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

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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.

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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 IFNγ), 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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