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

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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).

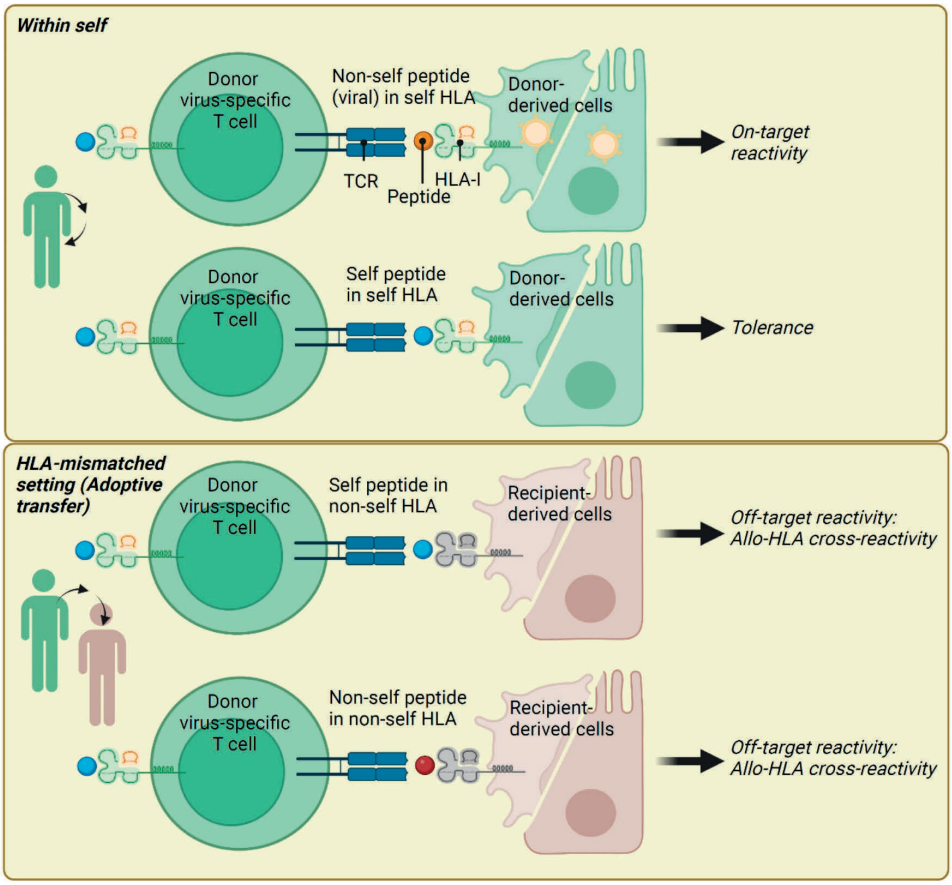

Figure 1. Peptide-HLA complexes as targets for T cells expressing a virus-specific TCR in an HLA-mismatched setting. Top panel; Non-self peptides (e.g. viral peptides) recognized in the context of donor-derived HLA molecules (self) induces on-target reactivity by donor-derived T cells expressing a virus-specific 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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