Designing T-cells with desired T-cell receptor make-up for immunotherapy

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Introduction

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STEM CELL TRANSPLANTATION

Allogeneic stem cell transplantation (allo-SCT) is a treatment option with curative potential for patients with various malignant and non-malignant hematological diseases[1]. Conventional myeloablative transplantation includes pre-transplantation conditioning with high dose chemo- and radiotherapy to eradicate residual disease and recipient (host) immunity in preparation for healthy donor-derived hematopoietic stem cells (graft). Allo-SCT is performed to replace the lethally damaged hematopoietic stem cells from the patient by donor hematopoietic stem cells that have the ability to proliferate and differentiate into mature blood cells and reconstitute the patient's hematopoietic system with donor-derived healthy blood cells.

Unfortunately, T-cells present in the stem cell graft from the donor can lead to severe damage to various tissues, named graft versus host disease (GvHD). GvHD is characterized by lesions of the skin, gut and the liver and is clinically subdivided in four degrees of severity. GvHD is one of the main causes of morbidity and mortality after allo-SCT. GvHD after allo-SCT can be inhibited by administering immunosuppressive agents that affect T-cell activation and proliferation. To prevent GvHD, T-cell depleted allo-SCT can be applied, resulting in a decreased incidence and severity of GvHD[2-4].

However, T-cell removal resulted in increased incidence of relapse of leukemia after allo-SCT and did not result in significantly improved overall survival[5,6]. In line with this finding was the association of the occurrence of GvHD with a decreased likelihood of relapse of the leukemia after allo-SCT[7,8]. These observations indicated that donor derived T-cells present in the stem cell graft not only mediate GvHD, but can also mediate a Graft versus leukemia (GvL) effect. Indirectly, the role of T-cells in GvL effect was demonstrated by the induction of remissions in patients after withdrawal of immunosuppression[9,10]. The observation that allo-SCT was associated with a lower risk of relapse and better disease-free survival than autologous SCT indicated that the T-cells mediating the GvL effect had to be from donor origin[11,12]. In addition, the finding of higher relapse rates in recipients of syngeneic compared to allogeneic transplants indicated that genetic disparities between patient and donor are necessary for the GvL effect[13,14]. The demonstration that infusions of lymphocytes from the original marrow donor without additional

chemotherapy could eradicate the recurrence of leukemia after allo-SCT provided the first direct evidence for a GvL effect[15,16].

The recognition that donor derived T-cells could mediate GvL activity laid the foundation for the subsequent development of non-myeloablative allo-SCT. The high intensity of myeloablative treatment aims at efficient killing of malignant stem cells. Regimen-related toxicity of the myeloablative treatment, however, limits this procedure to younger patients. The perception that donor T-cells were capable of efficiently eradicating leukemic cells resulted in development of reduced intensity conditioning regimens (RIC) in order to be able to perform nonmyeloablative allo-SCT in patients of older age, or with comorbidities[17-21]. These regimens do not eradicate all residual disease but result in sufficient immunoablation to permit engraftment of donor hematopoietic stem cells and induce a state of host-versus-graft tolerance that gives donor derived T-cells the opportunity to recognize and eliminate residual malignant stem cells. Although RIC regimens have been shown to permit engraftment with lower toxicity, GvHD is still an important complication, with considerable morbidity and mortality[22].

DONOR LYMPHOCYTE INFUSION / T-CELL BASED IMMUNOTHERAPY

The acknowledgement that donor derived T-cells have the capacity to specifically recognize and eradicate malignant cells initiated the development of T-cell based immunotherapy. After allo-SCT relapse of the hematological malignancy can occur that can be treated with donor lymphocyte infusion (DLI) from the original stem cell donor[23-25]. Treatment with DLI after allo-SCT can induce sustained complete remissions[23,24]. The best responses to DLI occur in patients with chronic myeloid leukemia (CML). Close to 80% of patients with relapsed chronic-phase CML after transplant will achieve a complete remission in response to unmanipulated DLI[15,23,24,26-28]. Patients with other malignancies respond less frequently to DLI[23,27,29-32]. Response rates of 25-50% have been reported in hematological malignancies like multiple myeloma (MM), chronic lymphocytic leukemia (CLL) and myelodysplasia (MDS). In acute lymhoblastic (ALL) and acute myeloid leukemia (AML), remissions have been documented even less frequently. Possibly, the time of donor T-cells to respond is too long in rapidly growing acute leukemia. Alternatively, the difference in responses to DLI may be due to intrinsic differences in susceptiblity of the diverse tumor types to adoptive immunotherapy.

Next to the beneficial GvL effect, induction of detrimental GvHD can be a severe complication of the application of DLI, especially in HLA-mismatched allo-SCT[33]. It remains challenging to separate GvL from GvHD. Individuals are genetically disparate due to a broad variety of single nucleotide polymorphisms (SNPs) that result in small differences in amino acid sequence of many proteins. Processing of these polymorphic stretches of amino acids that differ between patient and donor can lead to strong immune responses. Polymorphic peptides presented in the context of HLA-molecules able to elicit a donor immune response are defined as minor histocompatibility antigens (MiHAs) and

are thought to be the prime mediators of both GvL and GvHD after HLA-identical allo-SCT[34]. To selectively induce GvL, more defined T-cell populations with restricted anti-leukemic specificity should be used.

The possibility to isolate antigen-specific T-cells and reinfuse them to patients to reconstitute antigen-specific immunity has been demonstrated in immunodeficient bone marrow transplant recipients at risk for developing Cytomegalovirus (CMV) disease[35-39], Epstein-Barr virus (EBV) reactivation or development of EBV positive B cell lymphomas[40-43]. These opportunistic infectious diseases form a major clinical problem during the post-transplant period of immunodeficiency. It has been demonstrated that these viral diseases can be both prevented or cured by adoptive transfer of CMV-[35-39] and EBV-specific T-cells[40-43] isolated from the donor. This adoptive transfer was demonstrated not only to be effective but also to be safe without the induction of GvHD. Long-term persistence of the virus-specific donor T-cells could be demonstrated[44].

The isolation of therapeutic T-cells resulting in GvL without induction of GvHD has proven more difficult. Some patients with leukemia that were treated with MiHA-specific T-cells selected on bases of recognition of patient's normal hematopoietic and malignant cells but no recognition of non-hematopoietic cells like fibroblasts experienced exclusive GvL effect, whereas other patients suffered from GvHD without apparent GvL effect[45]. Previously, we reported the successful treatment of a patient with accelerated phase CML refractory to DLI infusion who received in vitro generated leukemia-reactive donor T-cells resulting in a molecular complete remission[46]. Based on this evidence that GvL can be separated from GvHD by using defined leukemia-reactive donor T-cells we have recently completed a phase I/II feasibility study analyzing the possibility of large scale in vitro generation of leukemia-reactive T-cells to treat patients with relapsed leukemia after allo-SCT[47]. Despite some evidence of clinical benefits, this technique is complex and very time-consuming and not feasible for every patient. In addition, it is now recognized that long in vitro culture periods negatively influence the in vivo functional activity of the T-cells[48-50].

In conclusion, adoptive transfer of donor derived T-cells with defined specificity directed against patient's malignant cells may be a potential strategy to separate the GvL effect from the GvHD. However, current approaches to obtain leukemia-specific donor T-cells are complex and time-consuming, and need to be customized for every patient.

T-CELLS

TCR rearrangement and selection

T-cells play a criticial role in protective immunity against different pathogens. Within the T-cell compartment, T-cells expressing the CD4 coreceptor and T-cells expressing the CD8 coreceptor can be distinguished that recognize peptides in the context of HLA class II or HLA class I molecules, respectively. T-cell precursors originating in the bone marrow migrate through the thymus where the definitive stages in T-cell development take place[51].

In the thymus selection takes place of T-cells expressing useful T-cell receptors (TCRs) able to engage self-HLA molecules[51], and lineage commitment to either CD4+ helper or CD8+ cytotoxic T-cells[52]. First, the rearrangement of TCR genes leading to TCR-cell surface expression is essential for progression during T-cell development. The TCR consists of a transmembrane heterodimer of TCRα and TCRβ chains linked with a disulfide bond. Each TCR locus consists of variable (V), joining (J), and constant (C) region genes, and the β chain locus also contains diversity (D) gene segments. Both TCR chains are the result of a complex process of random combination of different gene segments (V-D-J-C). The rearrangement of first the TCRβ and subsequently the TCRα genes result in the formation of TCRs with unique extracellular variable regions and a constant intracellular region[53]. During positive selection immature T-cells expressing TCRs with no or too low an affinity for self-HLA die by neglect, whereas immature T-cells with a TCR with intermediate affinity receive a survival signal. These immature T-cells subsequently commit to the CD4 or CD8 T-cell lineage with their precise lineage fate being determined by the HLA-restriction of their TCR. Immature T-cells that receive signals through HLA class II-restricted TCRs differentiate into CD4+ T-cells, whereas immature T-cells that receive signals through HLA class I-restricted TCRs differentiate into CD8+ T-cells. CD4+ and CD8+ T-cells then undergo negative selection resulting in elimination of T-cells with too high affinity to self-peptides in the context of self-HLA molecules.

Generally, the T-cells that end up in the periphery recognize via their unique TCR a particular conformation of an HLA molecule and antigenic peptide. The antigen-binding surface of a TCR is formed by three complementarity-determining regions (CDR) contributed by the TCRα and three contributed by the TCRβ chain. Whereas TCR CDR1 and CDR2 are well conserved throughout different TCRα and TCRβ subfamilies, the CDR3 region in contrast shows high diversity and plays a central role in peptide recognition[54]. The antigenic peptides that are recognized by T-cells, called epitopes, are derived from degraded proteins and can be presented in HLA class I or class II molecules[55]. The strength by which a T-cell binds to a target cell is called avidity. The T-cell avidity is determined by the affinity of the TCR for the antigen in the context of an HLA molecule, and additional interactions between T-cell and target cell via adhesion and costimulatory molecules that interact with different molecules on the target cells.

CD4 and CD8 coreceptors

Two molecules that play a role in enhancing the interaction between T-cell and target cell are the CD4 and CD8 co-receptors. CD4 and CD8 co-receptors are transmembrane proteins with extracellular domains that promote TCR engagement of HLA-ligands and, in addition, intracellular domains that enhance TCR signal transduction. The CD4 molecule is a coreceptor that enhances the overall avidity of the interaction between the T-cell and the target cell by binding to the β2 domain of the HLA class II molecule[56-58]. Whereas both the α and β chain of CD8 can cooperate to bind HLA class I molecules, the CD4 corececptor consists of one chain of which the N-terminal variable-like region makes

contact with the HLA-molecule. The CD8 co-receptor enhances binding between the T-cell and the target cell by binding to the alpha 3 domain of the HLA class I molecules[59,60]. While CD8 on peripheral T-cells mostly consists of disulfide-linked CD8αβ heterodimers, intestinal T-cells, γδ T-cells, and NK-cells express CD8αα homodimers[61-66]. CD8β protein requires association with CD8α for its stable expression at the cell surface. This is not due to the inability of CD8β molecules to form homodimers, which can be formed intracellularly, but these are unstable and rapidly degrade[67-69]. When expressed as cell-surface molecules, however, the coordinated binding of CD8αβ with TCR-engaged HLA class I is much stronger as compared to membrane-bound CD8αα[70-72]. Both CD4 and CD8 are molecules that promote signaling by HLA-restricted TCRs. The intracellular domains of CD4 and CD8 associate with the protein tyrosine kinase LCK which initiates TCR signal transduction when it is enzymatically activated[56,73-77]. By binding to the same peptide-HLA complexes that have engaged the TCR, CD4 and CD8 bring intracellular LCK, which is present in lipid rafts, into physical proximity with the cytosolic domains of the engaged TCR to initate signaling[59,78-80].

CD4+ and CD8+ T-cell functions

In response to antigen-recognition, several biological responses take place. The major effector functions of CD4+ helper T-cells are the secretion of cytokines acting on other T-cells and promoting CD8+ T-cell effector functions[81,82], as well as upregulation of CD40L promoting B-cell activation[83]. The major effector functions of CD8+ T-cells are the secretion of lytic granules that kill antigen positive target cells, as well as the production of cytokines[84]. In addition, after antigen-recognition, T-cells down-regulate their TCR resulting in a so called refractory period[85,86]. This refractory period enables T-cells to transcribe DNA, resulting in proliferation generating high numbers of antigen-specific T-cells and execution of different effector functions.

As mentioned before, CD4+ T-cells recognize peptides bound to HLA class II molecules. The major source of peptides that bind in HLA class II molecules are extracellular proteins. The major source of peptides that bind in HLA class I molecules and can be recognized by CD8+ T-cells are intracellular proteins found in the cytosol of antigen-presenting cells (APCs) or target cells. Although most CD8+ T-cells are cytotoxic T-cells recognizing antigens in the context of HLA class I and most CD4+ T-cells are helper T-cells recognizing antigens in the context of HLA class II, the existence of CD4+ T-cells with cytolytic capacity has been demonstrated previously[87]. In addition, CD8+ T-cells have been described recognizing an antigen in the context of HLA class I as well as an antigen in the context of HLA class II[88].

T-CELL DIFFERENTIATION

Antigen-encounter results in differentiation of naïve CD4+ and CD8+ T-cells not yet activated by antigen into either short- or long-lived effector and memory T-cells. Although T-cell responses quickly contract once the antigen is eliminated, memory T-cells survive and initiate larger and more efficient secondary

immune responses upon subsequent exposure to the antigen. The different naïve, effector and memory subsets can be distinguished both by their differential expression of several cell surface antigens and by their distinct functional properties[89-91].

In CMV- and EBV-specific immune responses different memory subsets are raised[92,93]. Even within one virus-specific memory response distinct memory subsets can be found. For example, the CD8+ memory T-cells specific for EBV lytic antigens predominantly have a more differentiated effector memory phenotype, consisting of mainly CD45RO+, CD27-, CD28-, CCR7- and CD62L- T-cells. CD8+ memory T-cells specific for EBV latent antigens predominantly have a central memory phenotype, consisting mainly of CD45RO+, CD28+, CCR7+ and CD62L+ T-cells[94]. Based on phenotypic characteristics CD8+ memory T-cells specific for CMV are mainly effector-type or late memory T-cells[95]. These experimental findings suggest that the memory phenotype reflects the frequency in which T-cells encounter their antigen and thus reflects the activation state in vivo of these T-cells .

MiHAs / HA-1 and HA-2

After HLA-identical allo-SCT donor derived T-cells recognizing MiHAs may induce both GvL effects, as well as GvHD[34]. Individuals are genetically disparate due to SNPs in the human genome that can result in small differences in amino acid sequence of several proteins. If these polymorphic peptides presented in the context of HLA on patient's cells elicit a strong immune response of donor derived T-cells these antigens are called MiHAs. Based on our current understanding of antigen-processing, different mechanisms can explain the great immunogenicity of MiHAs. First, if single or multiple amino acid substitutions are present within the peptide that is processed and bound into the groove of the HLA molecules and presented on patient-derived cells, these polymorphisms can be recognized as "foreign" by donor T-cells[96-100]. Second, polymorphisms in amino acids within the peptide that do not have direct contact with the TCR but are essential for binding of the peptide to the HLA class I molecules on the target cells may lead to differential expression of the peptide-HLA complex on the cell membrane between patient- and donor-derived cells[101-104]. If the peptide is not appropriately processed in donor cells and therefore cannot be presented in HLA molecules by cells from the donor but only by cells from the patient, donor T-cells will not have been educated to recognize this antigen as self, and a T-cell response against the epitope presented on patient's cells may occur. In addition, (partial) deletion of the gene coding for the protein involved has been described as a mechanism by which MiHAs can arise[105]. Polymorphic peptides of various lengths can also be presented in the context of HLA class II molecules. Although the mechanism by which peptides are processed and presented in HLA class II molecules is different from that of HLA class I and less clearly understood, several mechanisms for the generation of MiHAs are similar[106-109].

After allo-SCT the hematopoietic system will be of donor origin, whereas other tissues will still be of patient origin. Donor T-cells recognizing MiHAs ubiquitously expressed on several tissues may induce both GvL and GvHD. In contrast, donor

T-cells recognizing MiHAs exclusively expressed on cells of the hematopoeitic system will selectively induce GvL by eliminating all patient hematopoietic cells, including the malignant cells, but not donor hematopoietic cells. MiHAs that are expressed only on cells of the hematopoietic system, and are also expressed on leukemic precursor cells are optimal target antigens for a curative strategy without induction of GvHD. HA-2 and HA-1 were the first MiHAs described to be expressed solely on cells of the hematopoietic system and to be present on clonogenic leukemic precursor cells[103,110,111].

HA-2 is a HLA-A*0201-restricted epitope with a population frequency of 70%- 95%[112-114]. HA-2 is derived from a diallelic gene encoding a novel human class I myosin with selective high-level expression on hematopoietic cells[102,103,110]. Target cells recognized by HA-2-specific T-cells contained a single A-to-G transition at nucleotide 49 of the gene sequence that alters the sequence of the HA-2 epitope from YIGEVLVSM (HA-2M) to the immunogenic YIGEVLVSV (HA-2V). Although HA-2-specific T-cells are able to recognize both HA-2V and HA-2M variants when the synthetic peptides are exogenously pulsed onto HLA-A*0201+ target cells, experiments indicated that endogenously processed HA-2M peptide is not expressed on the cell surface[103]. It is not complete clear whether this failure of HA-2M to be presented is due to differently proteosomal cleavage or inefficient translation.

HA-1 is an epitope presented in the context of HLA-A*0201[97]. HA-1 is derived from a diallelic gene with a yet unknown function with selective hematopoietic expression, and, in addition, shows expression on epithelial cancer cells[110,111,115]. HA-1 has a population frequency of 35-69%[97,113,114]. A two nucleotide difference alters the sequence of the HA-1 epitope from VLRDDLLEA (HA-1R) into the immunogenic VLHDDLLEA (HA-1H)[97]. HA-1-specific T-cells required 10,000 times the concentration of exogenously pulsed HA-1R peptide compared to HA-1H peptide for HA-1-specific TNF-α production, and in concordance with this finding, HA-1H but not HA-1R transfected HeLa cells were recognized by HA-1-specific T-cells[97]. It was demonstrated that the HA-1R peptide is extremely rapidly dissociated from HLA-A*0201 when compared with the HA-1H peptide, and most likely, the HLA-A*0201/HA-1R complexes never reach the cell surface but already dissociate intracellularly[116].

Both MiHA HA-1 and HA-2 induce high-affinity T-cell responses and are shown to be induced frequently in vivo in HLA-A*0201+ patients that received allo-SCT[114,117-119]. Previously in MiHA HA-1 and/or HA-2 incompatible donor–recipient pairs a direct association between the emergence of MiHA HA-1 or HA-2 tetramer+ cytotoxic T-cells and the complete disappearance of malignant recipient cells was shown[120]. The observation that T-cell responses against HA-1 are capable of eliminating leukemic precursor cells capable of engrafting into immunodeficient NOD/SCID mice confirmed the ability of these T-cells to prevent outgrowth of leukemia[121].

In conclusion, differential expression of HA-1 and HA-2 in patients elicits high-affinity donor-derived T-cell responses recognizing these MiHAs as “foreign”. Since HA-1 and HA-2 are

exclusively expressed on cells of the hematopoietic system, these MiHAs are attractive target antigens for adoptive immunotherapy.

GENE THERAPY

TCR gene transfer

Adoptive transfer of T-cells is a strategy used to target both solid tumors and leukemia. Patients with relapsed hematological malignancies after allo-SCT can be successfully treated with donor lymphocyte infusion (DLI)[23,24], and patients with solid tumors can be effectively treated with tumor infiltrating lymphocytes (TILs) cultured from tumor tissue[122]. The beneficial GvL effect of DLI mediated by the recognition of MiHAs is, however, often accompanied by GvHD. Furthermore, isolation and expansion of TILs is feasible only for a fraction of patients with solid tumors. Since the antigen-specificity of a T-cell is purely defined by the TCRα and β chains, consequently, the transfer of TCR-chains into recipient T-cells can be used as a strategy to transfer T-cell immunity, as was demonstrated for the first time by the group of Steinmetz[123]. By introducing a well-characterized TCR, large numbers of T-cells with defined antigen-specificity can be obtained. Furthermore, TCR gene transfer allows the introduction of TCRs that have specificities that are not present in the endogenous T-cell repertoire of the recipient.

Different studies have shown the effectiveness of TCR transfer, both in vitro[123-128] and in vivo[129-131]. Recently, patients with advanced melanoma have been treated by adoptive transfer of lymphocytes genetically modified with a TCR specific for MART1(Melan-A)[130,132] and a TCR specific for gp100[132]. In the first clinical study, persistence of TCR gene-modified T-cells in individual patients was variable, and expression of the introduced MART-I-specific TCR was markedly lower than endogenous TCR expression [130]. Perhaps because of this, with a response rate of 4/31, clinical effectiveness of TCR gene transfer was clearly less than that of prior trials by the Rosenberg group that involved infusion of ex vivo expanded tumor-infiltrating lymphocytes (TILs) [122,133,134]. Also in the second clinical trial, although high affinity TCRs were used in this study, clinical response rate was still lower compared to the use of TILs. Although the clinical response rate was lower than anticipated, the results support the therapeutic potential of TCR gene-modified lymphocytes as an anti-tumor treatment.

Vector systems

Retroviral vectors based on Moloney Murine leukemia virus (MoMuLV) were the first viral vectors to be used in gene therapy trials and although various tools have been developed to deliver genes into human cells, genetically engineered retroviruses continue to be mostly used. Retroviruses are logical tools for gene delivery, since they introduce genes into the host cell genome, resulting in stable expression of the gene of interest. This integration in the genome will ensure that also upon proliferation of host cells, daughter cells will continu to express the gene of interest. For retroviral transduction, proliferation of the host cell is required. Therefore, lentivirus-based vectors were developed because they

could be used to deliver genes into nondividing cells. However, cytokine stimulation of quiescent human T-cells is still needed to transduce the T-cells using lentiviral vectors[135].

The most successful gene therapy trial using a Mo-MuLV-based retroviral vector was by Alain Fischer on children suffering from a fatal form of SCID, SCID-X1. This disease is an X-linked hereditary disorder characterized by an early block in the development of T- and NK-cells because of mutations in the γc cytokine receptor subunit. Hematopoietic stem cells (HSCs) from patients were stimulated and transduced ex vivo with a retroviral vector expressing the γc cytokine receptor subunit, and were then reinfused into the patients[136]. During a 10-month follow up, γc-expressing T- and NK-cells could be detected, and cell counts and function were comparable to age-matched controls. The selective proliferation advantage of the transduced lymphocyte progenitors due to expression of the γc cytokine receptor subunit contributed considerably to the success of this study. While 9 of 10 patients were successfully treated, 4 of the 9 children developed T-cell leukemia 31-68 months after gene therapy which were found to be due to insertional mutagenesis[137]. In 2 of these cases, blast cells contained activating vector insertions near the LIM domain-only 2 (LMO2) proto-oncogene. In two other patients, integrations near the proto-oncogene BMI1 and CCND2 were found. Chemotherapy led to sustained remission in 3 of the 4 cases of T-cell leukemia, but failed in the fourth. Successful chemotherapy was associated with restoration of polyclonal transduced T-cell populations. As a result, the treated patients continued to benefit from therapeutic gene transfer. Untill now, 20 SCID-X1 patients have been treated, with 5 children developing T-cell leukemia and the immunodeficiency corrected in 17 of the 20 patients[138].

Similarly, patients with adenosine deaminase (ADA)-deficiency SCID were treated with genetically corrected HSCs. ADA-SCID is a complex metabolic and immunological disorder, characterized by a severe immunodeficiency. Due to the absence of enzymatic activity of ADA, purine metabolites accumulate in plasma and cells, leading to lymphopenia, absent cellular and humoral immunity, failure to thrive, and recurrent infection. In 19 of the 27 patients treated with transduced HSCs the immunodeficiency was corrected[139]. In contrast to the SCID-X1 trial, none of the 27 patiens with ADA-deficiency treated with genetically modified HSCs showed any adverse effects up to 8 years after treatment[140].

Although the risk of insertional mutagenesis in retroviral integration has been subject to debate, in contrast to hematopoietic stem cells, retroviral vector integration into mature T-cells has been found to be safe. In the first clinical trial in the early nineties that attempted to treat patients suffering from ADA-deficiency with retrovirally transduced mature T-lymphocytes long-term reconstitution from transduced progenitor cells was observed at low levels, without in vivo clonal expansion or malignant transformation up to 4 years after treatment[141]. However, multiple infusions of corrected T-cells were required. Various studies have demonstrated that retroviral vector integration into mature T-cells has no consequence on the biology and function of transplanted T-cells, as demonstrated by

long-term engraftment of donor lymphocytes genetically engineered with the suicide gene thymidine kinase of herpes simplex virus (HSV-tk) after allo- SCT[142,143]. In addition, gene transfer to T-cells using retroviral constructs containing the marker gene truncated nerve growth factor receptor and subsequent infusion of more than 1011 transduced cells into 31 patients did not result in undesirable side effects[144]. Recently, the susceptibility of mature T-cells and hematopoietic stem cells to transformation after retroviral gene transfer with potent T-cell oncogenes was directly compared in an animal model[145]. All animals that received transplants of hematopoietic stem cells transduced with a T-cell oncogene developed leukemia/lymphoma. In contrast, none of the animals that received transplants of mature T-cells transduced with a T-cell oncogene developed a hematological malignancy.

These studies indicate that introduction of therapeutic genes using retroviral integration into mature T-cells is a safe strategy.

High affinity TCRs

TCRs introduced via gene transfer have to compete for cell surface expression with not only the endogenous TCR, but also with mixed TCR dimers that can be formed by pairing of the endogenous TCR chains with the introduced TCR chains (Figure 1). Therefore, gene transferred TCRs need to exhibit high affinity for their specific peptide-HLA complex. One strategy is to obtain TCRs that recognize foreign antigens in self-HLA. MiHA-TCRs like the HA-1- and HA-2-TCR derived from an immune response after allo-SCT of a HLA-A*0201 and HA-1/HA-2 positive patient with stem cells from a HLA-A*0201 positive but HA-1/HA-2 negative donor are examples of high-affinity TCRs recognizing foreign antigens in self-HLA. In contrast, TCRs recognizing tumor associated antigens (TAA) are mostly derived from T-cell responses against solid tumors and are directed against self-HLA molecules presenting peptides derived from self-proteins overexpressed in tumor tissue. Therefore, most of these TAA-specific TCRs are of low affinity. Several strategies have been explored to increase the affinity of TAA-specific TCRs, inducing variations in TCRα and β sequences and screening for TCRαβ complexes that exhibit improved binding affinity for the specific MHC-peptide combination[146-151].

Alternatively, chimeric antigen receptors (CARs) can be engineered that combine antigen-specificity with the high affinity of an antibody and T-cell activating properties in a single fusion molecule[152]. Generally, first generation CARs consisted of a single-chain antibody-derived antigen-binding motif that is coupled to signalling modules that are normally present in the TCR complex, such as the CD3ζ-chain. First generation CARs effectively redirected T-cell cytotoxicity, but failed to enable T-cell proliferation and survival upon repeated antigen exposure. Since then different second generation CARs have been engineered containing costimulatory signalling domains of CD28 or 4-1BB to reduce activation induced cell death (AICD) and improve persistence[153-158]. The value of second generation CARs has still to be validated in clinical trials.

Suicide genes

TCR gene transfer poses different safety issues, that might warrant the inclusion of a suicide gene. First, different strategies to improve affinity of TCRs might pose the risk of unwanted on-target toxicity. Recently, it has been described that administration of high affinity TAA-specific T-cells directed against the renal cell carcinoma antigen carboxy anhydrase IX (CAIX) resulted in severe cholestasis based on the overlooked CAIX expression by the bile duct epithelial cells[159]. Likewise, in the second clinical study of Rosenberg and colleagues that used a high-affinity MART-1-specific TCR on-target autoimmune destruction of melanocytes in ear, skin and hair that required treatment was observed in several patients[132]. Furthermore, in the case of unexpected off-target reactivity, inclusion of a suicide gene as a safety switch can abrogate unwanted toxicity directed against healthy tissue.

Several suicide genes or safety switches have been reported. HSV-tk is a well-established suicide gene that has been successfully used to control GvHD following DLI after allo-SCT[142,160,161]. Transfer of HSV-tk to DLI preserved the beneficial anti-tumor effect and allowed in vivo elimination of donor T-cells using ganciclovir if severe GvHD occured. In immunocompetent patients receiving HSV-tk gene modified DLI late after transplantation, however, gene modified lymphocytes rapidly disappeared due to induction of HSV-tk-specific immunity[162,163]. Another disadvantage of the HSV-tk suicide gene is that ganciclovir used to eliminate HSV-tk modified T-cells is first line therapeutic agent used in transplanted patients with CMV reactivations, a common complication after allo-SCT. Administration of ganciclovir to control CMV replication to patients after allo-SCT who received anti-leukemic TCR and HSV-tk modified T-cells will result in depletion of the TCR modified T-cells and terminate the beneficial anti-leukemic immune response.

Another well-studied suicide gene is the CD20 cell surface molecule[164,165]. CD20 is a transmembrane calcium channel that is believed to play a role in B-cell activation, proliferation and differentiation. It is first expressed on pre-B-cells and persists until later in differentiation, but is absent on terminally differentiated plasma cells. Since CD20 is already expressed on the cell surface of B-cells, it is unlikely that CD20 expressed on T-cells to function as a suicide gene will be immunogenic. We have demonstrated that human CD20 may be used as a safety switch in adoptive immunotherapy without affecting normal antigen-specific T-cell functions[166]. Rituximab is a therapeutic anti-CD20 antibody, which is widely used in the clinic, and upon ligation of CD20 triggers various effector mechanisms, including complement-dependent cytotoxicity (CDC). At present, only Rituximab and ganciclovir are available as clinical-grade therapeutic reagents.

TCR make up of host cells

In most TCR gene transfer studies unselected peripheral blood T-cells were used as host cells. Transfer of TCRs into an unselected pool of T-cells may lead to transduction into regulatory T-cells capable of impairing the anti-leukemic immune reaction. Furthermore, in a pool of T-cells with a diverse TCR repertoire, a

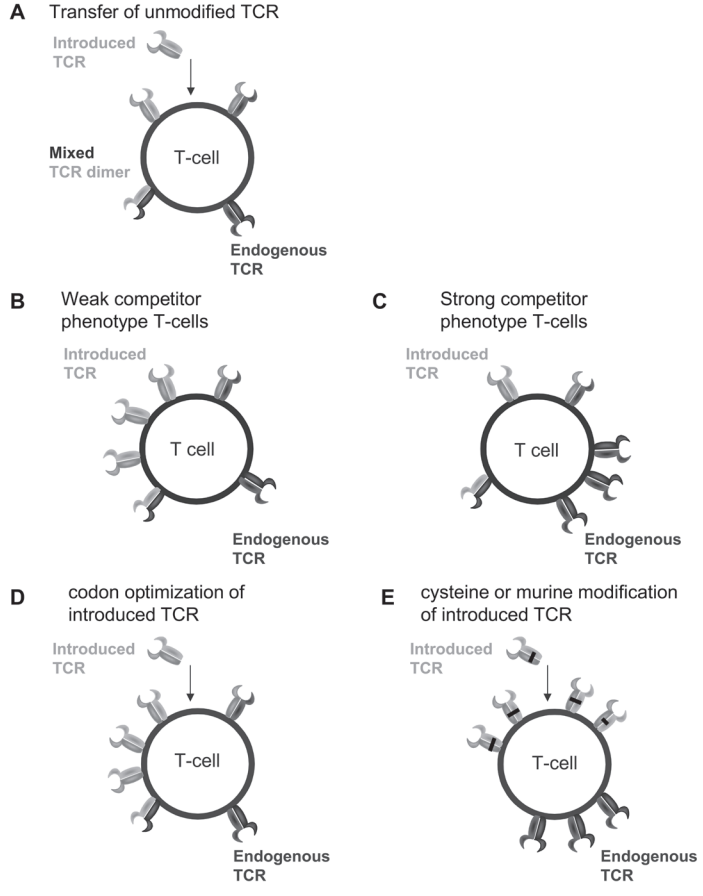

Figure 1. Simplified representation of TCR cell surface make up after TCR gene transfer in different T-cells using different strategies to improve cell surface expression of the introduced TCR.

*Figure 1: **(A)** Transfer of unmodified TCR will result in cell surface expression of the endogenous TCR, the introduced TCR and mixed TCR dimers composed of the introduced TCRα chain pairing with the endogenous TCRß chain and the endogenous TCRα chain pairing with the introduced TCRß chain. **(B)** T-cells with a weak competitor phenotype predominantly express the introduced TCR after TCR gene transfer, and to a lesser extent the endogenous TCR on their cell surface. **(C)** T-cells with a strong competitor phenotype predominantly express their endogenous TCR after TCR gene transfer, and to a lesser extent the introduced TCR on their cell surface. **(D)** Codon optimization is a strategy that improves cell surface expression of the introduced TCR by changing the nucleotide sequence to obtain optimal codon usage. This optimal codon usage results in identical amino acid sequence of the TCR chains, however, improves mRNA stability and translation efficacy of the introduced TCR chains, resulting in improved introduced TCR cell surface expression. **(E)** Inclusion of cysteine residues or murinization of the constant domains of the introduced TCR chains induces preferential pairing of the introduced TCR chains. Cell surface expression of the introduced TCR chains is improved since reduced numbers of mixed dimers are formed, resulting in less competition for cell surface expression. Additionally, forced preferential pairing might offer advantages for the introduced TCR of capturing more CD_3 complexes.*

high number of different mixed TCR dimers with unknown specificity can be formed due to pairing of the retrovirally introduced TCR chains with the endogenously expressed TCR chains, increasing the probability of the formation of autoreactive mixed TCR dimers. Theoretically, the introduction of a TCR into a T-cell will result in formation of two mixed TCR dimers, consisting of the endogenous TCRα chain pairing with the introduced TCRβ chain and vice versa (Figure 1A). Therefore, usage of unselected PBMCs with a broad TCR repertoire as host cells for TCR transfer will increase the risk of formation of mixed TCR dimers with a harmful off-target reactivity. An alternative strategy to prevent formation of mixed TCR dimers would be to transduce γδ-T-cells, since the γδ-TCR chains are not able to pair with αβ-TCR chains[167]. Human γδ-T-cells redirected with αβ-TCRs were fully functional in vitro and were capable of recognizing chronic myeloid leukemic cells. In addition, in murine studies we were able to show functional activity in vivo and persistence of the cells[168]. However, further analyses will be required to determine to what extent redirected γδ-T-cells and αβ-T-cells are different with respect to homing properties and specificity of the endogenous TCR. Another attractive strategy can be to transduce oligo- or monoclonal T-cell populations. Since most virus-specific T-cell populations consist of a restricted TCR repertoire[169-172], the number of different mixed

TCR dimers harboring harmful specificities will be limited. Another possible advantage of the use of virus-specific T-cells is the exclusion of regulatory T-cells from the pool of TCR modified lymphocytes that can possibly disturb the immune reaction. Furthermore, adoptive immunotherapy with EBV-specific T-cells in patients with post-transplant proliferative disease and CMV-specific T-cells as prophylaxis for CMV reactivation[61-63] in patients after SCT has proven to be a therapeutic strategy without toxicity or GvHD, and long-term persistence of these T-cells has been demonstrated[44]. Since EBV and CMV are examples of latent viruses, we hypothesize that due to frequent encounter with viral antigens and subsequent triggering of the endogenous TCR, TCR transferred virus-specific T-cells will survive for a prolonged period of time in vivo. Moreover, it was recently shown in a mouse model that tolerization of one TCR could be overcome by signaling via the other TCR. In this model the function of the tolerized self-tumor-reactive TCR of dual-T-cell receptor transgenic T-cells was rescued by proliferation induced via the virus-specific TCR, underlining the potency of TCR transfer into virus-specific T-cells[173]. In addition, expression of the transgene under regulation of a viral promotor is enhanced upon T-cell activation[174-177]. Using T-cells specific for latently present viruses may result in repetitive stimulation via the endogenous TCR and increased expression of the introduced TCR due to T-cell activation.

We have previously reported differences between TCRs in the capacity to compete for cell surface expression[178], and we described weak competitor phenotype TCRs exhibiting low cell surface expression (Figure 1B) and strong competitor phenotype TCRs (Figure 1C) exhibiting high cell surface expression after gene transfer. Probably interchain pairing of the introduced TCR and competition for CD3-complex formation may both play a role. Because the TCR is expressed only at the cell surface when noncovalently bound to the CD3 complex composed of CD3γ, CD3ε, CD3δ, and CD3ζ, correct assembly of all these subunits with TCRα- and β-chains is required to assure optimal membrane expression of the TCR-CD3 complex in T-cells[179-181]. Single subunits and partial receptor complexes redundant for the assembly process retain in the ER where these products are highly susceptible to proteolysis[182,183]. We speculate that weak and strong competitor phenotype can be explained by two mechanisms. Possibly, strong competitor phenotype TCRs have a higher interchain affinity, which results in rapid formation of TCRαβ complexes and hinders degradation of the single TCRα and β chains. Alternatively, strong competitor phenotype reflects the ability of particular TCR-chains to more efficiently capture CD3 and thus be preferentially transported to and expressed at the cell surface. Ideally, TCRs selected for the purpose of gene transfer should exhibit both high interchain affinity and a high TCR-CD3 intrinsic affinity to generate T-cells that preferentially express the transferred-TCR, resulting in a strong competitor phenotype. Alternatively, weak competitor phenotype T-cells could be selectively used as host cells. Recently, we have described that weak competitor phenotype of virus-specific T-cells is, to some extent, correlated with specificity[166]. However, selection of host cells with a weak competitor phenotype would minimize the pool of host cells useful for TCR gene transfer.

Furthermore, to ensure persistence of TCR modified T-cells, we would like to preserve the endogenous virus-specific TCR cell surface expression. Introduction of a strong competitor phenotype TCR into weak competitor phenotype virus-specific T-cells might result in loss of cell surface expression of the endogenous virus-specific TCR. Several strategies to improve expression of the introduced TCR have been described. mRNA and protein stability and translation efficacy of the introduced TCR chains can be enhanced by codon optimization[184] (Figure 1D). Furthermore, matched pairing of the introduced TCR chains can be facilitated by murinization[185-187] or introduction of cysteine residues in the constant regions of the introduced TCR chains, resulting in formation of an extra disulfide bond[188,189] (Figure 1E).

In conclusion, TCR gene transfer is a promising strategy to rapidly engineer therapeutically relevant amounts of anti-tumor specific T-cells. However, future application of TCR modified T-cells in clinical trials might benefit from increased knowledge how to improve cell surface expression of the introduced TCR and persistence of TCR modified T-cells.

Aim of the study

TCR gene transfer is a strategy that enables the rapid engineering of anti-leukemic T-cells with defined specificity, resulting in a so called 'off the shelf' therapy. An elegant strategy to promote persistence of TCR modified T-cells may be TCR gene transfer into CMV- and EBV-specific T-cells, which exhibit proper memory and effector phenotypes. Furthermore, these virus-specific T-cells do not induce GvHD after HLA identical allo-SCT, and can thus be safely administered. For efficient anti-leukemic reactivity of the introduced TCR coinciding with enhanced in vivo survival, a balance between cell surface expression of the introduced and endogenous TCR is required. The aim of this thesis was to optimize the efficacy of TCR gene transfer, study possibilities and restrictions of virus-specific T-cells as host cells for TCR gene transfer and characterize the occurrence of potentially harmful mixed TCR dimers and strategies to prevent their formation.

Since the introduced TCR chains have to compete for cell surface expression with the endogenous TCR, the introduced TCR chains are under control of a strong viral promotor, which, in contrast to the endogenous promotor, is constitutively active. In Chapter 2, we analyzed whether physiological TCR downregulation resulting in a protective refractory period was preserved in TCR modified T-cells. For this purpose, CMV- and EBV-specific T-cells were retrovirally transduced with the hematopoietic minor histocompatibility antigen HA-2-specific TCR (HA-2-TCR). TCR transduced T-cells were antigen-specifically triggered via either the introduced TCR or the endogenous virus-specific TCR. At various time points after stimulation TCR cell surface expression as well as TCR-responsiveness and activation induced cell death (AICD) was measured to analyze preservation of the protective refractory period.

TCR transfer into T-cells specific for persistent viruses may promote long-term persistence of TCR modified T-cells. When frequent encounter of viral antigens would lead to selective survival of TCR modified virus-specific T-cells predominantly expressing the endogenous TCR incapable of proliferating via

the introduced anti-leukemic MiHA-TCR, persistence in vivo of TCR modified virus-specific T-cells capable of controlling the hematological malignancy may fall short. In Chapter 3, we analyzed whether the dual-specificity of the TCR transferred T-cells after repetitive stimulation via either the introduced anti-leukemic TCR or the endogenous virus-specific TCR was preserved. Purified CMV-specific T-cells were transduced with the HA-2-TCR and either repetitively stimulated via the endogenous CMV-TCR to mimick a period of minimal residual disease (MRD) or via the introduced HA-2-TCR to mimic relapse, and preservation of dual-specificity was analyzed.

It has been described that introduction of TCR chains into T-cells results in mixed TCR dimer formation, consisting of the introduced TCR chains pairing with the endogenous TCR chains. Since the specificity of mixed TCR dimers is unpredictable, hazardous specificities may be formed. In Chapter 4, we investigated whether TCR transfer can lead to the generation of mixed TCR dimers exhibiting new detrimental reactivities. To address this issue we created T-cells expressing mixed TCR dimers. To be able to discriminate between the functionality of the endogenous TCR, the introduced TCR as well as mixed TCR dimers, we transduced different defined virus-specific T-cells with seven different well characterized antigen-specific TCRs and tested these for newly acquired reactivities against an HLA-typed EBV-LCL panel covering all prevalent HLA class I and II molecules, and against different normal cell subsets. Furthermore, we explored the introduction of cysteine residues in the constant domains of the introduced TCR resulting in formation of an extra disulfide bond as a strategy to avoid expression of neoreactive mixed TCR dimers.

The MiHA HA-1 is an attractive candidate antigen for clinical study, as it is exclusively expressed on hematopoietic cells. However, previously it has been demonstrated that HA-1-TCRs are poorly expressed after gene transfer. In Chapter 5 we therefore sought to improve HA-1-TCR expression after gene transfer. TCR-deficient jurkat-cells were used to study pairing capacities of the HA-1-TCR chains. The role of the CDR1 region of the always identical HA-1-TCR BV6S4 chain in low HA-1-TCRβ expression was analyzed by exchanging this region. Furthermore, two well described strategies, namely the inclusion of cysteine residues in the TCR constant domains and codon optimization were explored for improvement of HA-1-TCR cell surface expression after gene transfer in virus-specific T-cells known to possess endogenous TCRs which strongly compete for cell surface expression.

In Chapter 6 the results obtained in the studies are summarized, and the most optimal strategy for TCR gene transfer is discussed.