targeting the same antigenic epitope(47). One was a high probability that these PUB-I sequences can be generated during V-D-J recombination(28, 48). Furthermore, various nucleotide-sequences can result in the same TCR AA-sequences that further increase the probability(49). Selection *in vivo* by optimal antigen-specific proliferation may result in a dominant antigen-specific memory T-cell population(50). These determinants may also lead to TCRs that are highly-similar to the PUB-I sequence, although they were often not included in the analyses of such public T-cell responses. It has been shown that conserved AAs in the CDR3 loop provide a structural framework that is required for the maintenance of the three dimensional TCR-structure(51). A similar structural framework between the PUB-I and PUB-HS sequences can thus lead to a conserved engagement with the peptide/HLA complex(52). Our rationale is that the PUB-I and PUB-HS sequences are part of the same public T-cell response when the same peptide-HLA complex is targeted, the same variable gene is expressed to have identical CDR1 and CDR2 regions and contains the same conserved AAs in the CDR3 loop. With this set of rules, we were able to quantitatively analyze the public T-cell responses and showed that T cells expressing PUB-I TCRs together with T cells that express PUB-HS TCRs made up at least 41% of the total TCR-repertoire. To assess the role of the alpha chains in PUB-I and PUB-HS TCRs, we identified the CDR3α sequence usage of a selection of virus-specific T-cell populations that contained shared TCRβ sequences and the corresponding CDR3α sequences all showed to be identical or highly similar between individuals. However, the high percentages of shared TCR-sequences contradict the findings observed by Madi et al., 2017, where they performed immunization in mice with foreign ovalbumin (OVA)-derived peptide that resulted in dominant private TCR-repertoires and less public TCRs (53). Since virus-specific memory T-cell repertoires in the circulation are shaped based on antigen encounter and subsequent proliferation, the PUB-I and PUB-HS TCR-sequences most likely reflect highly functional T cells capable antigen-driven proliferation. For latent viruses such as CMV, EBV and AdV, virus-specific T cells frequently encounter antigen during episodes of viral reactivation. The presence of PUB-I and PUB-HS TCR-sequences for these virus-specific T cells could be rather high due to this frequent antigen encounter. However, multiple reports also observed shared

CDR3β sequences in T-cell populations specific for non-latent viruses such as Influenza, RSV, and SARS-CoV-2(16, 26, 54), suggesting that this phenomenon is not unique for latent viruses, although the unexpected high magnitude of PUB-I and PUB-HS TCR-sequences that we observed can be unique for latent viruses.

These percentages of PUB-I and PUB-HS TCRs within these virus specific T-cell responses may still be an underestimation since the prerequisite of the identification of a PUB-HS TCR was similarity to a PUB-I TCR that was present in at least 2 individuals. Highly similar TCRs with only mutual similarities without identity in at least 2 individuals were not included as PUB-HS TCRs. Therefore, some of the unique TCRs within the virus-specific T-cell repertoire may also be part of a public T-cell response. This was indeed illustrated by the growing percentages of PUB-I and PUB-HS sequences when including more sampled sequences(55). Although it was suggested that HLA polymorphisms might be a confounding factor that affect the sharing of TCRs(55, 56), we showed that our validation cohort with different HLA-backgrounds revealed frequencies of the PUB-I and PUB-HS TCRs with at least a similar magnitude. Our approach involved a short *ex vivo* expansion of the isolated virus-specific T cells that might have created a bias towards the expansion of the presently identified PUB-I and PUB-HS TCRs, indicating that the actual numbers of PUB-I and PUB-HS in unmanipulated peripheral blood may have even been higher.

In conclusion, our findings demonstrate that a large part of the virus-specific TCR-repertoire contains PUB-I and PUB-HS TCRs at high frequencies in multiple different individuals.. Because virus-specific memory T-cell repertoires in the circulation are shaped based on antigen encounter and subsequent proliferation, the PUB-I and PUB-HS TCR-sequences most likely reflect highly functional T cells capable of antigen-driven proliferation. Since it is plausible that the highly-similar TCRs with conserved motifs similarly dock to the peptide-HLA complex as the identical shared TCR-sequences, these PUB-I and PUB-HS sequences can be considered part of the same public T-cell response. Such public TCRs may then be utilized for diagnostic purposes or therapeutic benefit in TCR-gene transfer-based immunotherapy strategies to effectively control viral-reactivation in immunocompromised patients.

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Supplementary Table 1. Primer sequences.

Description	Name	Nucleotide sequence 5'►3'
cDNA primer TRB constant region reverse transcription	TRB_RT	CACGTGGTCGGGGWAGAAGC
cDNA primer SmartSeq2modified template switching oligo	SS2m_TSO	AAGCAGTGGTATCAACGCAGAGTACAT(G)(G){G}
PCR primer SmartSeq2modified forward	SS2m_For	GAGTTCAGACGTGTGCTCTTCCGATCTAAGCAGTGGTATCAACGCAGAGTACAT*G
PCR primer TRBC1 reverse	TRBC1_rev	CCTACACGACGCTCTTCCGATCTGTGGGAACACCTTGTTCAGGTCCT*C
PCR primer TRBC1 reverse	TRBC2_rev	CCTACACGACGCTCTTCCGATCTGTGGGAACACGTTTTTCAGGTCCT*C
Barcode primer SS2m region, forward, backbone	BC_R7xx_For	CAAGCAGAAGACGGCATACGAGAT nnnnnn GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C
Barcode primer TRBC region reverse, backbone	BC_R7xx_Rev	AATGATACGGCGACCACCGAGATCTACAC nnnnnn ACACTCTTTCCCTACACGACGCTCTTCCGATC*T

Abbreviations: TRB: T-cell Receptor Beta, SS2m: SmartSeq2Modified, TSO: Template Switching Oligo, TRBC: T-cell Receptor Beta Constant, For: Forward, Rev: Reverse, BC: Beta chain, nnnnnn: Identifier sequence
()=RNA, {}=LNA: Locked Nucleic Acid, *:phosphonothioate-binding

Supplementary Table 2. Identifier sequences.

Identifiers (For) Name	Identifiers (For) Seq	Identifiers (Rev) Name	Identifiers (Rev) Seq
BC_R701	ATCACG	BC_R725	ACTGAT
BC_R702	CGATGT	BC_R726	ATGAGC
BC_R703	TTAGGC	BC_R727	ATTCCT
BC_R704	TGACCA	BC_R728	CAAAAG
BC_R705	ACAGTG	BC_R729	CAACTA
BC_R706	GCCAAT	BC_R730	CACCGG
BC_R707	CAGATC	BC_R731	CACGAT
BC_R708	ACTTGA	BC_R732	CACTCA
BC_R709	GATCAG	BC_R733	CAGGCG
BC_R710	TAGCTT	BC_R734	CATGGC
BC_R711	GGCTAC	BC_R735	CATTTT
BC_R712	CTTGTA	BC_R736	CCAACA
BC_R713	AGTCAA	BC_R737	CGGAAT
BC_R714	AGTTCC	BC_R738	CTAGCT
BC_R715	ATGTCA	BC_R739	CTATAC
BC_R716	CCGTCC	BC_R740	CTCAGA
BC_R717	GTAGAG	BC_R741	GACGAC
BC_R718	GTCCGC	BC_R742	TAATCG
BC_R719	GTGAAA	BC_R743	TACAGC
BC_R720	GTGGCC	BC_R744	TATAAT
BC_R721	GTTTCG	BC_R745	TCATTC
BC_R722	CGTACG	BC_R746	TCCCGA
BC_R723	GAGTGG	BC_R747	TCGAAG
BC_R724	GGTAGC	BC_R748	TCGGCA

Supplementary Table 3. Occurrence and number of CDR3β amino-acid sequences that are shared between individuals.

Virus	Antigen	HLA	TRBV	CDR3	TRBJ	Occurrence (#)
CMV	pp50-VTE	A*01	TRBV20-1	CSARLLGGGQSYEQYF	TRBJ2-7	2/7
CMV	pp50-VTE	A*01	TRBV9	CASSVGQGSSYEQYF	TRBJ2-7	2/7
CMV	pp65-YSE	A*01	TRBV9	CASSVTGGTDTQYF	TRBJ2-3	2/6
CMV	pp65-NLV	A*02	TRBV7-6	CASSLAPGATNEKLFF	TRBJ1-4	5/8
CMV	pp65-NLV	A*02	TRBV7-6	CASSLAPGTTNEKLFF	TRBJ1-4	3/8
CMV	pp65-NLV	A*02	TRBV12-4	CASSSAYYGYTF	TRBJ1-2	2/8
CMV	IE1-VLE	A*02	TRBV7-3	CASSLGQGGVETQYF	TRBJ2-5	2/6
CMV	IE1-VLE	A*02	TRBV7-3	CASSPGQGGVETQYF	TRBJ2-5	2/6
CMV	pp65-TPR	B*07	TRBV7-9	CASSLIGVSSYNEQFF	TRBJ2-1	5/8
CMV	pp65-RPH	B*07	TRBV4-3	CASSPQRNTEAFF	TRBJ1-1	4/6
CMV	pp65-RPH	B*07	TRBV4-3	CASSPSRNTEAFF	TRBJ1-1	2/6
CMV	IE1-ELR	B*08	TRBV27	CASSSYRTDLNTEAFF	TRBJ1-1	2/5
CMV	IE1-QIK	B*08	TRBV9	CASSTQVSEPNTGELFF	TRBJ2-2	2/6
CMV	IE1-QIK	B*08	TRBV9	CASSVQRQTANTGELFF	TRBJ2-2	2/6
CMV	IE1-QIK	B*08	TRBV12-5	CASGPRAGAYNEQFF	TRBJ2-1	2/6
CMV	IE1-QIK	B*08	TRBV2	CASSGTGRLTMNTEAFF	TRBJ1-1	2/6
CMV	IE1-QIK	B*08	TRBV21-1	CASSKVAARVP-TLKLS	TRBJ1-1	2/6
CMV	IE1-QIK	B*08	TRBV7-9	CASSLTLAGNQPQHF	TRBJ1-5	2/6
EBV	BRLF1-YVL	A*02	TRBV10-1	CASSAGPDTQYF	TRBJ2-3	2/12
EBV	BRLF1-YVL	A*02	TRBV24-1	CATSDYGEDTQYF	TRBJ2-3	2/12
EBV	BRLF1-YVL	A*02	TRBV25-1	CASSEWTTDTQYF	TRBJ2-3	2/12
EBV	BRLF1-YVL	A*02	TRBV28	CASSKIMNTEAFF	TRBJ1-1	2/12
EBV	BRLF1-YVL	A*02	TRBV6-5	CASSQLLGSNQPQHF	TRBJ1-5	2/12
EBV	EBNA3A-RPP	B*07	TRBV4-1	CASSQDRLTGGYTF	TRBJ1-2	4/11
EBV	EBNA3A-RPP	B*07	TRBV4-1	CASSQDRLTGTQYF	TRBJ2-5	2/11
EBV	EBNA3A-RPP	B*07	TRBV4-1	CASSQEAFNYEQYF	TRBJ2-7	2/11
EBV	BZLF1-RAK	B*08	TRBV27	CASSSLNTEAFF	TRBJ1-1	8/17
EBV	BZLF1-RAK	B*08	TRBV27	CASSPLTDTQYF	TRBJ2-3	3/17
EBV	BZLF1-RAK	B*08	TRBV20-1	CSARDRGAENTGELFF	TRBJ2-2	3/17
EBV	BZLF1-RAK	B*08	TRBV20-1	CSARDRGGENTGELFF	TRBJ2-2	3/17
EBV	BZLF1-RAK	B*08	TRBV29-1	CSVGSGEGYEQYF	TRBJ2-7	3/17
EBV	BZLF1-RAK	B*08	TRBV4-1	CASSPGQGEGYEQYF	TRBJ2-7	3/17
EBV	BZLF1-RAK	B*08	TRBV7-9	CASSPTGAGNQPQHF	TRBJ1-5	3/17
EBV	BZLF1-RAK	B*08	TRBV27	CASSNLNTEAFF	TRBJ1-1	2/17
EBV	BZLF1-RAK	B*08	TRBV27	CASSDLNSPLHF	TRBJ1-6	2/17
EBV	BZLF1-RAK	B*08	TRBV27	CASSSLNSPLHF	TRBJ1-6	2/17
EBV	BZLF1-RAK	B*08	TRBV7-2	CASSLVLLGNSPLHF	TRBJ1-6	2/17
EBV	BZLF1-RAK	B*08	TRBV4-1	CASSRLAGDTDTQYF	TRBJ2-3	2/17
EBV	BZLF1-RAK	B*08	TRBV6-1	CASTGTASTDTQYF	TRBJ2-3	2/17
EBV	BZLF1-RAK	B*08	TRBV20-1	CSARDRGSENTGELFF	TRBJ2-2	2/17
EBV	BZLF1-RAK	B*08	TRBV20-1	CSARDRGTENTGELFF	TRBJ2-2	2/17
EBV	BZLF1-RAK	B*08	TRBV7-3	CASSSHSGINTGELFF	TRBJ2-2	2/17

Supplementary Table 3. Continued.

Virus	Antigen	HLA	TRBV	CDR3	TRBJ	Occurrence (#)	Virus	Antigen	HLA	TRBV	CDR3	TRBJ	Occurrence (#)
EBV	LMP2-FLY	A*02	TRBV6-5	CASSYQGGNYGYTF	TRBJ1-2	9/11	EBV	BZLF1-RAK	B*08	TRBV10-3	CATGLAGSTDTQYF	TRBJ2-3	2/17
EBV	LMP2-FLY	A*02	TRBV6-5	CASSRQGGNYGYTF	TRBJ1-2	7/11	EBV	BZLF1-RAK	B*08	TRBV4-1	CASSPGTGEGYEQYF	TRBJ2-7	2/17
EBV	LMP2-FLY	A*02	TRBV6-5	CASSLQGGNYGYTF	TRBJ1-2	5/11	EBV	BZLF1-RAK	B*08	TRBV7-2	CASSPGTGEGYEQYF	TRBJ2-7	2/17
EBV	LMP2-FLY	A*02	TRBV6-5	CASSGQGGNYGYTF	TRBJ1-2	4/11	EBV	BZLF1-RAK	B*08	TRBV7-2	CASSYHGSYEQYF	TRBJ2-7	2/17
EBV	LMP2-FLY	A*02	TRBV6-5	CASSKQGGGYGYTF	TRBJ1-2	3/11	EBV	BZLF1-RAK	B*08	TRBV7-6	CASSLAGEGYEQYF	TRBJ2-7	2/17
EBV	LMP2-FLY	A*02	TRBV6-5	CASSPQGGGYGYTF	TRBJ1-2	3/11	EBV	BZLF1-RAK	B*08	TRBV7-9	CASSSTGAGNQPQHF	TRBJ1-5	2/17
EBV	LMP2-FLY	A*02	TRBV6-5	CASSRQGGTYGYTF	TRBJ1-2	3/11	EBV	BZLF1-RAK	B*08	TRBV7-9	CASSSTGSGDQPQHF	TRBJ1-5	2/17
EBV	LMP2-FLY	A*02	TRBV6-5	CASSSQGGNYGYTF	TRBJ1-2	3/11	EBV	BZLF1-RAK	B*08	TRBV7-3	CASSLIASGGYNEQFF	TRBJ2-1	2/17
EBV	LMP2-FLY	A*02	TRBV6-5	CASSYSGGYYGYTF	TRBJ1-2	2/11							
EBV	LMP2-FLY	A*02	TRBV6-5	CASSDQGGGYGYTF	TRBJ1-2	2/11	EBV	EBNA3A-FLR	B*08	TRBV7-8	CASSLGQAYEQYF	TRBJ2-7	4/13
EBV	LMP2-FLY	A*02	TRBV6-5	CASSFQGGNYGYTF	TRBJ1-2	2/11	EBV	EBNA3A-FLR	B*08	TRBV7-8	CASSSGQAYEQYF	TRBJ2-7	4/13
EBV	LMP2-FLY	A*02	TRBV6-5	CASSPLGGAEGYTF	TRBJ1-2	2/11	EBV	EBNA3A-FLR	B*08	TRBV7-8	CASSTGQAYEQYF	TRBJ2-7	3/13
EBV	LMP2-FLY	A*02	TRBV6-5	CASSPQGGNYGYTF	TRBJ1-2	2/11	EBV	EBNA3A-FLR	B*08	TRBV4-3	CASSHGLAGILETQYF	TRBJ2-5	2/13
EBV	LMP2-FLY	A*02	TRBV6-5	CASSPQGGRDGYTF	TRBJ1-2	2/11	EBV	EBNA3A-FLR	B*08	TRBV4-3	CASSPTSGVAGELFF	TRBJ2-2	2/13
EBV	LMP2-FLY	A*02	TRBV6-5	CASSRQGGSYGYTF	TRBJ1-2	2/11	EBV	EBNA3A-FLR	B*08	TRBV4-1	CASSQGLAVSSYEQYF	TRBJ2-7	2/13
EBV	LMP2-FLY	A*02	TRBV6-5	CASSSQGGSNYGYTF	TRBJ1-2	2/11	EBV	EBNA3A-FLR	B*08	TRBV7-9	CASSWGPEQFF	TRBJ2-1	2/13
EBV	LMP2-FLY	A*02	TRBV6-5	CASSSQGGSYGYTF	TRBJ1-2	2/11							
EBV	LMP2-FLY	A*02	TRBV6-5	CASSYEGGYYGYTF	TRBJ1-2	2/11	EBV	EBNA3A-QAK	B*08	TRBV18	CAASRGCEPKTFST	TRBJ2-4	5/18
EBV	LMP2-FLY	A*02	TRBV6-5	CASSYQGGSYGYTF	TRBJ1-2	2/11	EBV	EBNA3A-QAK	B*08	TRBV28	CASSNLGVTELNTGELFF	TRBJ2-2	3/18
EBV	LMP2-FLY	A*02	TRBV6-5	CASNPQGGGGGYTF	TRBJ1-2	2/11	EBV	EBNA3A-QAK	B*08	TRBV5-1	CASSLELAVYNEQFF	TRBJ2-1	3/18
EBV	LMP2-FLY	A*02	TRBV6-5	CASNPQGGGNGYTF	TRBJ1-2	2/11	EBV	EBNA3A-QAK	B*08	TRBV5-1	CASSLETATEAFF	TRBJ1-1	3/18
EBV	LMP2-FLY	A*02	TRBV6-5	CASSYQGGNEQFF	TRBJ2-1	3/11	EBV	EBNA3A-QAK	B*08	TRBV5-1	CASSLETGGYGYTF	TRBJ1-2	3/18
EBV	LMP2-FLY	A*02	TRBV6-5	CASSLQGGNEQFF	TRBJ2-1	2/11	EBV	EBNA3A-QAK	B*08	TRBV27	CASSLYRDNQPQHF	TRBJ1-5	2/18
EBV	LMP2-FLY	A*02	TRBV6-5	CASTLQGGNEQFF	TRBJ2-1	2/11	EBV	EBNA3A-QAK	B*08	TRBV27	CASSPDRWETQYF	TRBJ2-5	2/18
							EBV	EBNA3A-QAK	B*08	TRBV28	CASSALSGLAGPGELFF	TRBJ2-2	2/18
EBV	LMP2-CLG	A*02	TRBV10-2	CASSEDGMNTEAFF	TRBJ1-1	3/10	EBV	EBNA3A-QAK	B*08	TRBV28	CASSKQGAPGHTGELFF	TRBJ2-2	2/18

Supplementary Table 3. Continued.

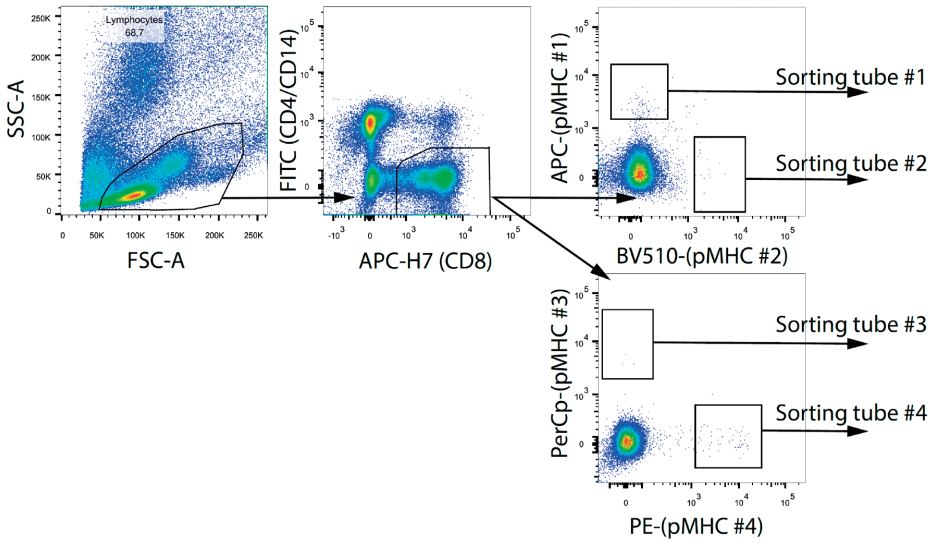
Virus	Antigen	HLA	TRBV	CDR3	TRBJ	Occurrence (#)	Virus	Antigen	HLA	TRBV	CDR3	TRBJ	Occurrence (#)
EBV	LMP2-CLG	A*02	TRBV10-2	CASSSDGMNTEAFF	TRBJ1-1	2/10	EBV	EBNA3A-QAK	B*08	TRBV28	CASSLLGARGLNEKLFF	TRBJ1-4	2/18
EBV	LMP2-CLG	A*02	TRBV10-2	CASSGDGMNTEAFF	TRBJ1-1	2/10	EBV	EBNA3A-QAK	B*08	TRBV28	CASSLLGTGGLSEKLFF	TRBJ1-4	2/18
EBV	LMP2-CLG	A*02	TRBV10-2	CASSQDGMNTEAFF	TRBJ1-1	2/10	EBV	EBNA3A-QAK	B*08	TRBV28	CASSQQGARSLSEKLFF	TRBJ1-4	2/18
EBV	LMP2-CLG	A*02	TRBV5-1	CASSLEGQASSYEQYF	TRBJ2-7	3/10	EBV	EBNA3A-QAK	B*08	TRBV4-2	CASSQDAGDRLAGVTGELFF	TRBJ2-2	2/18
							EBV	EBNA3A-QAK	B*08	TRBV5-1	CASSLETGDTQYF	TRBJ2-3	2/18
EBV	EBNA3C-LLD	A*02	TRBV19	CASSIALASEQYF	TRBJ2-7	2/7	EBV	EBNA3A-QAK	B*08	TRBV6-3	CASSLDPPGQSIRVNTGELFF	TRBJ2-2	2/18
EBV	BMLF1-GLC	A*02	TRBV29-1	CSVGTGGTNEKLFF	TRBJ1-4	6/10	AdV	HEXON-TDL	A*01	TRBV20-1	CSAPGQGTDTQYF	TRBJ2-3	8/12
EBV	BMLF1-GLC	A*02	TRBV20-1	CSARDRVGNTIYF	TRBJ1-3	5/10	AdV	HEXON-TDL	A*01	TRBV20-1	CSAPGQGTTEAFF	TRBJ1-1	4/12
EBV	BMLF1-GLC	A*02	TRBV20-1	CSARDGTGNGYTF	TRBJ1-2	3/10	AdV	HEXON-TDL	A*01	TRBV20-1	CSAPGQGTYEQYF	TRBJ2-7	3/12
EBV	BMLF1-GLC	A*02	TRBV20-1	CSARDRTGNGYTF	TRBJ1-2	3/10	AdV	HEXON-TDL	A*01	TRBV20-1	CSAPGQGSTEAFF	TRBJ1-1	3/12
EBV	BMLF1-GLC	A*02	TRBV29-1	CSVGAGGTNEKLFF	TRBJ1-4	3/10	AdV	HEXON-TDL	A*01	TRBV20-1	CSAPGQGEETQYF	TRBJ2-5	2/12
EBV	BMLF1-GLC	A*02	TRBV14	CASSQSPGGTQYF	TRBJ2-3	2/10	AdV	HEXON-TDL	A*01	TRBV20-1	CSAPGQGENTQYF	TRBJ2-3	2/12
EBV	BMLF1-GLC	A*02	TRBV20-1	CSARVGVGNTIYF	TRBJ1-3	2/10	AdV	HEXON-TDL	A*01	TRBV5-1	CASNDYDNEQFF	TRBJ2-1	2/12
EBV	BMLF1-GLC	A*02	TRBV29-1	CSAGSGGTNEKLFF	TRBJ1-4	2/10	AdV	HEXON-TDL	A*01	TRBV5-1	CASNLADDEQFF	TRBJ2-1	2/12
EBV	BMLF1-GLC	A*02	TRBV29-1	CSVGSGGTNEKLFF	TRBJ1-4	2/10	AdV	HEXON-TDL	A*01	TRBV10-3	CATQTGGSNQPQHF	TRBJ1-5	2/12
							AdV	HEXON-TDL	A*01	TRBV4-1	CASSQVVGQAHSPLHF	TRBJ1-6	2/12
EBV	BRLF1-YVL	A*02	TRBV20-1	CSAIGGSYNEQFF	TRBJ2-1	3/12	AdV	HEXON-TDL	A*01	TRBV6-6	CASSYPGNNSPLHF	TRBJ1-6	2/12
EBV	BRLF1-YVL	A*02	TRBV20-1	CSAPVPPYNEQFF	TRBJ2-1	2/12	AdV	HEXON-TDL	A*01	TRBV20-1	CSAR-ASVATSST	TRBJ2-7	2/12
EBV	BRLF1-YVL	A*02	TRBV20-1	CSARGTEFYEQYF	TRBJ2-7	2/12	AdV	HEXON-TDL	A*01	TRBV19	CATSSAAQETQYF	TRBJ2-5	2/12
EBV	BRLF1-YVL	A*02	TRBV28	CASSLFSNEQFF	TRBJ2-1	2/12							
							AdV	HEXON-KPY	B*07	TRBV10-3	CAINPGTAYGYTF	TRBJ1-2	2/8
							AdV	HEXON-KPY	B*07	TRBV18	CASSPGTPEQFF	TRBJ2-1	2/8

A total of 131 different shared identical CDR3β amino-acid sequences are shown. The number of T-cell populations that contain the shared identical CDR3β amino-acid sequences are shown per total number of T-cell populations of that respective specificity (#), reflecting the occurrence among donors.

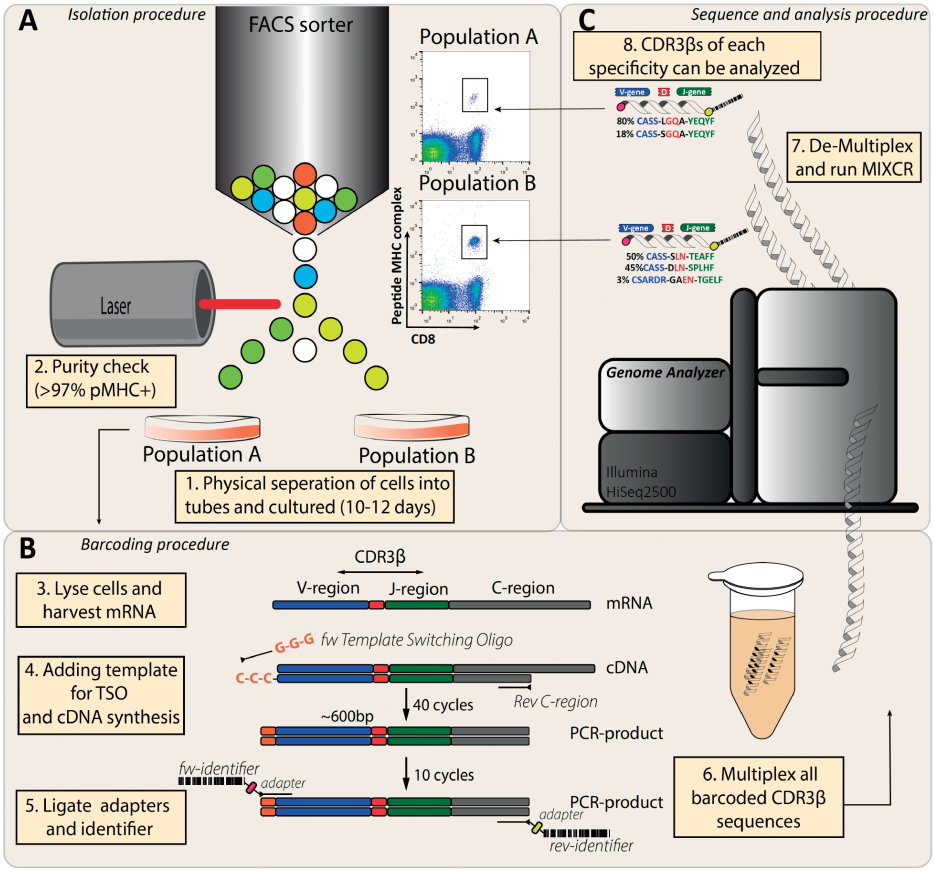
Supplementary Table 4. CDR3-alpha sequences of virus-specific T-cell populations with PUB-I and PUB-HS CDR3-beta sequences

Donor ID	Specificity	CDR3-beta	TRAV	CDR3-alpha	TRAJ
22	EBV-EBNA3A-RPP	CASSQDRLTGGYTF	TRAV24	CA**FS**SSNTGKLIF	TRAJ37
28	EBV-EBNA3A-RPP	CASSQDRLTGGYTF	TRAV24	CA**HG**SSNTGKLIF	TRAJ37
19	EBV-EBNA3A-RPP	CASSQDRLTGGYTF	TRAV24	CA**SS**SSNTGKLIF	TRAJ37
19	AdV-E1A-LLD	CSAR**A**GL**AE**TQYF	TRAV19	CALSDYGGYNKLIF	TRAJ4
28	AdV-E1A-LLD	CSAR**S**GL**SD**TQYF	TRAV19	CALSDYGGYNKLIF	TRAJ4
19	CMV-pp65-RPH	CASSPQRNTEAFF	TRAV23DV6	CAASIGNFGNEKLTF	TRAJ48
20	CMV-pp65-RPH	CASSPQRNTEAFF	TRAV23DV6	CAASIGNFGNEKLTF	TRAJ48
5	CMV-pp50-VTE	CSARLLGGGQSYEQYF	TRAV1-1	CAAPNNQGGKLIF	TRAJ23
7	CMV-pp50-VTE	CSARLLGGGQSYEQYF	TRAV1-1	CAAPNNQGGKLIF	TRAJ23
23	EBV-BRLF1-YVL	CSAIGGSYNEQFF	TRAV14DV4	CAMR**A**GGNFNKFYF	TRAJ21
18	EBV-BRLF1-YVL	CSAIGGSYNEQFF	TRAV14DV4	CAMR**S**GGNFNKFYF	TRAJ21
25	EBV-EBNA3A-CLG	CASSLEGQ**AS**SYEQYF	TRAV25	CAG**S**GAGSYQLTF	TRAJ28
29	EBV-EBNA3A-CLG	CASSLEGQ**GA**SYEQYF	TRAV25	CAG**L**GAGSYQLTF	TRAJ28
27	EBV-LMP2-FLY	CASS**S**QGGNYGYTF	TRAV17*01	CATEG**N**SGYSTLTF	TRAJ11*01
23	EBV-LMP2-FLY	CASS**Y**QGGNYGYTF	TRAV17*01	CATEG**D**SGYSTLTF	TRAJ11*01
29	EBV-LMP2-FLY	CASS**F**QGGNYGYTF	TRAV17*01	CA**S**EG**N**SGYSTLTF	TRAJ11*01

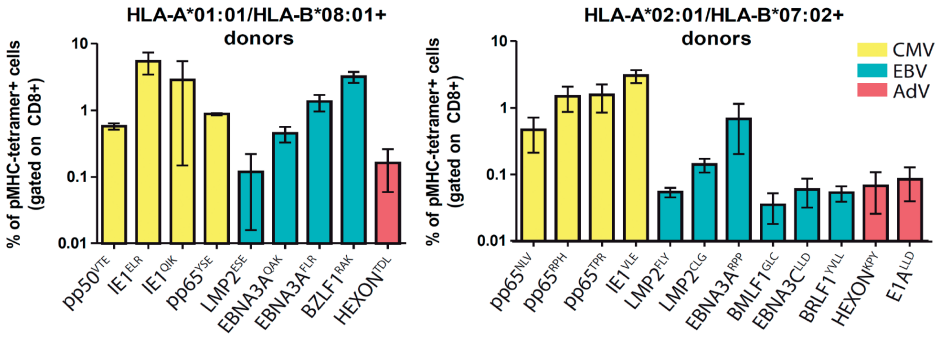
The CDR3-alpha usage of a selected number of virus-specific T-cell populations that contained a PUB-I or PUB-HS CDR3-beta sequence was analyzed. Amino-acids in bold represent differences between donors.



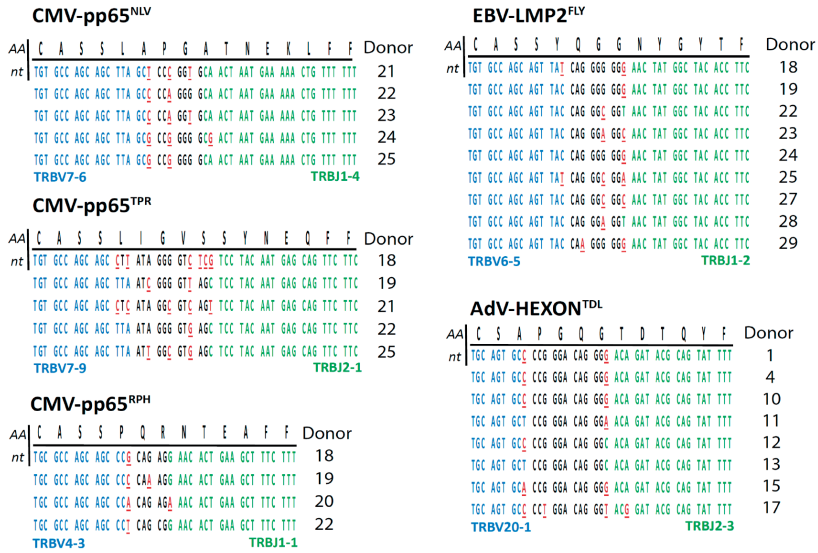
Supplementary Figure 1. Gating strategy for a 4-way single-pMHC-tetramer sort from PBMCs. In total, $30*10^6$ PBMCs were incubated with 4 different pMHC-tetramer complexes, followed by labeling with CD8, CD4 and CD14 monoclonal antibodies. Viable cells were gated based on FSC/SSC followed by gating of $CD8^{pos}$ and $CD4/CD14^{neg}$ T cells. Peptide-MHC-tetramer positive T cells were sorted simultaneously for 4 different specificities in bulk.



Supplementary Figure 2. Experimental setup to generate a library of CDR3β-sequences from virus-specific T-cell populations. **A**) A total of 190 virus-specific T-cell populations, restricted to HLA-A*01:01, HLA-A*02:01, HLA-B*07:02 or HLA-B*08:01 were isolated using 21 different peptideMHC-tetramers. **B**) Virus-specific T-cell populations were lysed and mRNA was harvested. In the first PCR step, primers specific for the C-region and template switching oligos were added to allow for cDNA synthesis and amplification. A second PCR step was performed with a single primer on each site, which adds unique forward and reverse identifiers (6 basepairs) to each PCR-product for each T-cell population. All 190 PCR-products were multiplexed and high-throughput sequenced. **C**) The library was de-multiplexed based on the unique identifiers. The CDR3β-region was determined using bi-directional readings with MIXCR.



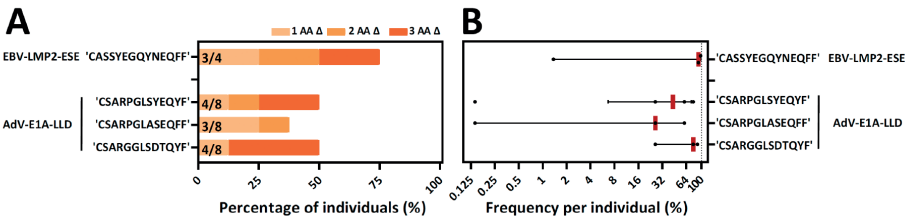
Supplementary Figure 3. Precursor frequencies of virus-specific T cells in healthy individuals. A total of 190 virus-specific T-cell populations, restricted to HLA-A*01:01, HLA-A*02:01, HLA-B*07:02 or HLA-B*08:01 were isolated using 21 different peptideMHC-tetramers. Percentages of CD8 positive pMHC-tetramer positive cells in the starting material are shown for each specificity. CMV and EBV-specific T-cell populations were only sorted from CMV and EBV-seropositive individuals, respectively.



Germline sequences

TRBV4-3	TGCGCCAGCAGCCAAGA	**TRBV7-9**	TGTGCCAGCAGCTTAGC	**TRBJ1-1**	TGAACACTGAAGCTTTCTTT	**TRBJ2-1**	CTCCTACAATGAGCAGTTCTTC
TRBV6-5	TGTGCCAGCAGTTACTC	**TRBV20-1**	TGCAGTGCTAGAGA	**TRBJ1-2**	CTAACTATGGCTACACCTTC	**TRBJ2-3**	AGCACAGATACGCAGTATTTT
TRBV7-6	TGTGCCAGCAGCTTAGC			**TRBJ1-4**	CAACTAATGAAAAACTGTTTTTT		

Supplementary Figure 4. **Identical shared CDR3β amino-acid sequences are found in different individuals with small nucleotide differences as a result of convergent recombination**. The CDR3β nucleotide sequences are shown per donor for 6 identical shared CDR3β amino-acid sequences. Underlined nucleotides in red resemble differences between the different individuals. Nucleotide sequences in blue and green represent perfect alignment with the germline sequences of the TRBV-gene and TRBJ-gene, respectively. The legend represents the germline sequences of (part of) the TRBV and TRBJ genes



Supplementary Figure 5. **Highly similar CDR3β amino-acid sequences in specific T-cell populations that did not contain an identical shared CDR3β amino-acid sequence**. **A**) For two specificities no identical shared CDR3β amino-acid sequences were found.. Individuals did contain highly similar sequences, and these were stacked with 1, 2 or 3 amino-acid differences. The occurrence, shown as percentages among healthy donors, is shown per CDR3β amino-acid sequence. The total number of different T-cell populations (different donors) for each specificity/CDR3β amino-acid sequence is shown at the inner-side of the y-axis. **B**) Shown is the sum of frequencies of the identical and highly similar (1,2 and 3 amino-acid differences) CDR3β amino-acid sequences per individual. Each dot is one individual, and the red-lines represents the medians with interquartile ranges
AA: amino-acids, nt: nucleotides, Δ: difference(s)


BENEFITS

CHAPTER 5

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

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ABSTRACT

Background

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

Methods

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

Results

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

Discussion

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Collection of donor peripheral blood mononuclear cells

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

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

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

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

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

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

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

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

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

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

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

TCR gene transfer into primary T cells and Jurkat E6 cells

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

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

Stimulator cells for functional analyses

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

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

Retroviral transduction of stimulator cells

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

Cytokine production assays to determine functionality

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

Flow-based activation assay of Jurkat E6 cells

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

Cytotoxicity assay

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

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

Quantitative polymerase chain reaction

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

RESULTS

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

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

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

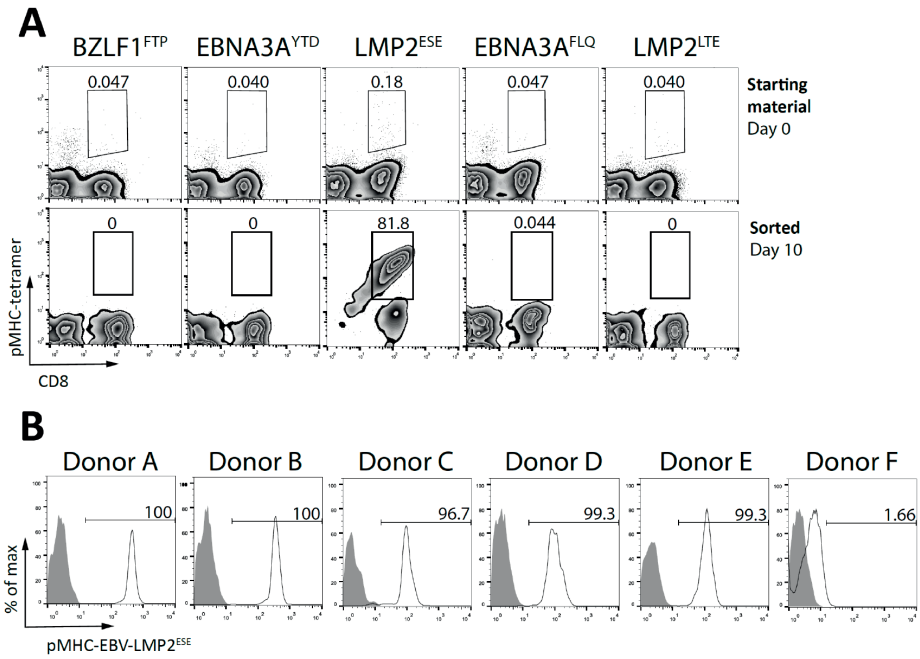

Figure 1. pMHC-tetramer staining of HLA-A*01:01-restricted EBV-specific T cells.
PBMCs from six healthy EBV^{pos} HLA-A*01:01^{pos} donors were incubated with different pMHC-tetramers that were predicted to be strong binders specific for EBV and restricted to HLA-A*01:01. **A**) Representative examples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

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

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

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

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

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

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

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

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

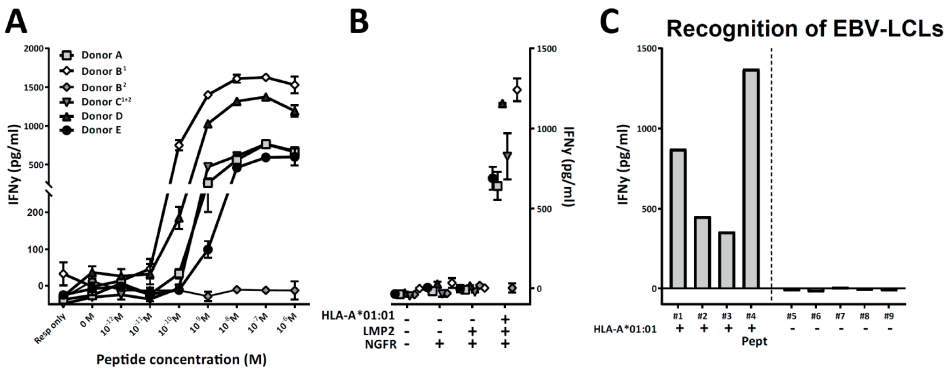

Figure 2. Recognition of exogenously and endogenously presented EBV-LMP2^{ESE} peptide by EBV-LMP2-specific T cells. **A**) Six different EBV-LMP2^{ESE}-specific T-cell populations were stimulated with HLA-A*01:01-transduced K562 cells loaded with titrated concentrations of the respective peptide for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

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

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

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

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

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

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

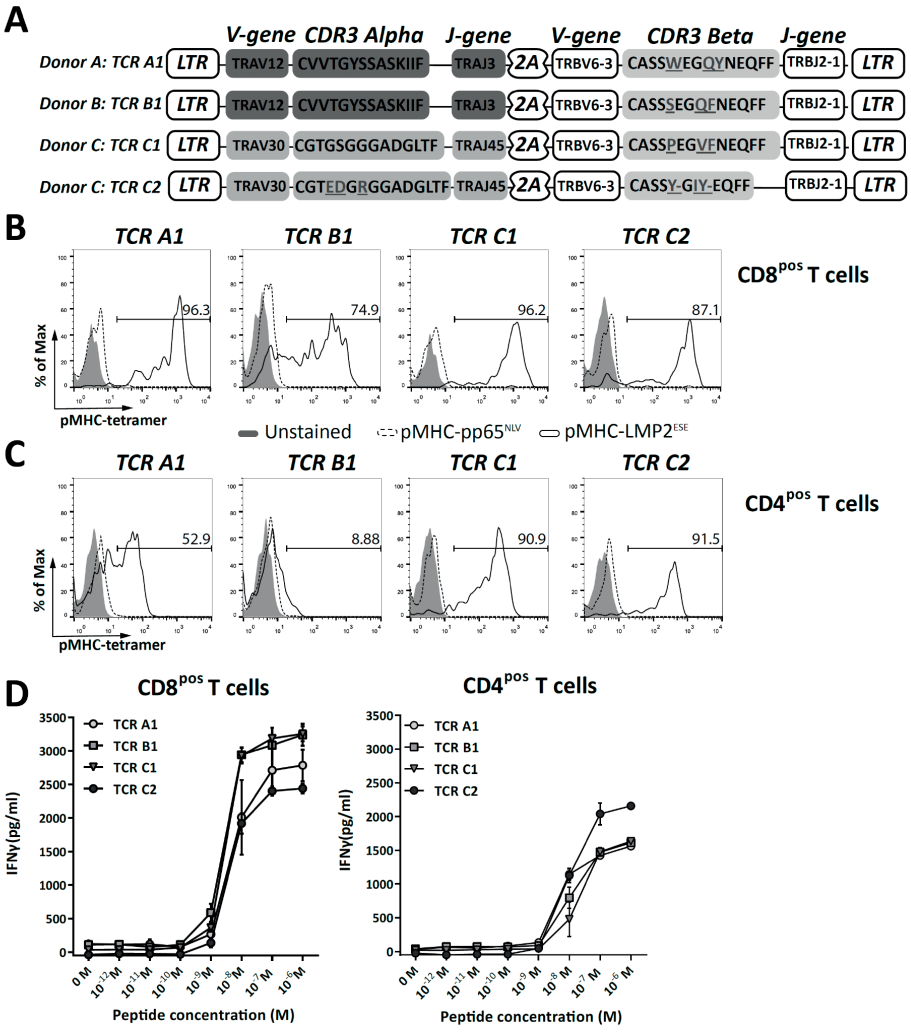

Figure 3. TCR gene transfer introduced EBV-LMP2^{ESE} specificity and reactivity into primary CD8^{pos} and CD4^{pos} T cells.

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

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

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

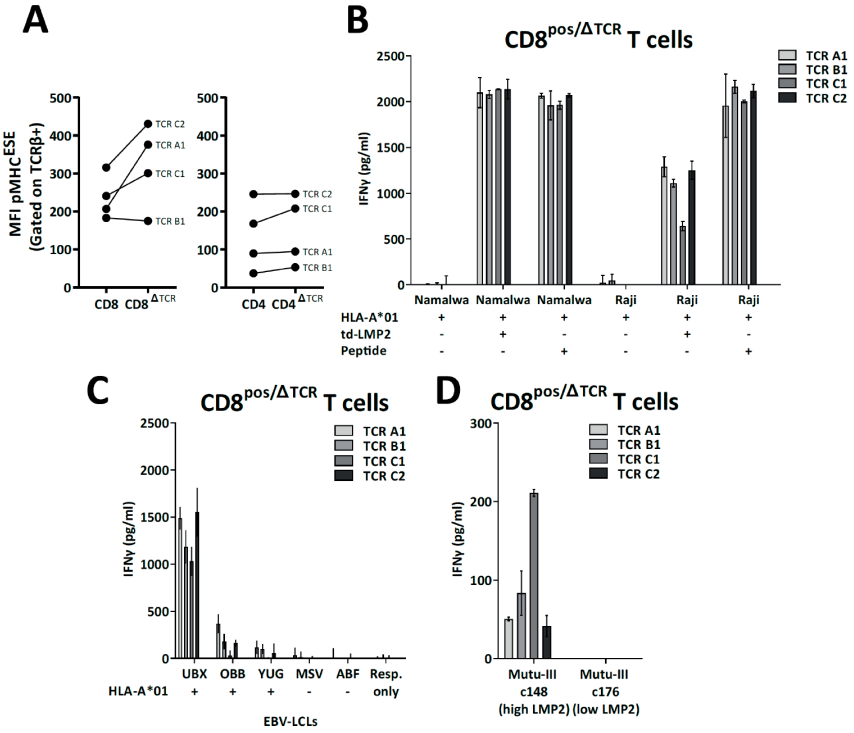

Figure 4: CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs effectively recognize endogenously processed and presented LMP2^{ESE} peptide.
A and **B**) Mean-Fluorescence Intensity (MFI) of pMHC-EBV-LMP2^{ESE} tetramer binding was assessed for CD8^{pos} (**A**) and CD4^{pos}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

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

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

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

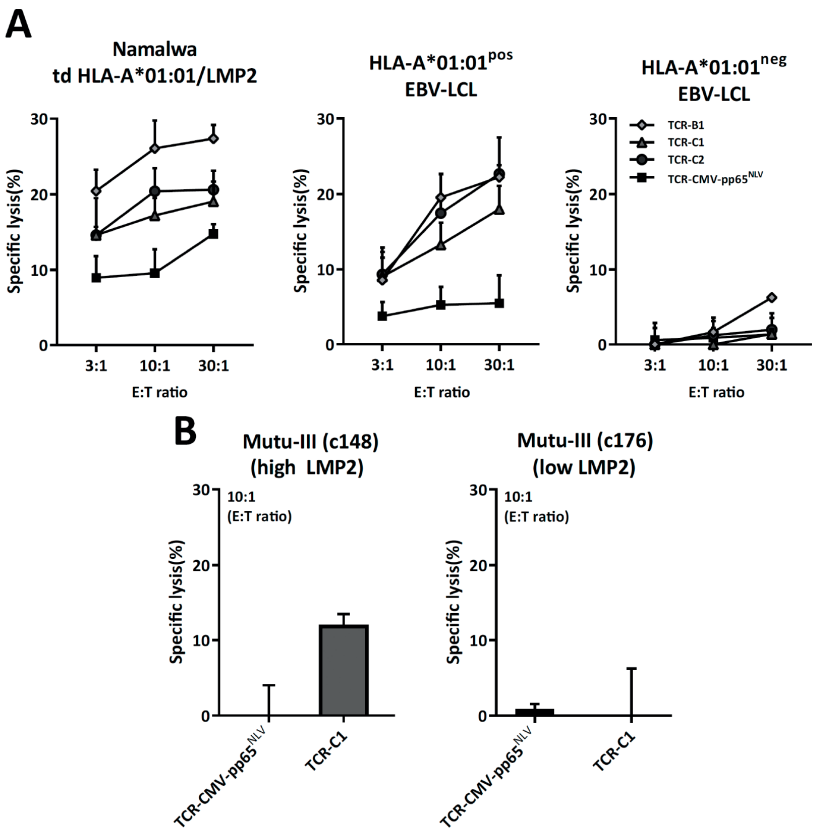

Figure 5. CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs lyse target cells presenting endogenous LMP2 peptide. **A**) Representative examples are shown of CD8$^{pos/\Delta TCR}$ T cells transduced with EBV-LMP2^{ESE}-specific TCRs that were tested for their lytic capacity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

DISCUSSION

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

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

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

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

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

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

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SUPPLEMENTARY MATERIAL

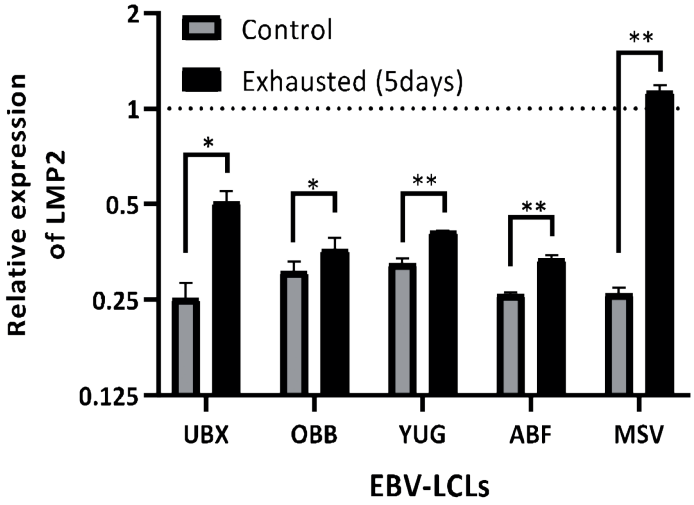
Supplementary Table 1. HLA-typing and EBV-serostatus of healthy donors.

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

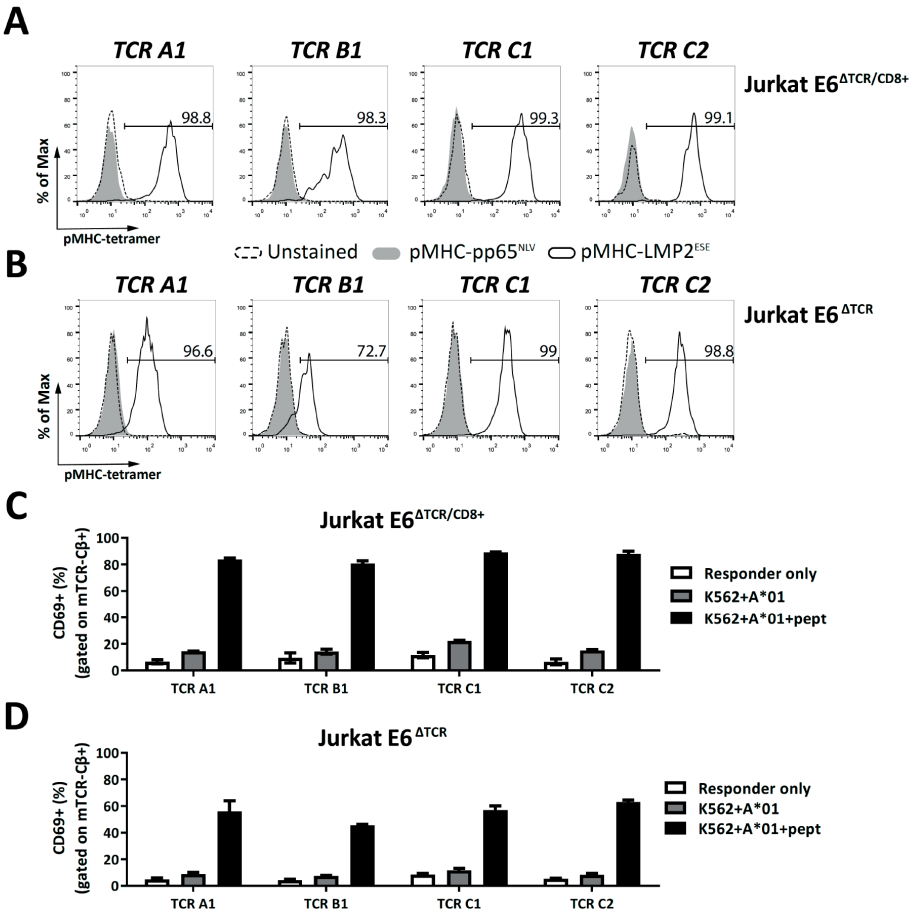
EBV-specific T cells restricted to HLA-A*01:01 were isolated from donors A-F. High resolution HLA-class-I typing was performed for all donors. Some HLA-class-II alleles were not determined, indicated by N.D.
[1]indicates donors used for isolation of $CD4^{pos}$ and $CD8^{pos}$ T cells for TCR-gene transfer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

Supplementary Table 2. Primers used for qPCR

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



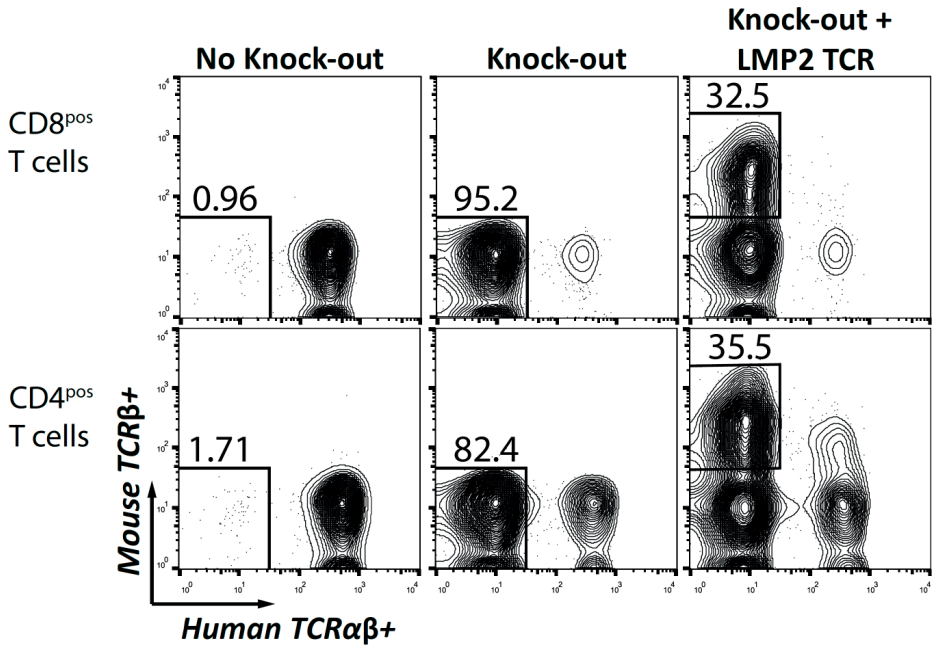
Supplementary Figure 1. Induced LMP2 expression in EBV-LCLs.
Five different EBV-LCLs were cultured under normal conditions and refreshed every 2-3 days or not refreshed for 5 days (exhausted) to induce LMP2 expression. LMP2 mRNA expression was determined by qRT-PCR. Expression shown was calculated as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



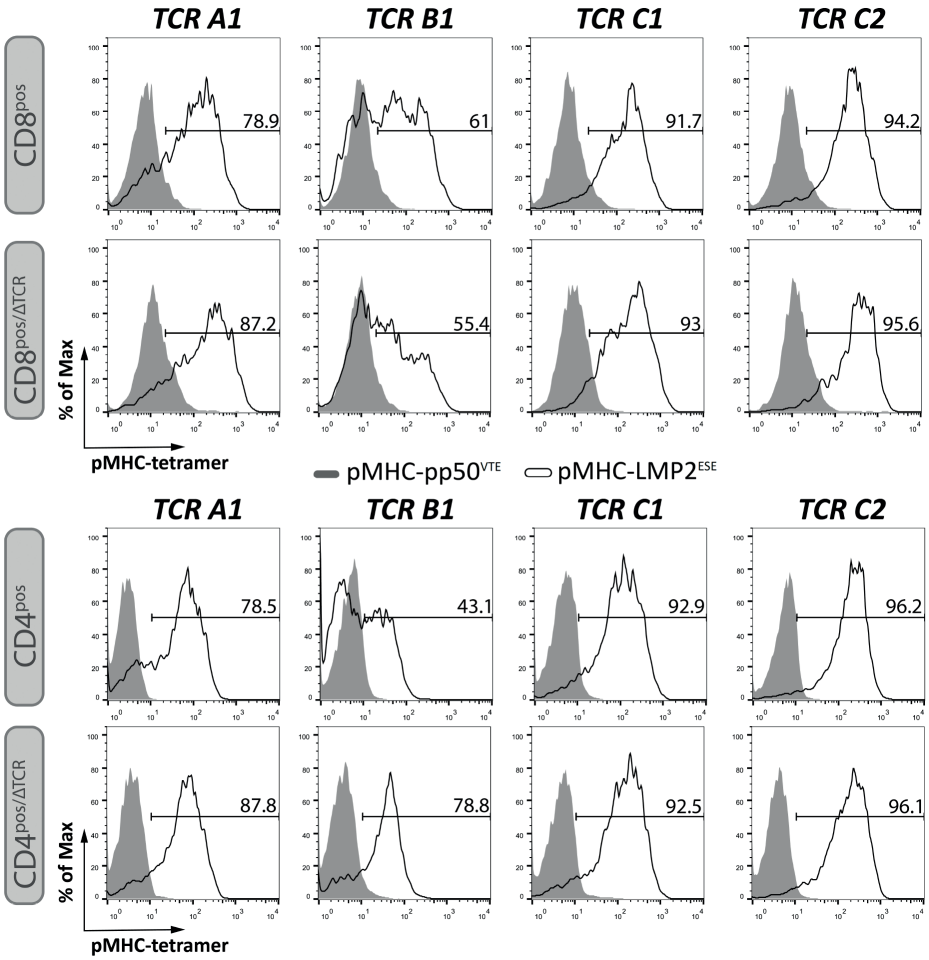
Supplementary Figure 2. TCR gene transfer introduced EBV-LMP2^{ESE} specificity and reactivity into Jurkat E6 cells.

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

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

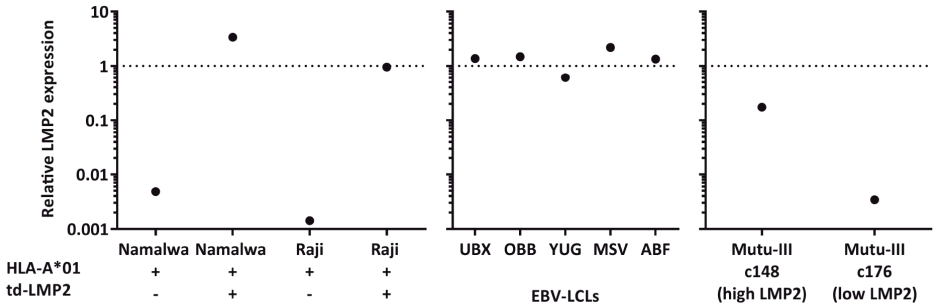


Supplementary Figure 3. Simultaneous CRISPR/Cas9 targeting of the endogenous TCRα and TCRβ. $CD8^{pos}$ and $CD4^{pos}$ T cells were isolated from healthy EBV^{neg} HLA-A*01:01^{pos} donor H, using MACS isolations. The endogenous TCR was knocked out using the CRISPR-Cas9 technology prior to introduction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

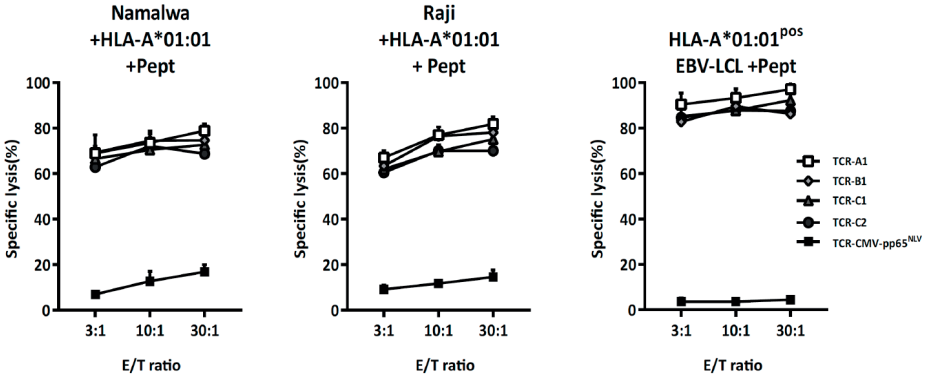


Supplementary Figure 4. pMHC-EBV-LMP2^{ESE} tetramer staining of CD8^{pos} and CD4^{pos} primary T cells with and without endogenous TCR knock-out(CRISPR/Cas9).

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



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



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


BENEFITS?

CHAPTER 6

Amino-acids at position 5 in the peptide/MHC binding region of a public virus-specific TCR are completely inter-changeable without loss of function

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ABSTRACT

Anti-viral T-cell responses are usually directed against a limited set of antigens, but often contain many T cells expressing different T-cell receptors (TCRs). Identical TCRs found within virus-specific T-cell populations in different individuals are known as public TCRs, but also TCRs highly-similar to these public TCRs, with only minor variations in amino-acids on specific positions in the Complementary Determining Regions (CDRs), are frequently found. However, the degree of freedom at these positions was not clear. In this study, we used the HLA-A*02:01-restricted EBV-LMP2^{FLY}-specific public TCR as model and modified the highly-variable position 5 of the CDR3β sequence with all 20 amino-acids. Our results demonstrate that amino-acids at this particular position in the CDR3β region of this TCR are completely inter-changeable, without loss of TCR function. We show that the inability to find certain variants in individuals is explained by their lower recombination probability rather than by steric hindrance.

INTRODUCTION

Human virus-specific $CD8^{pos}$ T cells express a heterodimeric alpha(α)/beta(β) T-Cell Receptor (TCR) that specifically recognizes a viral-peptide in the context of a human leukocyte antigen (HLA)-class-I molecule. The TCR-β chains have highly variable sequences due to recombination of the Variable (V) β genes with their corresponding Diversity (D) and Joining (J) genes and the nucleotides that are added at the V-D-J junctions(1). The TCRα-chains are generated similarly, with the exception of a D gene, resulting in V-J reading frames(2). Gene segment rearrangements could potentially generate a repertoire of 10^{15}-10^{20} unique TCRs(3). The Complementary Determining Regions (CDRs) are the sequences in the TCR that form loops and are responsible for the interaction with the peptide and HLA molecule. The CDR1 and CDR2 regions are fixed within the germline sequence of the V gene and their variability is based on the different V-genes (4, 5). The sequence at the recombined V-D-J and V-J regions encode for the CDR3 region, which is highly variable and greatly determines the specificity of the TCR. Although anti-viral T-cell responses are usually directed against a limited set of viral epitopes(6), they often contain many T cells expressing different TCRs. However, we and others have shown that identical TCRs directed against specific viral peptides can be found in different individuals, known as public TCRs(7-10).

It was recently shown by us and others that anti-viral TCR-repertoires also comprised receptors that were highly-similar to public TCRs and were restricted to the same HLA-molecule and specific for the same peptide(9, 11-15). These highly-similar TCRs were different from the public TCR on specific positions in the CDR3 regions. Key conserved residues in these CDR3 regions were identified as essential elements of TCR recognition(13). High variability of amino-acids was often found on positions at the site of V to D or D to J recombination, but not all 20 amino-acids were found at such a promiscuous position (12, 15). We recently identified an HLA-A*02:01-restricted public TCR with CDR3α [CATEGDSGYSTLTF] and CDR3β [CASSYQGGNYGYTF] that is specific for EBV-$LMP2^{FLY}$ and was found in 9 out of 11 EBV^{pos} HLA-A*02:01^{pos} individuals(16). In total, 10 other highly-similar TCRs were found, that were also specific for EBV-$LMP2^{FLY}$, with amino-acids being only different on position 5 of the CDR3β-sequence [CASSxQGGNYGYTF]. As not all amino-acids were found on this position, it is conceivable that specific rules may still limit the degree of sequence freedom in this location of the CDR3-region. Indeed, evidence was recently provided that positively charged and hydrophobic amino-acids in CDR3β sequences are disfavoured and that TCRs with cysteines in their CDR3 peptide-binding regions are clonally deleted (11). To test why such TCRs are disfavoured, fully human TCRs could be sequenced, altered to express a cysteine in their CDR3-peptide-binding region and transduced into primary T cells. However, transducing fully humanized TCRs into primary T cells might result in the mis-

pairing with endogenous TCRα and/or TCRβ-chains, resulting in unexpected specificity/reactivity(17) and competition for the TCR complex. Mutations of single amino acids to cysteines in each TCR Constant domain can lead to additional disulphide bonds, increasing preferential pairing(18). However, when human TCR Constant regions were replaced by their murine counterparts, an even further decreased expression of the hybrid TCRs was noted(19).

In this study, we used the public TCRαβ sequence specific for HLA-A*02:01-restricted EBV-LMP2^{FLY} as a model and systematically replaced the amino-acid at the highly-variable position 5 in the CDR3β sequence of this public TCR with all amino-acids to investigate whether specific rules apply to this highly-variable position. We transduced all 20 artificially generated TCRβ-sequence variants with the public TCRα-sequence in primary CD8^{pos} T cells of healthy EBV^{neg} HLA-A*02:01^{neg} individuals. We found that all variants remained specific for EBV-LMP2^{FLY} without major differences in functionality. Our results illustrate that amino-acids on position 5 of this public TCR are completely inter-changeable. We show that limitations in recombination probabilities likely restrict the appearance of specific amino-acids in the EBV-LMP2^{FLY}-specific TCR repertoires.

MATERIALS AND METHODS

Cell collection and culturing conditions

EBV-LMP2-FLYALALLL (LMP2^{FLY})-specific T-cell populations were isolated from peripheral blood mononuclear cells (PBMCs) from 11 healthy donors as described previously (20). In short, EBV-LMP2^{FLY}-specific T-cell populations were enriched by fluorescently activated cell sorted (FACS) using EBV-LMP2^{FLY}-specific peptide-HLA complexes. EBV-LMP2^{FLY}-specific T-cell populations were non-specifically expanded and purity was checked after 2 weeks of culture(20). Healthy donors (HLA-A*02:01^{neg} and EBV^{neg}) were selected to isolate primary CD4^{pos} and CD8^{pos} T cells for transduction of the artificially generated TCR-sequences. These primary CD4^{pos} and CD8^{pos} T cells were isolated using magnetic activated cell sorting (MACS) with CD4 and CD8 T-cell isolation kits with LS columns from Miltenyi Biotec (Bergisch Gladbach, Germany). Additional CD25-beads (Miltenyi) were added during CD4^{pos} T-cell isolation to deplete regulatory T cells. All primary T cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Basel, Switserland) additionally containing 5% heat-inactivated human serum (ABOS; Sanquin reagents, Amsterdam, The Netherlands), 5% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, USA), 2.7mM L-glutamine (Lonza), 100U/mL Penicillin (Lonza), 100μg/mL streptavidin (Lonza) and 100IU/mL recombinant-Interleukin-2 (IL-2; Chiron, Emeryville, USA), otherwise referred to as T-cell medium (TCM). EBV-LMP2^{FLY}-specific T-cell populations and primary T cells transduced with artificially generated TCRs

were expanded using TCM supplemented with 0.8µg/mL phytohemagglutinin (PHA; Oxoid Limited, Basingstoke, UK) and five-fold 35gy irradiated autologous or allogeneic PBMCs as feeder cells. The endogenous TCR of Jurkat E6 (Clone E6-1 ATCC®TIB-152) cells was knocked-out (ΔTCR) using a previously described approach(21, 22) and in specific experiments Jurkat E6 ΔTCR cells were transduced with CD8alpha/CD8beta (LZRS-plasmid). EBV-transformed lymphoblastic cell lines (EBV-LCLS) were generated according to standard protocols(23). EBV-LCLs, Jurkat E6 and K562 (ATCC® CCL-243) cells were cultured in stimulator medium consisting of IMDM (Lonza) supplemented with 10% FBS (Invitrogen), 100U/mL penicillin (Lonza), 100µg/mL streptavidin (Lonza) and 2.7mM L-glutamine (Lonza).

Generation of peptide-HLA-A*02:01 complexes

CMV-pp65-NLVPMVATV(pp65^{NLV}) and EBV-LMP2^{FLY} peptides were synthesized in-house using standard Fmoc chemistry. Recombinant HLA-A*02:01 heavy chain and human β2m light chain were in-house produced in Escherichia coli. MHC-class-I refolding was performed as previously described with minor modifications(24). MHC-class-I complexes were purified by gel-filtration using fast protein liquid chromatography (FPLC). Peptide-MHC (pMHC) tetramers EBV-LMP2^{FLY}/HLA-A*02:01 and CMV-pp65^{NLV}/HLA-A*02:01 were generated by labeling of biotinylated pMHC-monomers with streptavidin-coupled phycoerythrin (PE; Invitrogen, Carlsbad, USA). Complexes were stored at -80°C. Formation of stable pMHC-monomers was assessed using UVexchange technology(25) according to a previously described protocol(26).

Retroviral transductions and enrichments

The construct encoding the HLA-A*02:01 sequence was coupled to an IRES sequence with a truncated form of the nerve growth factor receptor (tNGFR) and was cloned into an LZRS plasmid. This construct was verified using reverse transcriptase polymerase chain reactions (RT-PCR) and Sanger sequencing. As an additional control, tNFGR only was cloned into an LZRS plasmid (mock). Retroviral transduction was performed as previously described (27). K562 wild type cell-lines were transferred to wells containing stable retroviral particles, generated using a puromycin selected stable ϕ-NX-amphotropic packaging cell-line, and incubated for 24 hrs at 37°C (28). Transduced cell-lines were subsequently enriched by Fluorescent Activated Cell Sorting (FACS) for expression of tNGFR using APC-labeled tNGFR antibodies (CD271; Southern Biotech Associations, Alabama, USA). In total, a median of $1*10^6$ cells (range $0.4\text{-}6*10^6$) FACS enriched EBV-LMP2^{FLY}-specific T cells were used to determine the TCR variable beta(β) sequence. TCR variable beta(β) sequences used by FACS enriched EBV-LMP2^{FLY}-specific T-cell populations were determined using ARTISAN PCR adapted for TCR PCR as previously described (16, 22, 29). CDR3β-sequences were built using MIXCR software with [default] settings and a limit of processing $10*10^6$ input sequences per FACS enriched EBV-LMP2^{FLY}-specific T-cell

population in combination with a bi-directional reading approach (5'-3' and 3'-5' read) (16, 30). As a cut-off, all sequences present above 0.1% in the enriched EBV-LMP2^{FLY}-specific T-cell populations were included in the analyses(16). T-cell clones were generated from EBV-LMP2^{FLY}-specific T-cell populations using limited dilution to determine the TCR variable alpha(α) sequences using SANGER sequencing. Twenty different retroviral vectors that contained the codon-optimized cys-cys TCR-constant-modified T-cell receptor α sequence: TRAV17, CDR3α [CATEGDSGYSTLTF] TRAJ11 and cys-cys TCR-constant-modified varying β sequence: TRBV6-5, CDR3β [CASSxQGGNYGYTF] TRBJ1-2 with all 20 different amino-acids on position 5 were constructed on MP71 backbones with murineTCR constant (mTCR-C) α/β sequences and joined by a P2A sequence as previously described(18, 22) and ordered from Baseclear (Leiden, The Netherlands). Codon-optimization was for all TCR-constructs the same, and only differed on position 5 of the CDR3 β sequence (**Supplementary Figure 1**). ϕ-NX-amphotropic packaging cells were transfected with MP71 vectors and pCL-ampho retrovirus packaging using FuGENE HD (Roche, Basel, Switzerland) according to the manufacturer's instructions and retroviral supernatant was harvested after 48 hours. Primary HLA-A*02:01^{neg} and EBV^{neg} CD4^{pos} and CD8^{pos} T cells were non-specifically activated for 48 hours using an autologous feeder mixture and PHA as described above, prior transduction. Primary T cells and Jurkat E6 cells were transduced with retroviral supernatant that contained the TCRα and TCRβ sequences in rectronectin-coated 24 wells-plates (100,000 cells per well). To determine the purity of MACS isolated primary CD8^{pos} and CD4^{pos} T cells, the cells were stained with PE-conjugated anti-CD4 or anti-CD8 (BD Pharmingen) for 20 minutes at 4°C. MACS enrichments using APC-labeled mTCR-Cβ antibodies (BD) and anti-APC-microbeads (Miltenyi) were performed in order to purify TCR-transduced populations. Transduction efficiencies and purities after MACS enrichments were assessed after 10 days of culturing by staining transduced cells with APC-labeled mTCR-Cβ-specific antibodies (BD) for 20 min at 4°C. Prior to mTCR-Cβ staining, cells were stained with PE-labeled HLA-A*02:01/pMHC-LMP2^{FLY}-specific pMHC-tetramers to determine their capacity to bind HLA-A*02:01/pMHC-LMP2^{FLY}-specific pMHC-tetramers. As a control, cells were stained with PE-labeled HLA-A*02:01/pMHC-CMV-pp65^{NLV}-specific pMHC-tetramers.

Functional assays

Interferon-γ (IFN-γ) production by TCR-transduced primary T cells was quantified using standard enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (Diaclone, Besançon, France). Responder T cells were co-cultured with stimulator cells at a ratio of 1:5 (responder: stimulator) for 16 hours at 37°C in TCM using 25IU/mL IL-2 instead of 100IU/mL IL-2. To measure activation of TCR-transduced Jurkat E6$^{\Delta TCR}$ cell-lines upregulation of activation marker CD69 was analyzed using flow cytometry. Responder TCR-transduced Jurkat E6$^{\Delta TCR}$ cells were stimulated with HLA-

A*02:01-transduced K562 cell-lines with or without exogenous peptide loading (10^{-6}M) at a ratio of 1:10 (responder:stimulator, R:S) in stimulator medium for 16 hours at 37°C. After O/N incubation, cells were washed twice before adding CD69-PE (Invitrogen), mTCR-Cβ-APC (BD) and CD8-PerCP (BD) monoclonal antibodies for 30 min at 4°C. All analyses were performed on a FACS Calibur (BD), and analyzed using Flowjo Software (TreeStar, Ashland, USA).

Data availability statement

The data that support the findings of this study are openly available on Mendeley at DOI: 10.17632/zzyrgzcwdp.1 , (https://data.mendeley.com/datasets/zzyrgzcwdp/1)

Ethics Approval

After informed consent according to the Declaration of Helsinki, healthy individuals (homozygously) expressing HLA-A*02:01 and HLA-B*07:02 were selected from the Sanquin database and the biobank of the department of Hematology, Leiden University Medical Center (LUMC).

RESULTS

Artificially generated TCR-sequences keep specificity despite amino-acid changes in the CDR3β-region

Analysis of HLA-A*02:01-restricted EBV-LMP2^{FLY}-specific TCR-repertoires, showed the presence in 9 out of 11 individuals of a public TCR-CDR3β region [CASSYQGGNYGYTF] (16, 31). T-cell clones that were generated from two individuals that expressed this public CDR3β sequence or highly-similar CDR3β-sequences (CASSPQGGGDGYTF, CASSRQGGSYGYTF, CASSGQGGGDGYTF), revealed to express a public CDR3α [CATEGDSGYSTLTF] as well. Moreover, 10 highly similar receptors were also found, which only differed at position 5 of the CDR3β. Also, highly similar receptors were found to differ at position 9/10 of the CDR3β sequence, but all 10 highly-similar receptors at least expressed the [NY] motif at positions 9/10. Since 9 amino-acids were never found at position 5 of the CDR3β, we asked whether these would interfere with the ability of the receptor to recognize EBV-LMP2^{FLY} in HLA-A*02:01. We first modeled the public TCR, with an [Y] on position 5 which was encoded by the germline TRBV6-5 gene (from here on referred to as wild type TCR), using a TCRmodeling algorithm to visualize position 5 in respect to HLA-A*02:01 and the FLYALALLL peptide (**Supplementary Figure 2**)(32). Although no formal crystal structure is known, position 5 seems to be at the spot whereby interaction with the peptide-HLA-complex cannot be ruled out, suggesting that the 9 highly similar CDR3β-sequences that contained amino-acids on position 5 that were not found could have resulted in a loss of specificity. To further test this, we

generated a TCR panel of this public TCR in which we substituted the Tyrosine [Y] at position 5 of the CDR3β-sequence with all 19 other amino-acids. We distinguished the public wild type CDR3β variant (Red) and 10 highly-similar CDR3β variants that were found *ex vivo* (Blue) in healthy individuals from the 9 CDR3β variants that were not found *ex vivo* (Orange) (**Figure 1A**). All artificially generated EBV-LMP2^{FLY}-specific TCRs were introduced into primary CD8^{pos} T cells isolated from peripheral blood of a healthy EBV-seronegative, HLA-A*02:01-negative donor. Transduction efficiencies ranged from 6-43% (**Supplementary Figure 3A: left**) and MACS enrichment, based on the presence of a murine-TCR Cβ epitope present in the transgenic TCRs(22, 33), yielded populations in which 84-99% T cells expressed the transgenic TCRs (**Supplementary Figure 3A: right**). Specific binding of HLA-A*02:01/pMHC-EBV-LMP2^{FLY} tetramers was demonstrated for all EBV-LMP2^{FLY}-specific TCR-transduced CD8^{pos} T-cell populations, regardless of whether the substituted amino-acids were found (**Figure 1B**) or not found *ex vivo* (**Figure 1C**). Although all TCRs were able to bind EBV-LMP2^{FLY}-specific pMHC-tetramer, heterogeneous binding was observed for TCR 13 [C], TCR 16 [D] and TCR 17 [E] transduced CD8^{pos} T cells, and low binding was observed for TCR 18 [K]. TCR-transduced CD8^{pos} T cells did not stain with a negative control pMHC-tetramer containing an irrelevant HLA-A*02:01-restricted CMV peptide (Representative examples; **Figure 1B and 1C**; pMHC-irr).

Heterogeneous EBV-LMP2^{FLY}-specific pMHC-tetramer binding could be indicative of low TCR avidity, with differences in functionality. To investigate the TCR avidity, we evaluated dependence on CD8 for binding to pMHC-EBV-LMP2^{FLY}-specific tetramers by introducing all 20 EBV-LMP2^{FLY}-specific TCRs into primary CD4^{pos} T cells (CD8^{neg}) isolated from a healthy EBV-seronegative, HLA-A*02:01-negative donor. TCR-transduction efficiencies ranged from 17-58% and further purification based on the expression of the introduced murine constant region resulted in pure TCR-transduced CD8^{neg} T cells (range 90-99%; **Supplementary Figure 3B**). Overall, a mean 1.9 (range 1.2-3.0) fold lower Mean Fluorescence Intensity (MFI) of EBV-LMP2^{FLY}-specific tetramer binding was observed for TCRs transduced in CD8^{neg} T cells compared to CD8^{pos} T cells (**Figure 1D**, **1E** and **Supplementary Figure 4A**). Surface expression of the introduced TCRs was similar in CD8^{neg} and CD8^{pos} T cells, as determined by the MFI of the murine TCR-Cβ (**Supplementary Figure 4B**). However, CD8^{neg} T cells transduced with TCR 3 [G] and TCR 16 [D] showed low and heterogeneous EBV-LMP2^{FLY}-specific pMHC-tetramer staining. Because the endogenous TCR was still present in these TCR-transduced T cells, competition for CD3 between the endogenous TCR and newly introduced TCR, could contribute to the heterogeneity in pMHC-tetramer binding. Therefore, we additionally transduced all EBV-LMP2^{FLY}-specific TCRs in CD8-transduced Jurkat E6 cells that did not express an endogenous TCR (ΔTCR). Heterogeneous EBV-LMP2^{FLY}-specific pMHC-tetramer staining was not observed in CD8^{pos} or CD8^{neg} Jurkat E6 cells (**Supplementary Figure 5**). Although CD8^{neg} Jurkat E6 cells transduced with TCR 3 [G] and TCR 16 [D]

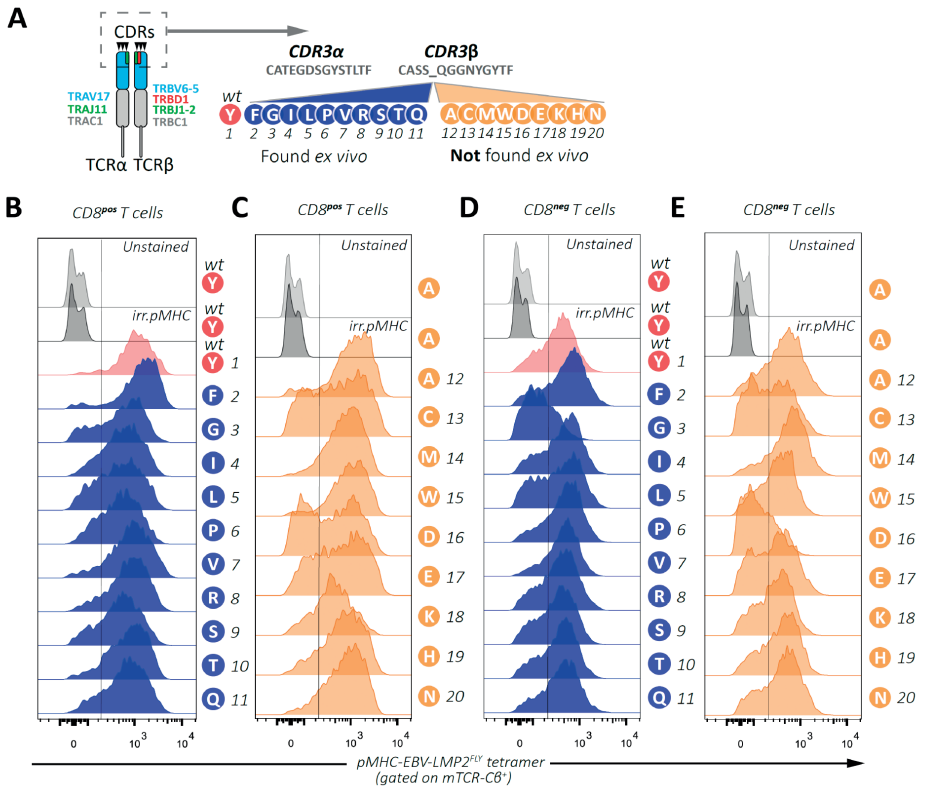

Figure 1. Amino-acid substitutions in the complementary determining region 3 of HLA-A*02:01-restricted EBV-LMP2-specific TCRs show overall maintenance of specificity in primary CD8^{pos} and CD8^{neg} T cells. Primary CD8^{pos} and CD4^{pos} (CD8^{neg}) T cells were isolated using MACS. TCR-transduced primary T cells were purified based on expression of murine-TCR-Cβ using MACS and expanded. Double positive CD4^{pos} CD8$^{pos}_{FLY}$ T cells were not observed in the analyses. **A**) The T-cell receptors of FACSorted memory EBV-LMP2^{FLY} -specific T-cell populations were sequenced from peripheral blood of 11 different donors. The identical (public) HLA-A*02:01-restricted EBV-LMP2^{FLY} -specific TCR with the CDR3β sequence CASSYQGGNYGYTF was found in 9 out of 11 individuals (Red). CDR3β sequences highly-similar to this public sequence were found that contained either one of 10 different amino-acids on position 5 of the CDR3β-sequence (Blue). Of all possible amino-acids, 9 amino-acids on position 5 of HLA-A*02:01-restricted EBV-LMP2^{FLY} -specific CDR3β sequences, were not found *ex vivo* (orange). **B** and **C**) Shown are histograms of specific HLA-A*02:01/pMHC-EBV-LMP2^{FLY} tetramer (red, blue and orange) or irrelevant HLA-A*02:01/pMHC-CMV-pp65^{NLV} tetramer (black) stainings of CD8^{pos} T cells transduced with TCRs that were found (**B**) or that were not found *ex vivo* (**C**). **D** and **E**) Shown are histograms of specific HLA-A*02:01/pMHC-EBV-LMP2^{FLY} tetramer (Red, Blue and Orange) or irrelevant HLA-A*02:01/pMHC-CMV-pp65^{NLV} tetramer (black) stainings of CD8^{neg} T cells transduced with TCRs that were found (**D**) or that were not found ex *vivo* (**E**).
Shown are data from one representative experiment (performed twice) using PBMCs from one donor.
Abbreviations: MACS, Magnetic Activated Cell Sorting. irr.pMHC, irrelevant pMHC tetramer. wt, wild type. CDR, Complementary Determining Region. TRAV/TRBV, T-cell Receptor Alpha/Beta Variable. TRBD, T-cell Receptor Beta Diversity. TRAJ/TRBJ, T-cell Receptor Alpha/Beta Joining. TRAC/TRBC, T-cell Receptor Alpha/Beta Constant

clearly bound EBV-LMP2^{FLY}-specific pMHC-tetramer, both pMHC-tetramers stained with the lowest MFI. Overall, these data show that all TCRs were able to bind EBV-LMP2^{FLY}-specific pMHC-tetramers in CD8^{pos} T cells and to some extent when transduced into CD8^{neg} T cells. Importantly, while some amino-acids (G and D) did diminish binding, this did not correlate with their prevalence in primary EBV-LMP2^{FLY}-specific repertoires, where variants with a G residue on position 5 were found.

Functionality of EBV-LMP2^{FLY}-specific TCR-transduced primary CD8^{pos} and CD8^{neg} T cells

To investigate whether the functional reactivity of EBV-LMP2^{FLY}-specific TCR-transduced primary CD8^{pos} and CD8^{neg} T cells would be affected by the amino-acid substitutions at position 5 of the CDR3β sequence, we performed a stimulation with HLA-A*02:01-transduced K562 cells exogenously loaded with varying concentrations of the EBV-LMP2^{FLY} peptide. Similar dose-dependent induction of IFNγ production was observed by CD8^{pos} T cells transduced with wild type [Y] EBV-LMP2^{FLY}-specific TCRs or all other 19 TCR variants with only minor differences in sensitivity to peptide dose (**Figure 2A and 2B**). Moreover, there was no clear difference in the sensitivity to peptide dose between TCR variants that were found *ex vivo* and those that were not. In fact, some of the latter even exhibited greater sensitivity than the wild-type [Y] TCR eliciting production of IFNγ at lower peptide concentrations (**Figure 2A and 2B**). Two TCR variants, TCR 3 [G] and TCR 16 [D], were less able to elicit production of IFNγ when expressed in CD8^{neg} T cells. TCR 3 [G] was only able to produce IFNγ at high peptide concentrations, while TCR 16 [D] seemed to be non-functional (**Figure 2C and 2D**). This reduced functionality of TCR 3 [G] and TCR 16 [D] was also observed when expressed in CD8^{neg}, but not in CD8^{pos} Jurkat E6$^{\Delta TCR}$ cells, as measured by upregulation of CD69 (**Supplementary Figure 6**). The limited functionality of TCR 3 [G] and TCR 16 [D] was in line with previous EBV-LMP2^{FLY}-specific pMHC-tetramer staining results. To investigate whether EBV-LMP2^{FLY}-specific TCR-transduced primary CD8^{pos} T cells were also capable of recognizing endogenously processed LMP2^{FLY}, we performed a stimulation with an HLA-A*02:01^{pos} EBV-transformed lymphoblastoid cell line (EBV-LCL). Similar induction of IFNγ production upon stimulation with the HLA-A*02:01^{pos} EBV-LCL was observed for CD8^{pos} T cells with EBV-LMP2^{FLY}-specific TCRs found *ex vivo* (**Figure 2E**) and with EBV-LMP2^{FLY}-specific TCRs not found *ex vivo* (**Figure 2F**).

No IFNγ production was observed upon stimulation with HLA-A*02:01 negative EBV-LCLs. In conclusion, there was no correlation between the presence or absence of specific TCR variants in the naturally occurring *ex vivo* HLA-A*02:01-restricted EBV-LMP2^{FLY}-specific TCR-repertoire and functionality. Although two TCR variants exhibited limited pMHC-tetramer staining and reduced functionality, especially in a CD8^{neg} context, one of these was found in the *ex vivo* repertoire, while the other was not found in FACS enriched and *in vitro* expanded EBV-LMP2^{FLY}-specific T-cell populations of the 11 individuals that were studied. This might suggest that these properties in CD8^{neg} T cells are not factors important for natural selection. Most importantly, all artificially generated TCR variants showed equal functionality in the presence of CD8, which is their natural context. We therefore conclude that the absence of specific amino-acids on position 5 in the *ex vivo* found HLA-A*02:01-restricted EBV-LMP2^{FLY}-specific "family" of public TCRs is not explained by constraints on TCR binding to the EBV-LMP2^{FLY}- HLA-A*02:01 complex.

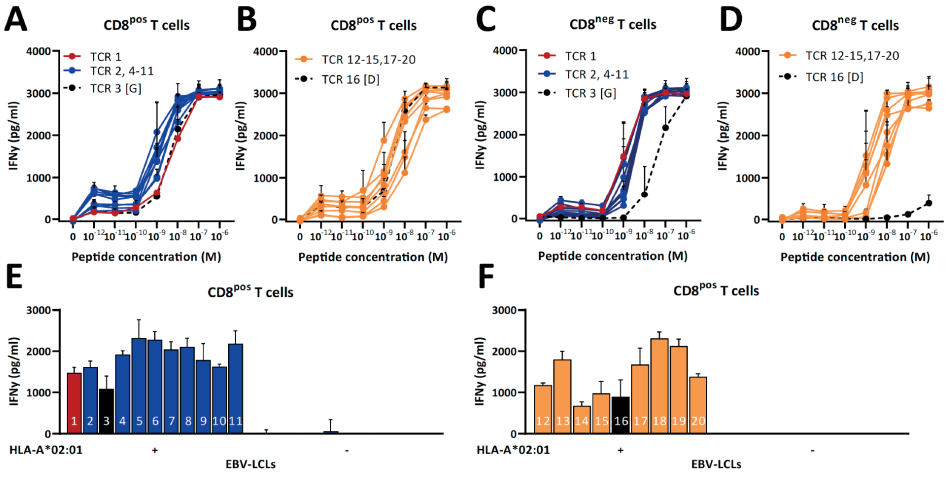

Figure 2. Functionality of EBV-LMP2^{FLY}-specific TCR-transduced primary CD8^{pos} and CD8^{neg} T cells. K562 cells were loaded with titrated concentrations of EBV-LMP2^{FLY} peptide for 16 hours and were used to investigate recognition by TCR-transduced primary CD8^{pos} and CD8^{neg} T cells. EBV-transformed B lymphoblastoid cell lines (EBV-LCLs) were used to study the recognition of endogenously produced EBV-LMP2^{FLY} peptide.IFNγ was measured by standard ELISA. **A** and **B**) Shown are responses of TCR-transduced CD8^{pos} T cells with TCRβ-sequences that were found (**A**) or that were not found *ex vivo* (**B**). **C** and **D**) Shown are responses of TCR-transduced CD4^{pos} (CD8^{neg}) T cells with TCRβ-sequences that were found (**C**) or that were not found *ex vivo* (**D**). **E** and **F**) IFNγ production upon stimulation with HLA-A*02:01 positive EBV-LCL is shown for TCR-transduced CD8^{pos} T cells with TCRβ-sequences that were found (**E**) or that were not found (**F**) *ex vivo*. HLA-A*02:01 negative EBV-LCLs were used as negative control.
Black symbols with dotted lines and black bars indicate TCRs that performed poorly when transduced into CD4^{pos} (CD8^{neg}) T cells.
Shown are data with means with standard deviations carried out in triplicate of one representative experiment (performed twice).

Occurrence of amino-acids on position 5 of the CDR3β correlate with the recombination probability

Despite the fact that the CDR3β sequence allows complete freedom to all amino-acids on position 5, 9 amino-acids were never found among a total of 251 different TCRs analyzed(16, 31), even though most of the other 11 amino-acids were found repeatedly in FACS-enriched and *in vitro* expanded EBV-LMP2^{FLY}-specific T-cell populations from different individuals, at frequencies ranging from 0.1%-42% (**Figure 3A**). We reasoned that the absence of these amino-acids might be a consequence of a bias in the recombination process(34, 35). To test this idea, we computed the recombination probability (pGen) of all 20 possible CDR3β-sequence variants. The recombination probability was calculated using the Optimized Likelihood estimate of immunoGlobulin Amino-acid sequences (OLGA) algorithm, which is able to compute the generation probabilities of TCR amino-acid sequences(36). The public wild type CDR3β-sequence (Red) and the 10 variants (Blue) that were found *ex vivo*, were significantly more likely to be generated than the variants containing one of the other 9 amino-acids at position 5 (Orange) (**Figure 3B**; p=0.014; unpaired *t* test, two-tailed). Furthermore, the high recombination probability correlated with the number of individuals with EBV-LMP2^{FLY}-specific T-cell populations that contained these CDR3β-sequences (**Figure 3C**). Therefore, we conclude that the bias against specific amino-acid usage on position 5 of the CDR3β is most likely explained by constraints imposed by the recombination process.

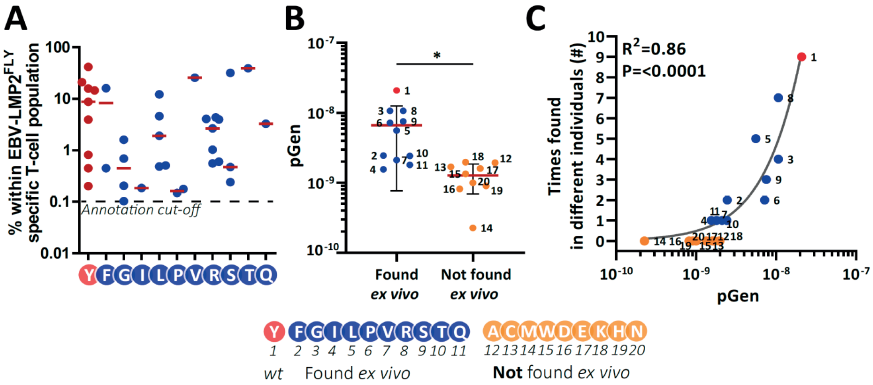

Figure 3. Optimized Likelihood estimate of immunoGlobulin Amino-acid sequences (**OLGA**) **shows that the variants containing CDR3β sequences that were not found *ex vivo* have a lower recombination probability**.**A**) Shown are frequencies of EBV-LMP2^{FLY}-specific TCRβ-sequences within each FACS enriched and expanded EBV-LMP2^{FLY}-specific T-cell population. Each dot represents a different individual. Only TCRβ-sequences above 0.1% were used. **B**) Shown are the generation probabilities (pGen) of EBV-LMP2^{FLY}-specific TCRβ-sequences that were found (Red and Blue) or that were not found *ex vivo* (Orange). **C**) The EBV-LMP2^{FLY}-specific TCRβ-sequences were identified from a total of 11 healthy donors. We investigated if the generation probabilities (pGen) correlated with the number of times that these sequences were found in peripheral blood of 11 different donors.
Statistical differences were assessed with the unpaired *t* test (**B**) and the Spearman's correlation coefficient *r* (**C**). Shown are the means (red lines) with standard deviations (error bars). *P<.05
Abbreviations: pGen, Recombination probability.

DISCUSSION

In this study, we investigated whether all amino-acids were possible at the highly-variable position 5 of the CDR3β sequence of a public HLA-A*02:01-restricted EBV-LMP2^{FLY}-specific TCR, independent of the amino-acid properties (e.g. charge, hydrophobicity and size). Position 5 of this CDR3β sequence [CASSxQGGNYGYTF], was recently identified as highly-variable since 11 different, but highly-similar TCRs, were found *ex vivo* in EBV-LMP2^{FLY}-specific T-cell populations from healthy individuals(16). It was reported before that TCRs with a cysteine [C] in the CDR3β sequence would be dysfunctional and CDR3β sequences with positively charged and hydrophobic amino-acids are disfavored(11). However, we demonstrated that the amino-acids on position 5 of the EBV-LMP2^{FLY}-specific CDR3β sequence were completely inter-changeable and they did not significantly influence the specificity or functionality as long as CD8 was present. Heterogeneous pMHC-tetramer staining was observed in TCR-transduced CD8^{pos} T cells, but not when these TCRs were transduced in Jurkat E6 cells, showing that the heterogeneous staining was not due to intrinsically insufficient pairing of the artificial TCRα and TCRβ-chains. Since there was no clear difference in functionality between variants, it is likely that certain CDR3β sequences were not found *ex vivo* in EBV-LMP2^{FLY}-specific T-cell populations due to limitations in the genetic recombination process. We indeed showed that CDR3β sequences that encode for the "missing" 9 amino-acids had a lower chance to be generated during V-D-J recombination (recombination probability(36)) compared to the 11 TCRβ sequences that were found in EBV-LMP2^{FLY}-specific T-cell populations *ex vivo*.

We and others have found many TCRs that are highly-similar to public TCRs and these highly-similar TCRs often differed on specific positions in the CDR3 region (13-16, 22, 31, 37). Position 5 of our public TCR [CASSYQGGNYGYTF] was not the only position that differed on a specific position compared to other highly-similar TCRs. Also position 9/10 [NY] showed high-variability, but not as much as position 5. Because the majority of different amino-acids were found on position 5 in combination with the [NY] sequence, we kept this part constant for all artificially generated TCRs.Whether only specific amino-acids are allowed on such variable positions in the CDR3 regions or whether such positions are completely inter-changeable with any amino-acid had not yet been investigated. It has been described that some CDR3 sequences have a higher likelihood to be generated by V(D)J-recombination, also known as the recombination probability (36, 38). Therefore, based on chance, some amino-acids could be less frequently observed in the CDR3-regions which could explain why not every variant was found. Indeed, for our public wild type TCR, the first 5 amino-acids are directly derived from the TRBV6-5 gene and the last 6 amino-acids are from the TRBJ1-2 gene without modifications, explaining why this variant has the highest generation probability and occurrence *in vivo.* Other variants that were found require additional trimming of nucleotides encoding

the tyrosine [Y] at the end of the TRBV6-5-gene. Therefore, based on chance, some variants might be more likely to be found. Increasing the number of individuals will most likely ultimately result in the identification of more TCR variants from the "not found" group of TCRs. From a TCR point of view to dock to the peptide-HLA complex, it may be assumed that the CDR3β-sequence only allows substitutions of amino-acids with similar propertiesor that position 5 does not interfere/interact with the peptide-HLA complex. However, the TCRmodel(32) used to visualize the TCR-peptide-HLA structure showed that positions 5, 6 and 7 are at the loop of the CDR3β region, which is known to interact with the peptide-HLA. However, important to note, this is not a formal crystal structure, but a model generated using the TCR model algorithm(32). For the wild type tyrosine [Y] amino-acid, we expected to find highly similar TCRs with amino-acids on position 5 with the same polarity [S,T,N and Q] or with the same aromatic structure [F]. However, positively charged [R] and non-polar [G,V,P,L and F] amino-acids were also found *ex vivo*. There was no correlation in charge, size or polarity of the amino-acids that were found *ex vivo* compared to those that were not. More strikingly, the hydrophobic amino acid tyrosine [Y] is very similar to phenylalanine [F] since they both contain an aromatic ring and are only differing by an -OH group. However, T cells expressing EBV-LMP2^{FLY}-specific TCRs with an [F] on position 5 of the CDR3β sequence were only found in 2 individuals, while T cells expressing TCRs with a [Y] on position 5 were found in 9 individuals, which correlates with the recombination probability, but not with the similary of the amino-acids. It has also been recently demonstrated that the CDR3α and even CDR1α contribute strongly to the peptide specificity(39, 40). It could be argued that minor alterations in the CDR3β are allowed as long as sufficient binding to the peptide-HLA is maintained by other CDR regions, thus belonging to one public TCR family.

Lower tetramer staining and functionality as observed for certain amino-acids in TCR-transduced CD8^{neg} T cells may indicate differences in TCR affinity, but this apparently is insufficient to result in reduced functional avidity of CD8^{pos} T cells expressing these TCRs. Stability of the TCR-CD4/CD8 coreceptor-MHC interactions and half-lives of the TCR-MHC complex are more likely to play a role in the differences observed. It is already known that CD8 can stabilize peptide/MHC-class-I binding of TCRs(41). In contrast, past studies and recent *in situ* measurements at intercellular junctions, show that CD4 does not stabilize interactions of TCRs with their natural peptide/MHC-class-II molecules(42, 43) This makes it unlikely that CD4 can stabilize interactions of TCRs with peptide/MHC-class-I molecules, although this has not been tested. Some of our generated TCR variants might therefore have a shorter half-life in CD4 T cells resulting in more heterogeneous/lower tetramer staining.

However, other rules may apply to the CDR3-region. It has recently been shown that positively charged and hydrophobic amino-acids in CDR3β sequences are disfavoured

and TCRs with cysteines in their CDR3 peptide-binding regions are clonally deleted(11). Although these amino-acids were mentioned to be disfavoured, the majority of the hydrophobic amino-acids and the positively charged amino-acid arginine [R] were found in the CDR3-regions of EBV-LMP2^{FLY}-specific TCRs. Interestingly, cysteines were found in the CDR3 regions of thymocytes that did not undergo thymic selection yet(11, 44). Why cysteines are not found in CDR3 regions of matured T cells is not completely understood, but we show that positively charged, all hydrophobic amino-acids and cysteine on position 5 of the CDR3β region of the EBV-LMP2^{FLY}-specific TCR did not impair the specificity and functionality. It might be that thymocytes with cysteines were negatively selected during thymic development because of a too strong TCR-signaling(44), which prevents maturation into autoreactive T cells. However, we did not find any autoreactivity of TCR-transduced T cells when stimulated with K562 cells that were transduced with HLA-A*02:01 when no peptide was added.

Our data show that the amino-acids on position 5 of the EBV-LMP2^{FLY}-specific CDR3β sequence, were completely inter-changeable in CD8 T cells and did not significantly alter the specificity and functionality. We show that the recombination probability drives the possibility to find all possible amino-acids on this position. This data implies that TCRs that are highly similar to the public TCR, and differ in amino-acids on such promiscuous positions in the CDR3β, should be considered as one public TCR family.

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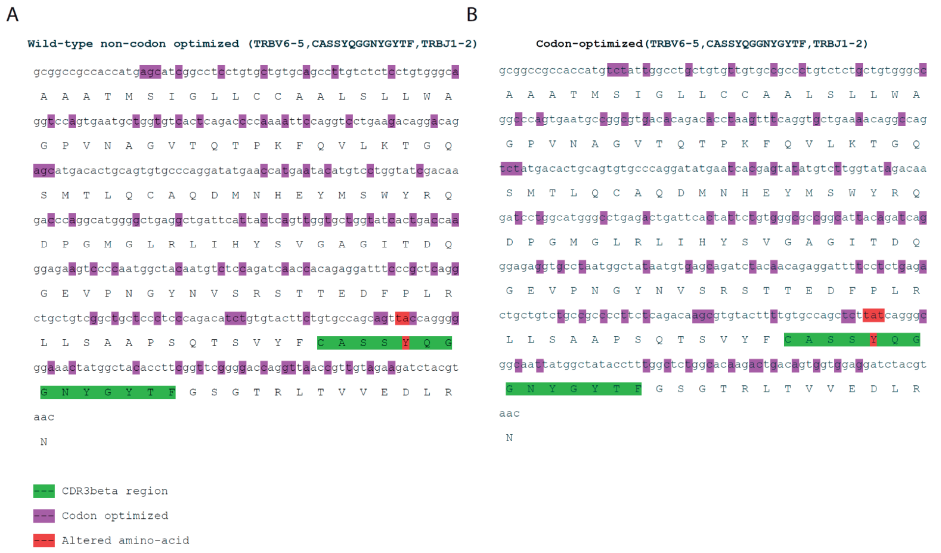
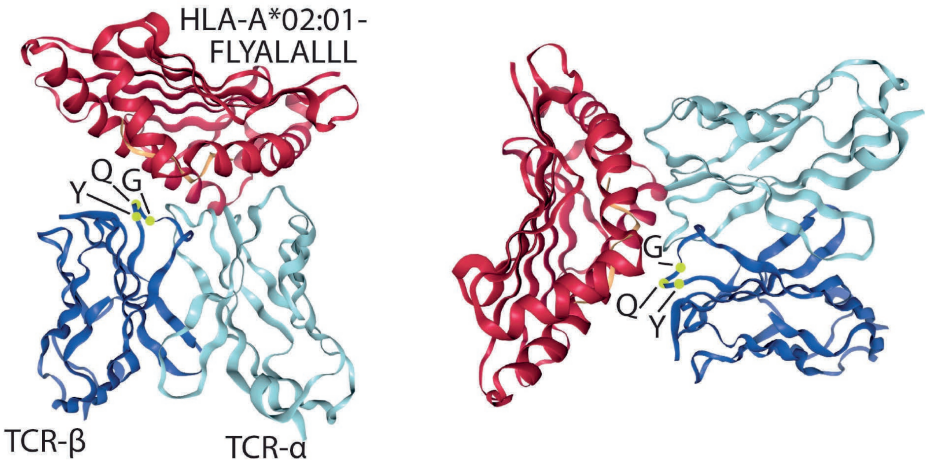
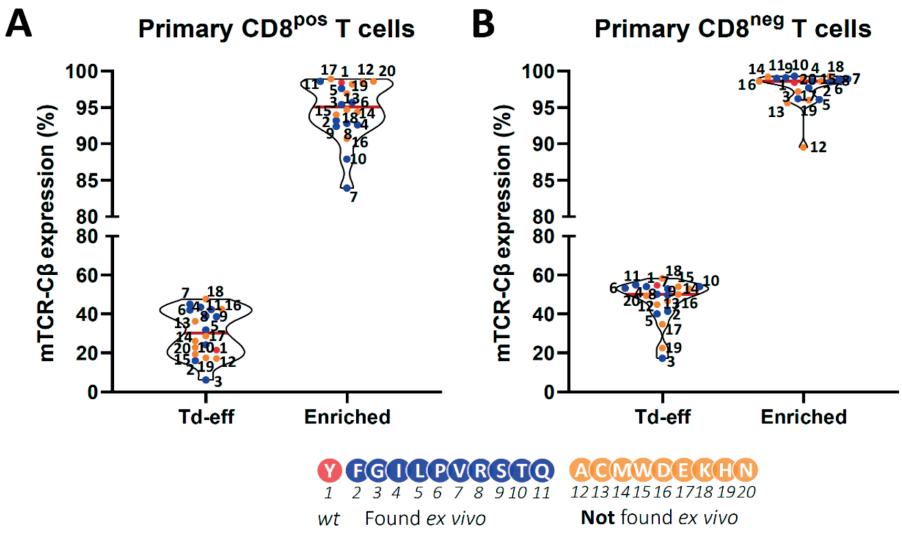


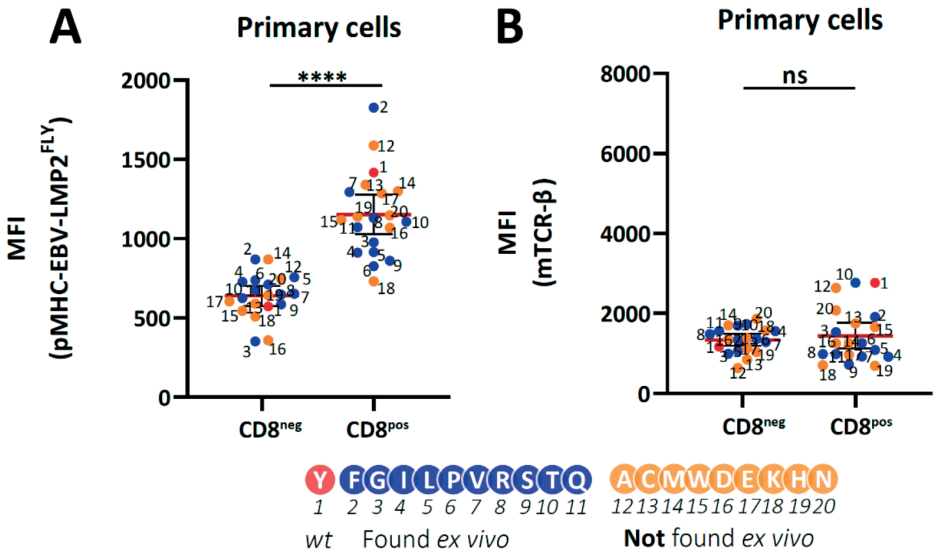
Supplementary Figure 1. Codon optimization of EBV-LMP2^{FLY}-specific TCRs. A) wild-type sequence of TCRβ (TRBV6-5-CASSYQGGNYGYTF-TRBJ1-2) expressing EBV-LMP2^{FLY}-specific T cells. B) codon-optimization of the CDR3β-CASSYQGGNYGYTF expressing EBV-LMP2^{FLY}-specific TCR. All other variants were made using this template and only the 3 red nucleotides were altered.



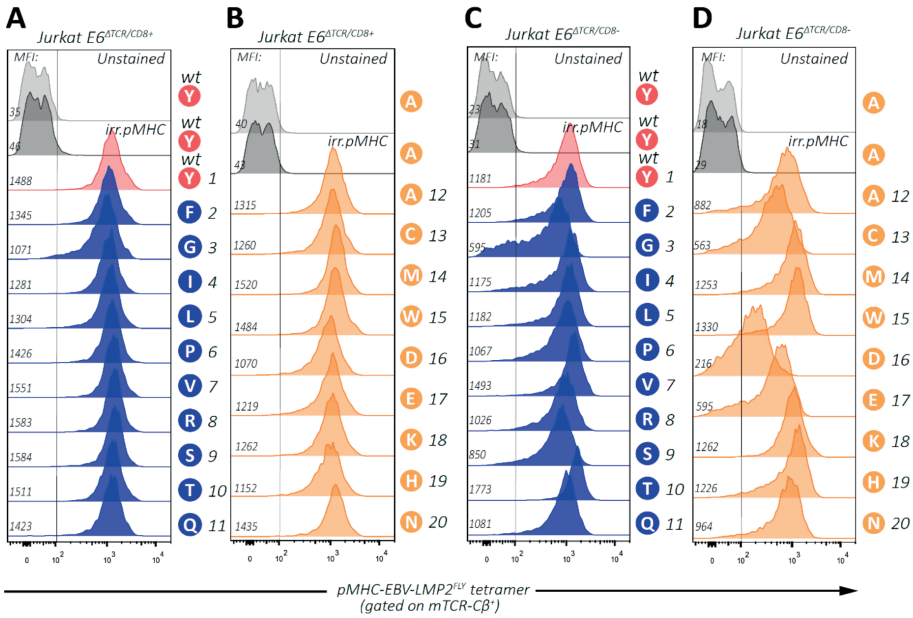
Supplementary Figure 2. TCR-pHLA model of EBV-LMP2^{FLY}-specific TCR expressing CDR3β-CASSYQGGNYGYTF. TCRmodel (https://tcrmodel.ibbr.umd.edu/) was used to generate the TCR-pHLA structure. The yellow dots represent positions 5 [Y], 6 [Q] and 7 [G] of the EBV-LMP2^{FLY}-specific TCR expressing CDR3β-CASSYQGGNYGYTF.



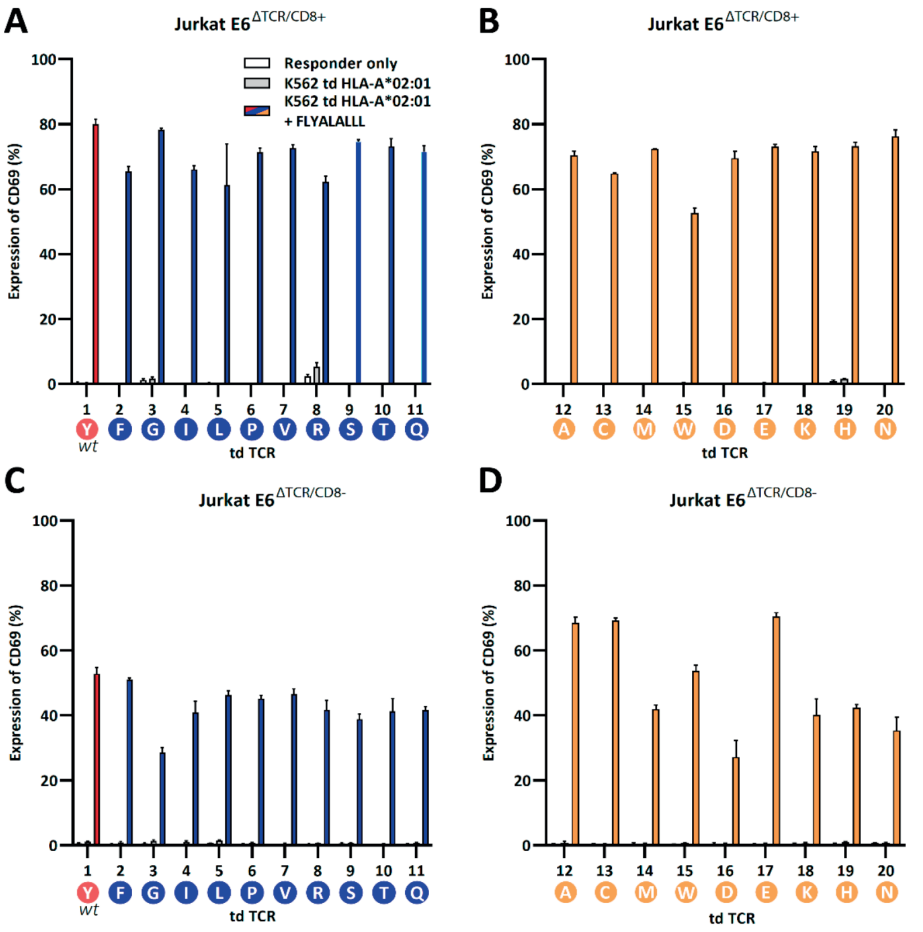
Supplementary Figure 3. Transduction efficiency of EBV-LMP2^{FLY}-specific TCRs and purity of enriched TCR-transduced primary T cells. Primary CD8^{pos} and CD4^{pos} (CD8^{neg}) T cells were isolated using Magnetic Activated Cell Sorting (MACS). Transduced primary cells were purified based on expression of murine-TCR-Cβ using MACS and expanded**.** The wildtype EBV-LMP2^{FLY}-specific TCRαβ-sequence is shown in red, additional TCRβ-sequences found *ex vivo* are shown in blue. TCRβ-sequences not found *ex vivo* are shown in orange. **A and B**) Shown is the transduction efficiency and purity after MACS enrichment of EBV-LMP2^{FLY}-specific TCR-transduced CD8^{pos} (**A**) and CD8^{neg} T cells (**B**).
Shown are medians (red) in violin plots with all individual samples.
Abbreviations: td-eff; transduction efficiency, wt; wildtype, mTCR-Cβ; murine TCR-constant β



Supplementary Figure 4. Introduced EBV-LMP2^{FLY}-specificity in primary T cells. Primary CD4^{pos} (CD8^{neg}) and CD8^{pos} T cells were isolated using Magnetic Activated Cell Sorting (MACS). Transduced primary cells were purified based on expression of murine-TCR-Cβ using MACS and expanded**.** The wildtype public EBV-LMP2^{FLY}-specific TCRαβ-sequence is shown in red, additional highly-similar TCRβ-sequences found *ex vivo* are shown in blue. TCRβ-sequences not found *ex vivo* are shown in orange **A)** Mean Fluorescence Intensity (MFI) of pMHC-EBV-LMP2^{FLY} tetramer binding was assessed for CD8^{neg} and CD8^{pos} T cells. **B**) Shown is the MFI of the introduced TCR in CD8^{neg} and CD8^{pos} T cells as measured by expression of murine-TCR-Cβ.
Statistical differences were assessed with the paired *t* test (**A and B**).
*Shown are means (red line) with 95% confidence intervals (error bars). ****P<.0001; ns; not significant >.05.*
Abbreviations; MFI; Mean Fluorescence Intensity, Td; Transduced, wt; wildtype



Supplementary Figure 5. Amino-acid substitutions in the complementary determining region 3 of HLA-A*02:01-restricted EBV-LMP2-specific TCRs show overall maintenance of specificity in Jurkat E6 cells. The endogenous TCRs of Jurkat E6 cells was knocked out using Crispr-Cas9 technology (ΔTCR). Jurkat E6$^{\Delta TCR}$ cells were transduced with CD8 to simulate CD8^{pos} T cells (Jurkat E6$^{\Delta TCR/CD8+}$). Transduced cells were purified based on expression of murine-TCR-Cβ using MACS and expanded**.** Twenty different TCR-constructs were designed with amino-acids substitutions at position 5 of the CDR3β-sequence. The wildtype EBV-LMP2^{FLY}-specific TCRαβ-sequence is shown in red, additional highly-similar TCRβ-sequences found *ex vivo* are shown in blue. TCRs 12-20 were not found *ex vivo* and are shown in orange. **A and B)**. Shown are histograms of specific HLA-A*02:01/pMHC-EBV-LMP2^{FLY} tetramer (red, blue and orange) or irrelevant HLA-A*02:01/pMHC-CMV-pp65^{NLV} tetramer (black) stainings of Jurkat E6$^{\Delta TCR/CD8+}$ cells transduced with TCRs that were found *ex vivo* (**A**) or that were not found *ex vivo* (**B**). **C** and **D**) Shown are histograms of specific HLA-A*02:01/pMHC-EBV-LMP2^{FLY} tetramer (red, blue and orange) or irrelevant HLA-A*02:01/pMHC-CMV-pp65^{NLV} tetramer (black) stainings of Jurkat E6$^{\Delta TCR/CD8-}$ cells transduced with TCRs that were found ex *vivo* (**C**) or that were not found ex *vivo* (**D**).
Abbreviations: irr.pMHC, irrelevant pMHC tetramer, MFI; Mean Fluorescent Intensity, wt; wildtype



Supplementary Figure 6. TCR gene transfer introduced EBV-LMP2^{FLY} specificity and reactivity into Jurkat E6 cells. The endogenous TCR of Jurkat E6 cells was knocked out using Crispr-Cas9 technology (ΔTCR). Jurkat E6$^{\Delta TCR}$ cells were transduced with CD8 to simulate CD8^{pos} T cells (Jurkat E6$^{\Delta TCR/CD8+}$). Transduced cells were purified based on expression of murine-TCR-Cβ using MACS and expanded. HLA-A*02:01-transduced K562 cell-lines were pulsed with 10^{-6} M of EBV-LMP2^{FLY}-specific peptide. **A** and **B**) EBV-LMP2^{FLY}-specific TCR-transduced Jurkat E6$^{\Delta TCR/CD8+}$ cells were tested for recognition of K562 cells transduced with HLA-A*02:01 and pulsed with and without EBV-LMP2^{FLY} peptide. The wildtype EBV-LMP2^{FLY}-specific TCRαβ-sequence is shown in red, additional highly-similar TCRβ-sequences found *ex vivo* are shown in blue (**A**). TCRβ-sequences not found *ex vivo* are shown in orange (**B**). **C** and **D**) CD8 co-receptor dependency was assessed by testing EBV-LMP2^{FLY}-specific TCR-transduced Jurkat E6$^{\Delta TCR/CD8-}$ for recognition of K562 cells transduced with HLA-A*02:01 and pulsed with and without EBV-LMP2^{FLY} peptide. The wildtype EBV-LMP2^{FLY}-specific TCRαβ-sequence is shown in red, additional highly-similar TCRβ-sequences found *ex vivo* are shown in blue (**C**). TCRβ-sequences not found *ex vivo* are shown in orange (**D**).

Shown are means with standard deviations of one experiment carried out in triplicate

Abbreviations: td; transduced, wt; wildtype

CHAPTER 7

Summary and Discussion

SUMMARY

Viral-reactivations are controlled by virus-specific memory T cells in healthy individuals, but this cellular immunity is impaired in patients that are immunocompromised, like patients receiving allogeneic stem cell transplantation (alloSCT) or patients that receive a solid organ transplant (SOT). For patients receiving alloSCT after conditioning therapy, donor-derived T cells are either depleted or suppressed in the period around the transplantation to reduce the risk of graft versus host disease (GVHD). Since T-cell depletion (TCD) from the graft also increases the incidence of relapse of the malignancy, TCD alloSCT can be followed by a donor lymphocyte infusion (DLI) to restore the graft versus leukemia (GVL) response, which may also restore viral immunity. However, between the moment of alloSCT and DLI, and during immune suppressive treatment for GVHD, patients experience a period of profound and prolonged T-cell deficiency in which they are at risk for developing infectious complications. In absence of a functional cellular immunity, latent viruses such as Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Adenovirus (AdV) can cause uncontrolled reactivations. Therefore, the major challenge in the field of alloSCT is to find a balance between the GVL effect, viral protection and GVHD. This thesis described different options to control viral reactivations in immunocompromised patients using adoptive transfer of virus-specific T cells or potential transduction of virus-specific TCRs and the risks associated with these approaches.

In order to explain potential treatment efficacy of adoptive transfer of stem-cell donor-derived virus-specific T cells, it is vital to determine the fate of the transferred T-cell populations. However, it is difficult to unequivocally distinguish progeny of the transferred T-cell products from recipient- or stem-cell graft-derived T cells that survived T-cell depletion during conditioning or stem-cell graft manipulation. In ***chapter 2***, we showed that by using mRNA sequencing of the T-cell receptor beta (TCRβ)-chains of individual virus-specific T-cell populations within prophylactically transferred virus-specific T-cell products, we were able to track multiple clonal virus-specific subpopulations in peripheral blood and could distinguish recipient- and stem-cell graft-derived virus-specific T cells from the progeny of the infused T-cell products. *In vivo* expansion of virus-specific T cells that were exclusively derived from the T-cell products was found to have similar kinetics as the expansion of virus-specific T cells that were part of the endogenous T-cell repertoire that was already present before the T-cell product infusion. Additionally, persistence of virus-specific T cells derived from the T-cell products could also be demonstrated in most patients who did not show viral reactivations.

In some situations, memory virus-specific T cells cannot be isolated from the stem-cell donor, for instance when stem-cell donors are seronegative for that particular virus.

For those patients without easy access to memory virus-specific T cells from their stem-cell donor, a third-party virus-seropositive donor source to obtain virus-specific memory T cells would allow rapid intervention to restore anti-viral immunity. However, third-party-derived T-cell products are in general only partially human leukocyte antigen (HLA)-matched with the patient. In ***chapter 3***, we used healthy donor-derived virus-specific T cells to investigate whether virus-specificity, HLA restriction and/or HLA background could predict the risk of allo-HLA cross-reactivity. These virus-specific T cells were stimulated *in vitro* against a panel of EBV-transformed lymphoblastoid cell-lines (EBV-LCLs) covering 116 allogeneic HLA-molecules. The allo-HLA cross-reactivity was confirmed using HLA-class-I and HLA-class-II negative K562 cells that were retrovirally transduced with single HLA-class-I alleles of interest. HLA-B*08:01-restricted T cells showed the highest frequency and diversity of allo-HLA cross-reactivity, regardless of virus-specificity, and this allo-HLA cross-reactivity was skewed towards multiple recurrent allogeneic HLA-B molecules. Additional thymic selection for a second HLA-B allele in heterozygous donors could significantly influence the level of allo-HLA cross-reactivity mediated by HLA-B*08:01-restricted T cells. When the secondary HLA-B allele was also part of a unique HLA superfamily like HLA-B*08:01, no difference in the frequency and diversity of allo-HLA cross-reactivity was observed. However, when the secondary HLA-B allele was part of a large HLA superfamily, a significant reduction was observed in frequency and diversity of the allo-HLA cross-reactivity mediated by HLA-B*08:01-restricted virus-specific T cells. These results illustrate that the degree and specificity of allo-HLA cross-reactivity by T cells follow rules.

Another approach to decrease the risk of off-target reactivities mediated by third-party-derived T-cell products would be to selectively enrich for T cells that express TCRs that are safe or have a limited off-target reactivity. Ideally, virus-specific T-cell populations expressing TCRs that are common and expressed by different individuals (known as public TCRs) could be used for this. Therefore, in ***chapter 4*** we quantitively analyzed the TCR-repertoires of CMV, EBV and AdV-specific T cells from healthy individuals. We specifically determined the magnitude, defined as prevalence within the donor population and frequencies within individuals, of public TCRs and TCRs that are highly-similar to these public TCRs. We found that almost one third of all CMV, EBV and AdV-specific TCR nucleotide-sequences represented public TCR amino-acid sequences and we found an additional 12% of TCRs that were highly similar to the public TCR by maximally differing 3 amino-acids. We illustrated that these public and highly-similar TCRs were structurally related and contained shared core-sequences in their TCR-sequences. We found a prevalence of combined public and highly-similar TCRs of up to 50% among individuals homozygously expressing the common haplotypes HLA-A*01/B*08 and HLA-A*02/B*07 and frequencies of combined virus-specific public and highly-similar TCRs making up more than 10% of each virus-specific T-cell population. These findings

were confirmed using an independent TCR-database of virus-specific TCRs derived from individuals heterozygously expressing HLA-A*01:01, HLA-A*02:01, HLA-B*07:02 or HLA-B*08:01. We therefore concluded that the magnitude of the contribution of public and highly similar TCRs to these virus-specific T-cell responses is high. We hypothesized that the TCRs that are highly-similar to the public TCRs with conserved motifs similarly dock to the peptide-HLA complex as the public TCR-sequences. Therefore, the highly-similar sequences can be considered part of the same public T-cell response. Such TCRs may be utilized for diagnostic purposes or therapeutic benefit in TCR-gene transfer-based immunotherapy strategies to effectively control viral-reactivation in immuno-compromised patients.

Although the TCRs that are highly-similar to public TCRs were frequently found, (with only minor variations in amino-acids on specific positions in the complementary determining region 3 [CDR3]), the degree of freedom of amino-acids at these positions and the effect on peptide/HLA binding remained unclear. To investigate this, we used an HLA-A*02:01-restricted EBV-Latent Membrane Protein 2 (LMP2)^{FLY}-specific public TCR as model and systematically replaced the amino-acids at the highly-variable position 5 in the CDR3β sequence with all 20 possible amino-acids in ***chapter 5***. We demonstrated that amino-acids at this particular position were completely inter-changeable, without loss of TCR function. We showed that the inability to detect certain variants of this TCR within the T-cell repertoire of random individuals was explained by their lower recombination probability rather than by steric hindrance.

Virus-specific TCR-gene transfer allows rapid intervention to restore antiviral immunity in patients for whom there is no virus-seropositive stem cell donor available. This approach may also be used for patients with virus-associated malignancies, like EBV-associated malignancies of latency type II/III that express EBV antigens (LMP1/2). Intriguingly, T cells recognizing any EBV-derived peptide in the common HLA allele HLA-A*01:01 had not been found thus far. Therefore, in ***chapter 6***, we aimed to isolate HLA-A*01:01-restricted EBV-LMP2-specific T cells and sequence their TCRs. HLA-A*01:01-restricted EBV-LMP2-specific T cells were isolated and their TCRs were characterized. TCR gene transfer into primary T cells resulted in specific pMHC tetramer binding and reactivity against HLA-A*01:01 positive EBV-LMP2-expressing cell lines. We transduced these EBV-LMP2-specific TCRs in primary T cells whereby the endogenous TCR was knocked out (ΔTCR) using CRISPR-Cas9 technology to mitigate potential mis-pairing of the introduced TCR with the endogenous TCR. After endogenous TCR knock out, CD8+/ΔTCR T cells modified to express EBV-LMP2-specific TCRs showed maintenance of functionality by IFN-γ secretion and cytotoxicity toward EBV-LMP2-expressing malignant cell lines. This HLA-A*01:01-restricted EBV-LMP2-specific TCR could potentially be used in future TCR gene therapy to treat EBV-associated latency type II/III malignancies.

DISCUSSION

Adoptive virus-specific T-cell therapy to boost viral immunity

Reactivation of latent viral infections can be a life-threatening complication in patients during the early immune compromised phase after alloSCT. In these patients, the memory virus-specific T cells that are responsible for control of reactivations are either deleted due to TCD alloSCT or suppressed due to the use of immunosuppressive agents. Eventually, new primary virus-specific T-cell responses will develop from the engrafted donor stem cells, but this takes time. During the immune compromised phase after TCD alloSCT, administration of unmodified DLI can be given to restore viral immunity(1, 2). However, early DLI can also result in GVHD since inflammatory conditions and the presence of patient-derived antigen presenting cells (APCs) can provoke a profound alloreactive donor-derived T-cell response(3-5). Despite advances in pharmacotherapeutic approaches, use of antiviral medication is limited by toxic side effects, development of resistant variants and inability to provide long-term protection(6-8). Therefore, immunotherapeutic strategies to accelerate reconstitution of virus-specific immunity after alloSCT remain a powerful alternative to conventional drugs. Selection and transfer of virus-specific T-cell populations from the donor, with exclusively beneficial effects such as control of viral-reactivations, is highly desirable for these immunocompromised patients.

Adoptive virus-specific T-cell therapy can be applied either in a prophylactic, pre-emptive or therapeutic setting. In a therapeutic setting, virus-specific T-cell products are created and given to patients already suffering from viral disease. Under these circumstances, only patients that are diagnosed with viral disease receive a personalized virus-specific T-cell product. In this case, the virus-specific T cells need to be available directly after diagnosis in sufficient numbers to immediately target virus-infected cells *in vivo*. Unfortunately, this ideal scenario for the treatment of viral disease after alloSCT is not realistic, because T-cell products cannot always be generated in time and effectiveness is not guaranteed. Ideally, virus-specific T-cell products are created and given to patients that have a high risk of developing viral disease (persistent viral reactivation), but are not yet suffering from viral disease. Instead of this approach, other strategies are being investigated, including pre-emptive infusion of seropositive stem-cell donor-derived virus-specific T-cell products into patients in case of persistent viral reactivation before clinical manifestations of the viral reactivation develop. Alternatively, prophylactic infusion of seropositive stem-cell donor-derived virus-specific T-cell products could be given to all seropositive patients. If this would be feasible, effective and affordable, monitoring of viral reactivation could even be reduced or abolished. In patients without a seropositive stem cell donors who are at risk for viral complications, pre-emptive infusion of seropositive third-party donor-derived virus-specific T-cell products may be applied.

Using either approach, only the immunocompromised phase after alloSCT need to be bridged, since a new protective endogenous T-cell repertoire will generally develop from the donor stem cells. Although cells from the myeloid lineage often normalize within 2 to 4 weeks after alloSCT(9, 10), rebuilding the adaptive T-cell mediated immune repertoire takes much longer. Complete recovery depends on the efficiency of *de novo* T-cell education in the thymus. The output of the thymus depends in turn on several factors including, but not restricted to, disease status, patient age, source and composition of the graft, type of conditioning, and presence of GVHD(11-17). Although a precise time span has so far not been calculated for humans, even under favorable conditions it takes, at least several months to produce naive T cells from donor stem cells. Morevover, a plateau level of thymic output is reached only after at 1 to 2 years after alloSCT(16). The immunodeficient period generally needs to be bridged for roughly a year in order to develop a full spectrum of memory virus-specific T cells that can control future reactivations.

The progeny of prophylactically infused virus-specific T-cell products from seropositive donors

In a pre-emptive or prophylactic setting, the intention is to prevent viral complications by the infusion of virus-specific T cells prior to disease development. To establish treatment efficacy in this setting, it is important to determine whether product-derived virus-specific T cells expand in the presence of viral antigens and survive/persist in the absence of viral antigen. In this setting, the fate of the individual transferred virus-specific T-cell populations needs to be tracked. However, it is difficult to unequivocally distinguish progeny of the transferred T-cell products from recipient- or stem-cell graft-derived T cells that survived T-cell depletion during conditioning or stem-cell graft manipulation. In ***chapter 2***, where stem-cell donor-derived virus-specific T-cell products were prophylactically transferred to recipients of alloSCT, we used mRNA sequencing of the TCRβ-chains of the individual virus-specific T-cell populations within T-cell products to track multiple clonal virus-specific subpopulations in peripheral blood. This enabled us to distinguish recipient- and stem-cell graft-derived virus-specific T cells from the progeny of the infused T-cell products. In contrast to the peptideMHC-tetramer technology that was previously used for this study(18), TCR sequencing of purified viral antigen-specific T-cell populations allowed us to track these with high sensitivity and specificity in peripheral blood of patients after infusion. It also made it possible to distinguish donor-derived virus-specific T cells that were already present in the patient before infusion from those exclusively derived from the infused T-cell products. Virus-specific TCR sequences from T cells in the adoptively transferred products that were exclusively found in peripheral blood of the patient after infusion of the products were documented in all patients infused with CMV-specific T cells. Additionally, in 80% of patients infused with EBV-specific T cells and in 47% of patients infused with AdV-specific

T cells, T cells were found that were exclusively derived from the T-cell product. Product-derived CMV-specific T cells could be detected in the same patients using either TCR-sequencing or pMHC-tetramers. However, product-derived EBV-specific T cells could be detected in all 8 patients with positive viral-loads during follow-up using TCR-sequencing and only in 2 patients using pMHC-tetramers(18). Additionally, using TCR-sequencing, expansion of EBV-specific T cells could already be detected before positive viral-loads were detected, while using pMHC-tetramers, expansion of EBV-specific T cells could only be detected from 2 to 4 weeks after detection of viral loads onwards. In line with previous studies(19-21), this shows that the relatively low numbers of *ex vivo* isolated T cells have enough time to rapidly expand to large numbers *in vivo* and might be sufficient for virus control. In the pre-emptive setting, infusions take place immediately after viral DNA load detection. Although the intention of the study setup was prophylactic, CMV-specific T cells were transferred while positive CMV viral loads were already present in 4 out of 5 patients. Therefore, the response from the adoptively transferred CMV-specific T cells in these 4 patients, could be interpreted as results from a pre-emptive situation. In this example, only direct expansion of CMV-specific T cells could be investigated in the presence of viral antigen and not persistence in absence of viral antigen.

Many studies show associations with decrease in viral-loads and appearance of virus-specific T cells after transfer of stem-cell donor-derived T-cell products(22-29). However, it is difficult to unequivocally distinguish progeny of transferred T-cell products from recipient- or stem-cell graft-derived T cells that survived T-cell depletion during conditioning or stem-cell graft manipulation. This makes it difficult to also prove contribution to the control of virus-reactivation by product-derived virus-specific T cells. With TCR-sequencing, product-derived sequences could be distinguished from the patient, but also from sequences that were already present in the patient before infusion. Contribution of product-derived T cells could be assessed with more certainty, but only randomized, placebo-controlled prospective trials can provide definitive proof of the efficacy of transferred virus-specific T cells. To investigate whether adoptive transfer of virus-specific T cells in a pre-emptive setting is indeed effective, a multinational, randomized, placebo-controlled phase III clinical study (TRACE) has recently been initiated. The aim of that study is to examine whether refractory viral reactivations in patients after alloSCT can be controlled/treated with the infusion of a multi-virus-specific T-cell product from their seropositive stem-cell donor.

Adoptive transfer of virus-specific T-cell products for patients transplanted with a seronegative donor

The donor used for adoptive transfer of virus-specific T cells is preferentially the seropositive stem-cell donor. However, since in adults the prevalence of CMV and EBV seropositivity in the Western population is about 50% and 90%, respectively, significant

numbers of alloSCT recipients are transplanted with CMV-seronegative donors(30, 31). In this setting, only naïve virus-specific T cells are present in peripheral blood of the donor. Although the frequency of virus-specific naïve T cells is very low, generating a T-cell product from a virus naïve donor may be doable(32). However, if patients already experience CMV reactivation at the moment of adoptive transfer, infusion of low numbers of naïve virus-specific T cells is not likely to be effective, because time is required for adequate priming and expansion until appropriate cell numbers are reached to fight the virus-infected cells. Naïve virus-specific T cells can be stimulated and expanded *in vitro*, but this takes a considerable amount of time and it is not clear whether *in vitro* priming renders the same quality of T cells compared to *in vivo* priming after viral infection. A possible other strategy, is the manufacturing of T-cell products derived from third-party seropositive healthy subjects(33-35). The advantage of virus-specific T cells from third-party donors is that a bank can be created of stored T-cell lines, which are immediately available 'off-the-shelf' for patients encountering viral complications(33). However, due to the limited size of such banks it remains very difficult to match the third-party donor-derived off-the-shelf product for multiple HLA alleles with the recipient and/or hematopoietic stem cell donor, since such T-cell biobanks often consist of T-cell products generated against immunogenic peptides restricted to a few common HLA-molecules.

Because off-the-shelf T-cell products are difficult to fully match for multiple HLA alleles with the patient, effectivity of the T-cell product is the main concern. Since the virus-specific T cells from the third-party donor will only recognize viral peptides in the HLA complexes that are present within the third-party donor, the virus-specific T-cell products from this third-party donor will not be functional in a fully HLA-mismatched setting. Therefore, at least 1 HLA molecule should be matched between the donor of the off-the-shelf T-cell product and the patient to allow for an efficient anti-viral response mediated by the third-party donor-derived product upon adoptive transfer. However, since most of the off-the-shelf products are generated using overlapping peptide stimulation and enrichment for activated T cells (e.g. production of IFNy), it is difficult to assess what proportion of the T-cell product contains virus-specific T cells that are restricted to the HLA allele(s) that are matched between the third-party donor and the patient. This may cause a situation where the majority of virus-specific T cells that are present in the product, would not be functional in the patient due to the HLA mismatches. As a result of this uncertainty, off-the-shelf virus-specific T-cell products are predominantly generated and transferred to patients that share a common HLA allele such as HLA-A*02:01, since it is known that multiple immunogenic viral peptides can be presented by this common HLA molecule (in the Caucasian population). Generating T-cell banks directed against viral peptides presented in one of these common HLA alleles allows for adoptive transfer to many patients that express at least one of these common HLA molecules. However, patients that are not Caucasian are less likely to express these HLA alleles and are

therefore less likely to benefit from these banked virus-specific T cells.

Another alternative for the generation of third-party donor-derived virus-specific T-cell products, is to use a registered HLA-typed virtual bank of seropositive healthy donors. A blood bank, like Sanquin in the Netherlands, contains a repository that consists of a large number of healthy donors, and many are at least HLA-class-I typed with known seropositivity for CMV. From this virtual repository, donors with the best HLA-match with the patient can be selected. This way, generating virus-specific T-cell products that target a large number of relevant immunogenic antigens may be feasible. However, in contrast to the off-the-shelf third-party donor-derived T-cell products, such products still need to be generated ad-hoc. This approach therefore takes more time before the patient/recipient receives a virus-specific T-cell product. Such products will still likely be only partially HLA matched, although fewer HLA alleles will likely be mismatched compared to already generated off the shelf third-party donor-derived T-cell products. In any scenario, partially HLA-matched virus-specific T cells have a risk of mediating off-target reactivities. Additionally, the other way around, alloreactive T cells of the stem-cell donor within the patient may reject transferred HLA-mismatched third-party donor-derived T cells, hampering the persistence and efficacy of adoptively transferred third-party donor-derived virus-specific T cells.

Risk of off-target reactivity mediated by third party virus-specific T cells

Adoptive transfer of partially HLA-matched virus-specific T cells from healthy third-party donors from a repository is a potential strategy to temporarily provide anti-viral immunity to patients receiving alloSCT. However, these third party donor-derived virus-specific T cells have not been tolerized by thymic negative selection against the non-matched HLA molecules that are present within the patient or presented by cells from the stem-cell donor, thereby implying the risk of off-target toxicity due to allo-HLA cross-reactivity directed against the mismatched HLA alleles(36). This toxicity includes the risk of graft rejection by alloreactive T-cell responses of third-party donor T cells to donor stem cells. Furthermore, GVHD can be induced when third-party donor-derived T cells recognize tissue cells of the patient. Additionally, in recipients of solid organs, allo-HLA cross-reactivity might also be a major trigger of graft rejection, as shown by the association between viral reactivation, increase in virus-specific T cells and graft rejection(37, 38). It had been demonstrated that third-party donor-derived virus-specific T cells can exert allo-HLA cross-reactivity directed against mismatched HLA molecules *in vitro*(39-42). However, these anti-viral T-cell responses did not show clear predictable, but rather seemingly random cross-reactivities against mismatched HLA molecules. In ***chapter 3***, we used virus-specific T cells from healthy donors as model to investigate whether virus-specificity, HLA restriction and/or HLA background could predict the risk of allo-HLA cross-reactivities. Although no specific recurrent allo-HLA cross-reactivities

could be found based on virus-specificity, HLA-restriction turned out to be a good predictor of the magnitude and diversity. Contrary to the previous reports(39-42), this suggests that the degree and specificity of the allo-HLA cross-reactivities follow rules. These rules rely on the peptides that can be presented by each HLA molecule and similarities or differences between mismatched HLA molecules, which determine the magnitude and diversity of the allo-HLA cross-reactivity. The number and types of peptides that can be presented by each HLA molecule is known as the peptidome of that particular HLA molecule. Every HLA molecule has a different peptidome, but some HLA molecules belong to an HLA superfamily of molecules with shared amino-acids on anchor residues. For instance, HLA*01:01 and HLA-A*26:01 have a shared anchor amino acid threonine on position 2 and Valine on position 9 of the peptide binding motif. We hypothesized that the magnitude of the allo-HLA cross-reactivity is dependent on the peptidome of the HLA molecule to which the virus-specific T cell is restricted and the peptidome of the other HLA molecules (HLA background) of that individual (e.g. that have driven the thymic selection). As shown in ***chapter 3***, the levels of allo-HLA cross-reactivities mediated by HLA-A*01:01, HLA-*02:01 and HLA-B*07:02-restricted T cells were relatively limited, which might be explained by the large superfamilies that these HLA-A and HLA-B molecules belong to. Whenever the peptidome of an HLA molecule has no similarities with the mismatched HLA molecules and belongs to a so called unique or unclassified "HLA-superfamily", T cells restricted to this unclassified HLA molecule may have a high chance to be allo-HLA cross-reactive, since negative thymic selection for peptide-HLA complexes from an HLA superfamily has not taken place. Luckily, the majority of individuals have two different HLA alleles per HLA-class-I group (e.g. HLA-A). In many individuals, the second HLA allele within the same major HLA-class-I group (e.g. A, B and C) reduces alloreactivity of that persons' T-cell repertoire, but only if the other HLA allele does not also belong to a unique or unclassified HLA superfamily. Indeed, as shown in ***chapter 3***, when neither HLA-B alleles belongs to an HLA superfamily, like in HLA-B*08:01/HLA-B*13:02 heterozygous or homozygous HLA-B*08:01 individuals, HLA-B*08:01-restricted virus-specific T-cell populations contained high frequencies and diversity of allo-HLA cross-reactive cells. It seemed that this allo-HLA cross-reactivity was predominantly skewed towards multiple recurrent allogeneic HLA-B molecules. This suggests that this mainly applies within each major HLA-class-I group. Intriguingly, studies in the field of organ transplantations also showed a significant increase of acute graft rejections in recipients that expressed HLA-B*08:01(43, 44).

Despite these results, only low rates (~5%) of off-target toxicity/de novo GVHD were observed in stem-cell recipients that were treated with partially HLA-matched virus-specific T cells(33, 45-47). There are several potential reasons for this discrepancy. Since we tested the allo-HLA cross-reactive potential against a multitude of common and rare HLA-class-I molecules, the most obvious explanation is that the transferred virus-specific

T cells are allo-HLA cross-reactive against a particular mismatched HLA molecule that is not expressed by the patient. It is also important to note that "off-the shelf" partially HLA-matched products are predominantly generated to cover the most common HLA alleles HLA-A*01:01, HLA-A*02:01 and HLA-B*07:02. HLA-A*02:01-restricted virus-specific T cells have most widely been used and these possess an intrinsic low-risk of off-target reactivity, as was shown in ***chapter 3***. It stands to reasons that his likely skewed the results in favour of low rates of off-target toxicity. Other potential explanations for this discrepancy between the off-target reactivities observed *in vitro* and *in vivo* are: (1) rapid rejection of the virus-specific T cells, not allowing them to persist or expand and generate an allo-response(34), (2) removal of the in vitro off-target (>10% cytotoxic) virus-specific T cells from the product prior to administration to the patient and/or selection of T-cell products that do not show in vitro allo-HLA reactivity(46), (3) senescence and impaired cytokine production due to extensive culturing of the virus-specific T cells prior to adoptive transfer(48), (4) weak adhesion molecule expression (i.e., ICAM-1) by the target organ(49), (5) low T-cell numbers of cross-reactive virus-specific T cells administered and/or limited *in vivo* proliferation, or (6) suppression or deletion of infused T cells due to immunosuppressive medication or recently received antibodies against T cells, like anti-thymocyte globulin (ATG) (50). Importantly, almost all above mentioned reasons, also have an effect on the efficacy of the transferred virus-specific T cells. In ***chapter 3***, we showed that the HLA background of the third-party donor was shown to be another reason for this potential discrepancy. When partially HLA-matched virus-specific T cells are adoptively transferred in the future, the HLA background should be considered according to a predefined order. Donors should be selected that have the highest degree of HLA-class-I match, and those HLA alleles that are not matched should be part of the same superfamily of the mismatched HLA allele. Donors with HLA alleles (within the major HLA-class-I group) that are part of a unique HLA superfamily or are undefined should be avoided. Because the majority of undefined and unique HLA superfamilies fall within the major HLA-B group, priority to HLA matching within this HLA group is advised. We propose that if full matching for HLA-B is not possible, third-party donors should be selected from a virtual repository that express HLA-B molecules that are part of the same superfamily. A similar approach can then be applied for HLA-A to reduce the chance for a broad off-target toxicity in clinical application of third-party donor-derived T-cell products. When 'off the shelf' products from third-party donors are used, the options to select donors/products with specific HLA backgrounds are very limited and therefore this approach would be mainly relevant for third-party donors selected from a virtual bank.

Public TCRs as library for TCR-gene transfer

Another approach that would circumvent the search for donors with the right requirements and possibly reduce the chance for a broad off-target toxicity, would be

to determine the sequence encoding the virus-specific TCR. Then viral transduction can be utilized to introduce such a functional virus-specific TCR into polyclonal donor-derived T cells, thereby redirecting their specificity(51). Selecting virus-specific TCRs that are found in multiple different individuals, referred to as public TCRs, would be less likely to cause broad off-target toxicity. TCRs, are generated during thymopoiesis, whereby thymocytes mature and will express TCR-α and TCR-β chains by rearrangement of different germline elements (variable, diversity and joining). Insertion of template-independent nucleotides between the recombined segments (junctional region) results in a significant increase in variability. The sequence around these junctions encodes for the CDR3, a loop that reaches out and interacts with the peptide embedded in an HLA molecule, together with the loops of the CDR1 and CDR2 regions, which are fixed within the germline variable gene sequence. It has been calculated that the number of different TCRs that can theoretically be generated ranges between 10^{15}-10^{20} (52). Although virus-specific memory T-cell populations, that are shaped after control or clearance of the infection, only target a limited number of viral peptides, the multiple viral-peptides that are targeted in the various HLA alleles make it theoretically unlikely that individuals would frequently share the exact same virus-specific TCR (public TCR). Public TCR sequences have been found in dominant virus-specific memory T-cell populations from different individuals. However, no consensus has thus far been reached when a TCR can be called a public TCR. Currently, TCRs are reported to be "public" when they are found in different individuals and contain: 1) the exact same nucleotide-sequence, 2) different nucleotide-sequences but the same amino-acid sequence, or 3) when TCRs only have minor amino-acid differences. In ***chapter 4*** and ***chapter 5***, we shed more light on the promiscuity and magnitude of public TCRs and what their contribution was to the total TCR-repertoire. In ***chapter 4***, we quantitatively analyzed TCR-repertoires of virus-specific memory T-cell populations, and determined the prevalence within the population of public TCRs and of TCR-sequences that are highly-similar to these public TCR-sequences. Additionally, the frequencies of public TCRs and of TCR-sequences that are highly-similar to these public TCR-sequences within individuals were investigated. The majority of studies only looked into exact TCR matches, which could lead to an underestimation by excluding highly-similar TCRs in their analyses(53, 54). We defined all TCR-sequences that would generate the exact same amino-acid sequence as public identical TCR (PUB-I) and defined TCRs with minor amino-acid differences, but highly-similar to a public identical TCR, as public Highly-Similar TCR (PUB-HS). These two families of TCR-sequences can be considered as the same public T-cell response if they follow a certain set of rules: (1) the same peptide-HLA complex is targeted, (2) the same variable gene is expressed to have identical CDR1 and CDR2 regions and (3) the same conserved amino-acids are expressed in the CDR3 loop. With this, we showed that the contribution of PUB-I TCRs to the total repertoire was already high and that PUB-HS TCRs followed the above rules, marking them as being part of the same public T-cell response. When these rules are

applied, it was demonstrated that almost half of the TCR-repertoire contained TCRs that were part of this public response. We were able to detect motifs in TCRs restricted to a single peptide-HLA complex that were unique for that TCR-peptide-HLA combination and not shared with TCRs restricted to other peptide-HLA complexes. We hypothesized that the type of amino-acids at the promiscuous positions that are not part of the motif might not be important for the specificity and/or three-dimensional structure of the TCR as long as there was an amino-acid present at such a promiscuous position, irrespective which amino-acid. By modifying the highly-variable position 5 of the CDR3β sequence of a public TCR with all 20 amino acids, in ***chapter 5*** we demonstrated that indeed amino acids at this particular position are completely inter-changeable, without loss of TCR function. In line with previous findings, it was shown that the recombination probability correlated with the number of times a public TCR was found in multiple individuals(55, 56). These TCRs are often found in healthy individuals that are able to control a specific virus. T cells expressing these TCRs are therefore expected to be effective and suitable for use in a therapeutic setting. With the extensive number of TCRs that were found in ***chapter 4***, this library of TCRs could be used in ***chapter 2*** to look *in vivo* whether T cells with virus-specific public TCRs are expanding in response to viral reactivation. Here, we found expansion of virus-specific T cells that contained public TCRs coinciding with disappearance of viral loads in multiple patients.

Combined knowledge obtained about the potential therapeutic relevance of public TCRs (or highly-similar TCRs) in ***chapter 2***, their potential off-target reactivities studied in ***chapter 3*** and a library of public TCRs generated in ***chapter 4***, could be applied for generating a virtual library of virus-specific TCRs for future therapeutic use in immunodeficient patients. We did not specifically looked into the safety of these public TCRs individually. However, the virus-specific T-cell populations (e.g. HLA-A*02:01-restricted EBV-LMP2^{FLY}) from which these TCRs were derived, did not show any allo-HLA cross-reactivity when tested against a panel of EBV-LCLs expressing allogeneic HLA molecules in ***chapter 3***. Contrary to this example, some virus-specific T cells expressing public TCRs were shown to have a predictable allo-HLA cross-reactivity, like the already known allo-HLA-B*44 cross-reactive HLA-B*08:01-restricted EBV-EBNA3A^{FLR}-specific TCR(57) (TRBV7.8), the newly identified allo-HLA-B*35 cross-reactive HLA-B*08:01-restricted EBV-EBNA3A^{FLR}-specific TCR (TRBV4-3, not published) and the newly identified allo-HLA-B*35 cross-reactive HLA-A*02:01-restricted EBV-EBNA3C^{CLG}-specific TCR. Since also a public EBV-EBNA3A^{FLR}-specific TCR was found without demonstrating allo-HLA cross-reactivity, generating a library of TCRs with exclusively beneficial on-target effects might therefore be feasible for every targetable viral-antigen, including HLA-B*08:01-restricted-EBV-EBNA3A^{FLR}. The virus-specific TCRs that do exhibit allo-HLA cross-reactivity can also be used, as long as patients are avoided that express the HLA-alleles with which they cross-react.

Viral antigens as targets for immunotherapy to treat virus-associated malignancies

One of the earliest described viruses that was shown to be associated with different types of malignancies was EBV. Malignancies associated with this virus often exhibit one of the 3 latency phases of EBV. In latency phase III, EBV expresses all viral proteins (e.g, EBV nuclear antigen 1–3 (EBNA1–3) and LMP 1 and 2), while in latency phase I only EBNA1 is expressed(58). The inability to control EBV-reactivations can occur as a rare complication in healthy individuals, often referred to as chronic active EBV (CAEBV) infection(59). CAEBV infection may progress to the development of several malignancies of lymphoid origin including Burkitt lymphoma, Hodgkin lymphoma, B-, T-, and natural killer cell lymphomas, posttransplant lymphoproliferative disorder (PTLD) as well as epithelial malignancies like nasopharyngeal carcinoma and gastric carcinoma(60, 61). Each of these malignancies is associated with different latency phases. In alloSCT recipients, the inability to control EBV-reactivations occurs more often and was shown to lead to EBV-driven post-transplant lymphoproliferative disease (PTLD)(62). The risk for PTLD is increased for recipients that receive a graft that is T-cell depleted, HLA-mismatched, from an unrelated donor or when the patient is elderly(63-65). Especially absence of EBV-specific T cells, and thereby absence of control/suppression, is known to be associated with development of PTLD. Introduction of the B-cell targeting anti-CD20 monoclonal antibody Rituximab has significantly improved PTLD-related mortality(63). However, if patients do not respond to Rituximab, they have a poor prognosis.

An alternative strategy for the prevention or treatment of PTLD is infusion of donor-derived EBV-specific T cells prepared *in vitro*. However, since EBV-associated malignancies are associated with different latency phases, a product needs to be generated that is specific for EBV-derived antigens that are also expressed during the latency phase that corresponds with the malignancy. EBV-driven PTLD is associated with latency phase III, resulting in expression of all immunogenic antigens by EBV-infected B cells. Treatment of PTLD with EBV-specific T cells has been proven successful and promising after allogeneic hematopoietic stem cell transplantation(66-68). Recently, results from a phase III multicenter study (ALLELE-study) were released whereby Tab-cel, an off-the-shelf allogeneic EBV-specific T-cell immunotherapy, showed promising results for patients with PTLD that were not responding to rituximab(69). Recently, marketing authorization approval by the European Medicines Agency (EMA) was obtained for this allogeneic T-cell immunotherapy Tabelecleucel (tab-cel®). This could open the door for more future T-cell based immunotherapies, including the use of HLA-matched third-party donors from a virtual repository. Recently, adoptively transferred EBV-LMP1/2–specific T cells were used to treat EBV+ latency type II lymphomas, that mainly express LMP1 and LMP2(70, 71). However, the majority of these described antigens are restricted to common (predominantly Caucasian) HLA molecules like HLA-A*02:01 and HLA-B*07:02. While patients expressing HLA-A*01:01 and/or HLA-B*37:01 have an increased risk of

developing EBV-associated malignancies(72), strikingly, EBV-specific T cells recognizing any of the EBV antigens in the context of the common HLA-A*01:01 molecule had not been characterized to date. With algorithms (netMHC) that could predict the binding of LMP1 or LMP2-derived peptides in HLA-A*01:01, we showed in ***chapter 6*** that HLA-A*01:01-restricted EBV-specific T cells existed. Although these cells were present, they were of such low frequency that they would need extensive culture periods for "off-the-shelf" therapy. However, it is also possible to determine the sequence encoding the TCR and utilize viral transduction to introduce such a functional virus-specific TCR into polyclonal donor-derived T cells, thereby redirecting their specificity(51). We demonstrated that the specificity and functionality of HLA-A*01:01-restricted EBV-LMP2-specific TCRs was maintained with TCR gene transfer. One concern of TCR gene therapy is the mispairing of the introduced and endogenous TCR chains, leading to the production of a TCR with undefined specificity, which yields a potential risk of GVHD. CRISPR-Cas9 gene-editing technology has been successfully used for the knockout of the endogenous TCR alongside retroviral insertion of the new TCR in primary T-cell populations(73), and was also shown in ***chapter 6*** to be successful. This approach prevents the potential GVHD caused by mispairing. Together with the described TCRs in ***chapter 4***, a library of TCRs with exclusively on-target effects might be a future approach to introduce these TCRs into donor-derived polyclonal T cells with endogenous TCR knockout to target any viral-antigen, either in the setting of viral-reactivation, or virus-associated malignancies.

In this thesis the risks and potential benefits of adoptively transferred (third-party-derived) virus-specific T cells were investigated. The results described in this thesis allow for the implementation of a registered HLA-typed virtual repository of seropositive healthy donors as source for third-party virus-specific T cells using specific rules to select donors to minimize potential off-target reactivities. Future research should investigate the risk of allo-HLA cross-reactivity of virus-specific T cells obtained from other HLA backgrounds than the ones studied here. Fundamental knowledge obtained about virus-specific public TCRs and private TCRs could open up the field of using these TCRs in potential future TCR-gene transfer therapies, or as diagnostic tool to monitor virus-specific T-cell responses. However, more emphasis on virus-specific T cells restricted to non-Caucasian HLA molecules should be focused on to make this more broadly applicable. Future research should also expand the knowledge that has currently been obtained about viral antigens as targets for immunotherapy to treat virus-associated malignancies.

In conclusion, we showed that allo-HLA cross-reactivity mediated by virus-specific T cells is not as unpredictable as previously thought. It follows rules allowing us to make a risk assessment of potential third party donors based on the HLA background and

HLA restriction of the third-party donor-derived T cells. Furthermore, the majority of virus-specific T-cell populations demonstrated to express public TCRs that can be used to track virus-specific T cells *in vivo*, while they are also good candidates for future TCR-gene transfer strategies as demonstrated by the expansion in patients with CMV/EBV reactivations.

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ADDENDUM A

Nederlandse samenvatting
Abbreviations
List of publications and conference presentations
Curriculum vitae
Dankwoord

NEDERLANDSE SAMENVATTING

Immuungecompromitteerde patiënten en virale infecties

Virussen zijn pathogenen die een gastheercel nodig hebben om te reproduceren. Afhankelijk van het type virus worden virusdeeltjes verspreid wanneer de gastheercel doodgaat of wanneer virusdeeltjes de gastheercel verlaten zonder deze dood te maken. De antivirale immuunrespons die dan volgt, kan opgedeeld worden in een vroege niet-specifieke fase en een antigen-specifieke fase waarbij T en B cellen een belangrijke rol spelen. B cellen differentiëren in plasmacellen en produceren virus-specifieke antilichamen die vrije virusdeeltjes kunnen herkennen en neutraliseren. T cellen kunnen daarentegen virusdeeltjes herkennen die gepresenteerd worden op het oppervlak van virus-geïnfecteerde cellen. Deze vorm van immuniteit is beperkt functioneel of zelfs afwezig bij immuungecompromitteerde patiënten. Bijvoorbeeld bij patiënten die een allogene stamceltransplantatie (alloSCT) hebben ondergaan voor de behandeling van een hematologische maligniteit. De grootste uitdaging in het veld van alloSCT is een balans vinden tussen het optreden van het gunstige graft versus leukemia (GVL; transplantaat versus leukemie) effect en graft versus host disease (GvHD; transplantaat versus gastheer ziekte). Bij GvHD worden weefsels zoals de darmen, lever, longen of huid van de patiënt aangevallen door T cellen van de donor. Strategieën om GvHD tegen te gaan, bestaan uit het geven van immunosuppressieve medicatie of het verwijderen van donor T cellen uit het stamceltransplantaat. Deze T cellen kunnen dan op een later moment alsnog gegeven worden om het GVL effect te bewerkstelligen. Deze strategieën leiden allebei tot een tijdelijk gecompromitteerde immuniteit, waardoor virale infecties niet goed onder controle kunnen worden gehouden. De virale pathogenen die de meeste complicaties geven in patiënten na alloSCT, zijn cytomegalovirus (CMV), Epstein-Barr virus (EBV) en humaan Adenovirus (AdV). Deze virussen kunnen persisteren in gastheercellen na de eerste infectie en kunnen op een later moment reactiveren vanuit hun latente fase.

Controle van virus reactivatie in immuungecompromitteerde patiënten

CMV, EBV en AdV zijn virussen die na primaire infectie latent aanwezig kunnen blijven in verschillende type cellen. Ook in gezonde individuen worden deze virussen niet volledig opgeruimd, maar worden ze wel onder controle gehouden door de antivirale T cellen. Bij immuungecompromitteerde patiënten zorgen CMV, EBV en AdV wel frequent voor problemen, omdat functionele antivirale T cellen onvoldoende aanwezig zijn, terwijl deze virussen nog wel latent aanwezig zijn. Antivirale medicatie zoals ganciclovir, foscarnet, cidofovir en letermovir kunnen worden gebruikt om de virus replicatie te remmen. Deze middelen hebben echter alleen een tijdelijk effect en moeten herhaaldelijk worden toegediend. Het komt in sommige gevallen ook voor dat patiënten niet meer reageren op deze medicatie. Aangezien het herstel van virus-specifieke T cellen na allo-

SCT noodzakelijk is voor adequate controle van virale infecties, is het overbrengen van functionele virus-specifieke T cellen van de stamceldonor naar de ontvanger/patiënt een aantrekkelijke strategie om de antivirale immuniteit sneller terug te krijgen in patiënten na alloSCT. Als de stamceldonor in het verleden in contact is gekomen met deze virussen, heeft het immuunsysteem van de stamceldonor een adequate antivirale immuniteit opgebouwd. De aanwezige virus-specifieke T cellen van de donor van de oorspronkelijke stamceltransplantatie kunnen dan geselecteerd worden uit perifeer bloed van de donor. Er zijn inmiddels verschillende manieren om deze T cellen te selecteren, waarbij de resultaten van een aantal exploratieve klinische studies laten zien dat deze cellen veilig toegediend kunnen worden aan de betreffende patiënt zonder ontwikkeling van GvHD. Daarnaast werden na toediening van de geselecteerde donor T cellen aan de patiënt toegenomen frequenties van virus-specifieke T cellen waargenomen in perifeer bloed van de ontvanger/patiënt. Deze strategie kan echter alleen reproduceerbaar worden uitgevoerd wanneer de stamceldonor ooit eerder in contact is geweest met het virus, gemeten door aanwezige antistoffen in het bloed (seropositief), en daardoor geheugen T cellen gericht tegen dit virus heeft gegenereerd.

Wanneer een stamceldonor dus niet eerder in contact is geweest met het virus, kunnen er niet reproduceerbaar virus-specifieke T cellen geïsoleerd worden. Een andere vervangende stamceldonor die wel seropositief is, is vaak niet makkelijk vindbaar. De zoektocht naar een geschikte stamceldonor begint namelijk met de vergelijking van de humane leukocyten antigenen (HLA) typering van de patiënt en de potentiële stamceldonoren. Doordat deze HLA moleculen polymorf zijn, en daardoor grotendeels heel verschillend zijn tussen individuen, is het moeilijk om een match tussen stamceldonor en patiënt te vinden. Deze HLA moleculen komen tot expressie op het oppervlakte van cellen en zijn verantwoordelijk voor de presentatie van peptiden (antigenen) die door T cellen kunnen worden herkend. Voordat T cellen deze peptiden in HLA moleculen kunnen herkennen, worden T cellen opgeleid in een klier die de zwezerik (thymus) genoemd wordt. Tijdens deze thymus-selectie, worden T cellen die in staat zijn peptiden net genoeg te herkennen in de context van het eigen (autoloog) HLA geselecteerd en “vrijgegeven” in het bloed. T cellen die peptiden in eigen HLA te sterk of helemaal niet herkennen, worden verwijderd. Belangrijk hierbij is dat T cellen die vreemd (allogeen; allo) HLA goed zouden kunnen herkennen niet onderworpen worden aan dit selectieproces, omdat deze vreemde HLA moleculen niet tot expressie komen in de eigen thymus. De receptor waarmee T cellen het gepresenteerde peptide kunnen herkennen in een HLA molecuul, heet de T-cel receptor (TCR).

Indien virus-specifieke T cellen niet uit de eigen stamceldonor geïsoleerd kunnen worden, kan er worden gezocht naar een andere geschikte seropositieve gezonde donor (derde partij). Een mogelijke bron voor gezonde seropositieve donoren is de bloedbank.

Donoren van deze derde partij kunnen dan gebruikt worden om virus-specifieke T cellen uit het bloed te isoleren en toe te dienen aan de patiënt, waardoor de antivirale immuniteit in de patiënt hersteld kan worden. Deze derde partij donor zal alleen grotendeels niet gematcht zijn voor de HLA moleculen van patiënt en/of stamceldonor.

Ongewenste reactiviteit door gedeeltelijke HLA match

Gedeeltelijke HLA match tussen patiënt, stamceldonor en de derde partij donor kan leiden tot ongewenste reactiviteit van T cellen van de derde partij donor gericht tegen cellen van de patiënt en/of de stamceldonor, die HLA moleculen tot expressie brengen die vreemd (allogeen) zijn voor de T cellen van de derde partij donor. Andersom kunnen de T cellen van de stamceldonor het virus-specifieke T-cel transplantaat van de derde partij donor afstoten waardoor er geen ongewenste reactiviteit kan plaatsvinden, maar ook geen adequate controle van de virale reactivatie. In allebei de gevallen is er geen thymus selectie geweest van de allogene HLA moleculen. Virus-specifieke T cellen afkomstig van een derde partij donor herkennen met hun TCR een viraal antigen in de context van het autologe HLA, maar wanneer diezelfde TCR een ander peptide herkent in de context van het allogene HLA, dan kan dit leiden tot ongewenste reactiviteit in de vorm van GVHD of transplantaatafstoting. Deze vorm van ongewenste reactiviteit wordt ook wel allo-HLA kruis-reactiviteit genoemd. Wanneer virus-specifieke T cellen van een derde partij donor niet voldoende HLA gematcht worden met de patiënt, kan dit dus tot allo-HLA kruis-reactiviteit leiden. Als we zouden kunnen voorspellen welke allogene HLA moleculen herkend kunnen worden door virus-specifieke T cellen bij een gedeeltelijke HLA match, kunnen we specifieke donoren, virus-specifieke T-cel populaties of TCRs selecteren met een minimaal risico op allo-HLA kruis-reactiviteit.

Dit proefschrift

Het onderzoek in dit proefschrift was gefocust op de opties die gebruikt kunnen worden om virale reactivaties onder controle te houden in immuungecompromitteerde patiënten door middel van toediening van virus-specifieke T cellen of genetische introductie van virus-specifieke TCRs en de risico's die daarmee gepaard gaan.

Om de effectiviteit te verklaren van virus-specifieke T cellen afkomstig van stamceldonoren, is het van belang om het lot van de toegediende virus-specifieke T cellen in de patiënt te bepalen. Voorheen was het alleen moeilijk om de toegediende virus-specifieke donor T cellen te onderscheiden van T cellen afkomstig van de patiënt of van donor T cellen uit het stamceltransplantaat die de conditionering hebben overleefd. In ***hoofdstuk 2*** hebben we van virus-specifieke T cellen afkomstig van stamceldonoren (T-cel producten) de sequenties van de TCRs van alle individuele virus-specifieke T-cel populaties achterhaald en daarmee deze T cellen kunnen traceren in het bloed van de individuele patiënten na toediening van deze T-cel producten. Met behulp van de

sequenties van de TCRs konden we onderscheid maken tussen virus-specifieke T cellen die alleen afkomstig waren uit het T-cel product, T cellen afkomstig van de patiënt, of T cellen die al aanwezig waren voor toediening van het virus-specifieke T-cel product (T cellen uit het stamceltransplantaat). De expansie van virus-specifieke T cellen tijdens een virale reactivatie verliep met eenzelfde kinetiek voor T cellen die exclusief afkomstig waren uit het toegediende T-cel product als voor virus-specifieke T cellen die al aanwezig waren in de patiënt voorafgaand aan de infusie van het virus-specifieke T-cel product. Daarnaast hebben we kunnen aantonen dat virus-specifieke T cellen die exclusief afkomstig waren uit het virus-specifieke T-cel product, ook konden persisteren in afwezigheid van een virale reactivatie.

In sommige situaties kunnen geheugen virus-specifieke T cellen niet geïsoleerd worden uit perifeer bloed van de stamceldonor. Bijvoorbeeld wanneer de stamceldonor de virusinfectie niet eerder heeft doorgemaakt (seronegatief). Voor patiënten met een seronegatieve stamceldonor, zou een virus-seropositieve derde partij donor een snelle interventie mogelijk maken om de antivirale immuniteit te herstellen door middel van infusie van derde partij donor afkomstige virus-specifieke geheugen T cellen. Echter, T-cel producten van een derde partij zijn over het algemeen slechts gedeeltelijk HLA gematcht met de patiënt. De virus-specifieke T cellen van een derde partij donor zullen dus een andere opmaak van HLA moleculen hebben (HLA typering) ten opzichte van de patiënt. De HLA typering classificeert HLA-klasse-I moleculen HLA-A, -B en -C en HLA-klasse-II moleculen HLA-DR, -DQ- en -DP. Uiteindelijk kunnen er 12 verschillende HLA moleculen tot expressie komen per individu. De virus-specifieke T cellen van de derde partij donor moeten op zijn minst voor 1 van de 12 mogelijke HLA moleculen gematcht zijn met de patiënt, anders kunnen de virus-specifieke T cellen geen virus-geïnfecteerde cellen opruimen, aangezien deze de virale peptide zullen presenteren in autoloog HLA. In ***hoofdstuk 3*** hebben we virus-specifieke T cellen van gezonde donoren geïsoleerd en getest om te onderzoeken of de virus-specificiteit, HLA restrictie en/of HLA typering van de T cellen bepalende factoren waren om het risico op allo-HLA kruis-reactiviteit te kunnen voorspellen. Om de allo-HLA kruis-reactiviteit te testen werden de virus-specifieke T cellen *in vitro* gestimuleerd met getransformeerde onsterfelijke cellijnen (EBV-LCLs) van 24 verschillende individuen. Deze individuen waren zo uitgekozen dat deze in totaal 116 verschillende allogene HLA moleculen tot expressie brengen. Vervolgens gebruikten we HLA-klasse-I/II negatieve K562 cellen om HLA allelen van interesse middels genetische modificatie te introduceren. Met 40 verschillende genetisch aangepaste K562 cellijnen waarbij elke cellijn 1 specifiek HLA molecuul tot expressie bracht, konden we bevestigen welke HLA moleculen herkend werden bij de allo-HLA kruis-reactivatie tegen de EBV-LCLs. HLA-B*08:01-gerestricteerde virus-specifieke T cellen lieten de hoogste frequentie en diversiteit van allo-HLA kruis-reactiviteit zien, onafhankelijk van hun virus-specificiteit. De allo-HLA kruis-reactiviteit was voornamelijk gericht tegen een specifieke

groep van allogene HLA-B moleculen. Door heterozygote donoren te selecteren die naast HLA-B*08:01 ook positief waren voor 1 van deze allogene HLA-B moleculen, bewezen we dat de frequentie en diversiteit van HLA-B*08:01-gerestricteerde virus-specifieke T cellen dan significant lager werd. De thymus selectie in deze heterozygote donoren heeft ervoor gezorgd dat deze allo-HLA kruis-reactieve virus-specifieke T cellen verwijderd werden. Daarentegen waren HLA-B*08:01-gerestricteerde virus-specifieke T cellen van heterozygote donoren die naast HLA-B*08:01 ook positief waren voor een allogeen HLA-B molecuul die niet herkend werd, nog steeds in dezelfde mate allo-HLA kruis reactief tegen een grote groep van allogene HLA-B moleculen. Deze resultaten lieten zien dat de frequentie, diversiteit en specificiteit van allo-HLA kruis-reactiviteit wetmatigheden volgen.

Het selectief verrijken voor T cellen met een TCR, die beperkte of geen allo-HLA kruis-reactiviteit laat zien, zou een andere optie zijn om het risico van ongewenste reactiviteit te verminderen van T-cel producten afkomstig van een derde partij donor. In een ideale setting, zouden virus-specifieke T cellen gebruikt kunnen worden met TCRs die veelvoorkomend zijn en in verschillende individuen te vinden zijn. De kans dat dit soort virus-specifieke T cellen allo-HLA kruis reactief zijn tegen een grote groep van allogene HLA moleculen is dan namelijk klein. Dit soort TCRs worden ook wel public TCRs genoemd. Om die reden hebben we in ***hoofdstuk 4*** de TCR repertoires van CMV, EBV en AdV-specifieke T cellen van gezonde individuen kwantitatief onderzocht. Hierbij is de prevalentie van een public TCR en TCRs die daar sterk op lijken binnen de groep van gezonde individuen bestudeerd. Daarnaast is er gekeken naar de frequentie van een public TCR en TCRs die daar sterk op lijken binnen ieder individu. Bijna 1/3 van alle CMV, EBV en AdV-specifieke TCR nucleotide sequenties konden vertaald worden naar een public TCR aminozuur sequentie. Daarbovenop had 12% van de TCRs een sterk vergelijkbare sequentie die maximaal 3 aminozuren verschilden. We lieten in dit onderzoek zien dat deze public TCRs en sterk vergelijkbare TCRs structureel gerelateerd waren en een gedeeld motief hadden in de TCR sequentie. Gecombineerd kwamen deze public TCRs en sterk vergelijkbare TCRs in meer dan 50% binnen de groep van gezonde individuen voor en de frequentie binnen een virus-specifieke T-cel populatie was meer dan 10%. We concludeerden dat de bijdrage van public TCRs en sterk vergelijkbare TCRs een groot aandeel had in virus-specifieke T-cel responsen. Dit soort TCRs zouden gebruikt kunnen worden als diagnostische tool of als toekomstig therapeutisch middel door de genetische code van deze TCRs te introduceren in immuun cellen om virale reactivaties onder controle te houden in immuungecompromitteerde patiënten.

In ***hoofdstuk 4*** werden frequent TCRs gevonden die sterk vergelijkbaar waren met public TCRs, maar waarbij alleen kleine variaties van aminozuren gevonden werden op specifieke posities in de TCR sequentie. Het bleef echter onduidelijk wat de toegestane

mogelijkheden/'vrijheid' van verschillende aminozuren (20 verschillende) waren op deze specifieke posities en wat dat voor invloed had op de specificiteit. Om te onderzoeken of de diversiteit van de verschillende aminozuren op een positie met grote variatie invloed had op de specificiteit, hebben we ***in hoofdstuk 5*** een HLA-A*02:01-gerestricteerde EBV-specifieke public TCR als model gebruikt en daarbij systematisch het aminozuur op positie 5 van de Complementary Determining Region 3 (CDR3) vervangen voor alle 20 mogelijke aminozuren. We hebben laten zien dat de aminozuren op die positie van de TCR volledig vervangbaar waren voor elk van de 20 aminozuren, zonder verlies van TCR functie en specificiteit. De reden waarom sommige varianten niet eerder gevonden werden in het T-cel repertoire van gezonde individuen kon verklaard worden door de verminderde kans om die sequenties te maken tijdens het generen van die TCR sequenties in de thymus in plaats van beperkingen in binding.

Voor patiënten met een seronegatieve stamceldonor zou de antivirale immuniteit snel hersteld kunnen worden door middel van infusie van derde partij donor afkomstige virus-specifieke geheugen T cellen. Deze strategie zou ook gebruikt kunnen worden voor patiënten met virus-geassocieerde maligniteiten, zoals EBV-geassocieerde maligniteiten, waarbij EBV antigenen zoals LMP1 of LMP2 hoog tot expressie komen. Virus-specifieke T cellen gericht tegen deze antigenen zijn echter in kleine getallen aanwezig of nog niet eerder gedetecteerd binnen bepaalde HLA restricties, zoals bijvoorbeeld HLA-A*01:01. Dit maakt het lastig om LMP1 en/of LMP2-specifieke virus-specifieke T cellen te isoleren en direct toe te dienen bij deze patiënten. Een alternatief zou zijn om de genetische code van LMP1 en/of LMP2 virus-specifieke TCRs te introduceren in T cellen van een donor. Patiënten die HLA-A*01:01 positief zijn hebben een vergroot risico op het ontwikkelen van EBV-geassocieerde maligniteiten en zouden kunnen profiteren van een EBV-specifiek T-cel product dat een LMP1 of LMP2 antigen herkent in de context van HLA-A*01:01. Vreemd genoeg waren er tot nu toe nog nooit T cellen gevonden die een EBV afkomstig antigen herkennen in de context van HLA-A*01:01. Daarom probeerden we in ***hoofdstuk 6*** EBV-LMP1 en EBV-LMP2-specifieke T cellen te isoleren uit HLA-A*01:01 positieve donoren ende genetische codes van deze TCRs te achterhalen. HLA-A*01:01-gerestricteerde EBV-LMP2-specifieke T cellen werden succesvol geïsoleerd en hun TCRs werden gekarakteriseerd. Introductie van de sequentie van EBV-LMP2-specific TCRs in primaire T cellen resulteerde in een populatie van T cellen die specifiek waren voor EBV-LMP2 en reactiviteit lieten zien tegen HLA-A*01:01 positieve cellijnen die EBV-LMP2 tot expressie brachten. Hierna hebben we deze EBV-LMP2-specifieke TCRs in primaire T cellen geïntroduceerd, waarbij de endogene TCR werd uitgeschakeld (ΔTCR) met behulp van CRISPR-Cas9-technologie om het potentiële misparen van de geïntroduceerde TCR met het endogene TCR te beperken. Na het uitschakelen van de endogene TCR bleken deze T cellen die gemodificeerd waren om EBV-LMP2-specifieke TCRs tot expressie te brengen hun functionaliteit te behouden en lieten ze cytotoxiciteit

zien tegen kwaadaardige cellijnen die EBV-LMP2 tot expressie brachten. Deze HLA-A*01:01-gerestricteerde EBV-LMP2-specifieke TCR zou mogelijk gebruikt kunnen worden in toekomstige TCR-gen-therapie voor de behandeling van patiënten met EBV-geassocieerde latente type II/III maligniteiten die LMP2 tot expressie brengen.

Het onderzoek in dit proefschrift heeft aangetoond dat allo-HLA kruisreactiviteit, gemedieerd door virus-specifieke T-cellen, niet zo onvoorspelbaar is als eerder werd gedacht. Het volgt regels waardoor we een risicobeoordeling kunnen maken van potentiële geschikte derde partij donoren op basis van de HLA achtergrond en HLA restrictie van de T cellen van de derde partij donoren. Bovendien toonde de meerderheid van de virus-specifieke T-cel populaties aan dat ze publieke TCRs tot expressie brachten die kunnen worden gebruikt om virus-specifieke T cellen *in vivo* te volgen, terwijl ze ook goede kandidaten zijn voor toekomstige TCR-gentransfer strategieën, zoals aangetoond door de expansie bij patiënten met CMV/EBV reactivaties.

ABBREVIATIONS

AdV	Adenovirus
AlloSCT	Allogeneic stem cell transplantation
APCs	Antigen presenting cells
APC	Allophycocyanin
B2m	β2-microglobulin
BD	Beckton dickinson
BM	Bone marrow
CAEBV	Chronic active EBV
CAR	Chimeric Antigen Receptor
CD	Cluster of differentiation
CMV	Cytomegalovirus
CDR1,2,3	Complementary determining region 1,2,3
DC	Dendritic cell
DLI	Donor lymphocyte infusion
DLBCL	Diffuse large B-cell lymphoma
EBV	Epstein-Barr virus
EBV-LCLs	EBV-transformed lymphoblastoid cell-lines
FBS	Fecal bovine serum
EBNA1-3	EBV Nuclear Antigen 1-3
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FACS	Fluorescent activated cell sorting
FITC	Fluorescein isothiocyanate
FL	Follicular lymphoma
GC	Gastric carcinoma
GMP	Good manufacturing practise
GvHD	Graft versus host disease
GVL	Graft versus leukemia
HIV	Human Immunodeficiency Virus
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
IFN-γ	Interferon gamma
IL-2	Interleukin-2
IMDM	Iscove's modified dulbecco's medium
LMPs	Latent membrane proteins
LUMC	Leiden university medical center
MACS	Magnetic activated cell sorting
MHC	Major histocompatibility complex

NGFR	Nerve growth factor receptor
NK	Natural killer
NPC	Nasopharyngeal carcinoma
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PerCP	Peridinin-chlorophyllprotein
pMHC	Peptide major histocompatibility complex
PHA	Phytohemagglutinin
PTLD	Post-transplant lymphoproliferative disease
PUB-HS	Public highly-similar
PUB-I	Public identical
Rt-PCR	Reverse transcriptase polymerase chain reactions
TCD	T-cell depletion
TCR	T-cell receptor
TRAV	T-cell receptor alpha variable
TRAJ	T-cell receptor alpha joining
TRBD	T-cell receptor beta determining
TRBV	T-cell receptor beta variable
TRBJ	T-cell receptor beta joining
TSO	Template switching oligo

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

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Third party virus-specific T-cells for treatment of viral reactivations in immune compromised patients and risks of allo-HLA cross-reactivity

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Huisman W , Leboux DAT, Hageman L, Amsen D , Falkenburg JHF, Jedema I
Third party virus-specific T-cells for treatment of viral reactivations in immune compromised patients and risks of allo-HLA cross-reactivity

American Society of Hematology (ASH) Annual Meeting 2018, San Diego, USA (poster presentation)
Huisman W , Leboux DAT, van der Maarel LE, Hageman L, Amsen D , Falkenburg JHF, Jedema I
The Scope of Allo-HLA Cross-Reactivity by (Third Party) Virus Specific T Cells is Surprisingly Affected by HLA Restriction Rather than Virus Specificity

American Society of Hematology (ASH) Annual Meeting 2018, San Diego, USA (poster presentation)
Huisman W, van der Maarel LE, Hageman L, de Jong RCM, Amsen D, Falkenburg JHF, Jedema I
Isolation and Validation of the First Functional HLA-A*01:01 Restricted EBV-LMP2 Specific T Cells For Treatment of EBV Associated Type II/III Lymphomas

European Society for Blood and Marrow Transplantation (EBMT) Annual Meeting 2019, Frankfurt, Germany (oral presentation)
Huisman W , Leboux DAT, van der Maarel LE, Hageman L, Amsen D , Falkenburg JHF, Jedema I
The Scope of Allo-HLA Cross-Reactivity by (Third Party) Virus-Specific T-Cells is Surprisingly Affected by HLA Restriction Rather than Virus Specificity
Winner Best Young Abstract Award

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Off-Target HLA Cross-Reactivity by (Third Party) Virus-Specific T Cells is Surprisingly Affected by HLA Restriction and HLA Background but not by Virus Specificity
Winner Abstract Achievement Award

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The First Functional HLA-A*01:01-Restricted EBV-LMP2-Specific T-cell Receptors For TCR Gene Therapy Of Patients With EBV-Associated Type II/III Malignancies
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The First Functional HLA-A*01:01-Restricted EBV-LMP2-Specific T-cell Receptors For TCR Gene Therapy Of Patients With EBV-Associated Type II/III Malignancies

CURRICULUM VITAE

Wesley Huisman werd geboren op 16 juni 1991 in Zoetermeer. In 2010 haalde hij zijn atheneum diploma aan het Erasmus College te Zoetermeer, waarna hij startte met de studie Biologie aan de Universiteit van Leiden. Voor aanvang van de masteropleiding Biologie waar hij zich specialiseerde voor cellulaire biologie, deed hij 7 maanden wetenschappelijk onderzoek op de afdeling Celbiologie aan de Universiteit van Leiden. Hier onderzocht hij welke type prothese materialen bacteriële infecties kon voorkomen door gebruik te maken van infectie modellen in zebravissen. Tijdens de masteropleiding voor cellulaire biologie deed hij vervolgens 9 maanden wetenschappelijk onderzoek op de Afdeling Hematologie op het Laboratorium voor Experimentele Hematologie in het Leiden University Medical Center (LUMC) waarbij er werd onderzocht of een dendritische cellijn (DCOne®) die tumor geassocieerde antigenen (TAAs) tot expressie brachten als 'vaccin' kon worden gebruikt om T cellen te richten tegen die deze TAAs. Vervolgens heeft hij 10 maanden lang wetenschappelijk onderzoek gedaan op de afdeling Genetica in het Erasmus Medisch Centrum. Hier heeft hij onderzocht of een speciale type spierstamcellen, genoemd reserve cells, verrijkt konden worden *in vitro* en als therapie worden gebruikt bij spierziekten. Hij heeft dit onderzocht in verschillende muis modellen waarbij spierschade was toegebracht.

In oktober 2015 is hij gestart met zijn promotieonderzoek op het Laboratorium voor Experimentele Hematologie van de Afdeling Hematologie van het LUMC onder begeleiding van prof. dr. J.H.F. Falkenburg en dr. I. Jedema. Dit onderzoek was in samenwerking met dr. D. Amsen van Sanquin, als onderdeel van een Product and Process Development of Cellular Products (PPOC) project. In het kader van de verschillende studies bezocht hij diverse congressen in binnen- en buitenland waar hij meerdere presentaties gaf. De resultaten van de projecten zijn beschreven in dit proefschrift.

Op 1 mei 2021 is hij gestart als onderzoeker bij de afdeling Leiden University Center of Infectious Diseases (LU-CID) in het LUMC waarbij hij onderzoek doet naar de wisselwerking van (antigen-specifieke) T cellen die zich in de bovenste luchtwegen bevinden en T cellen die zich in het perifere bloed bevinden.

DANKWOORD

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