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

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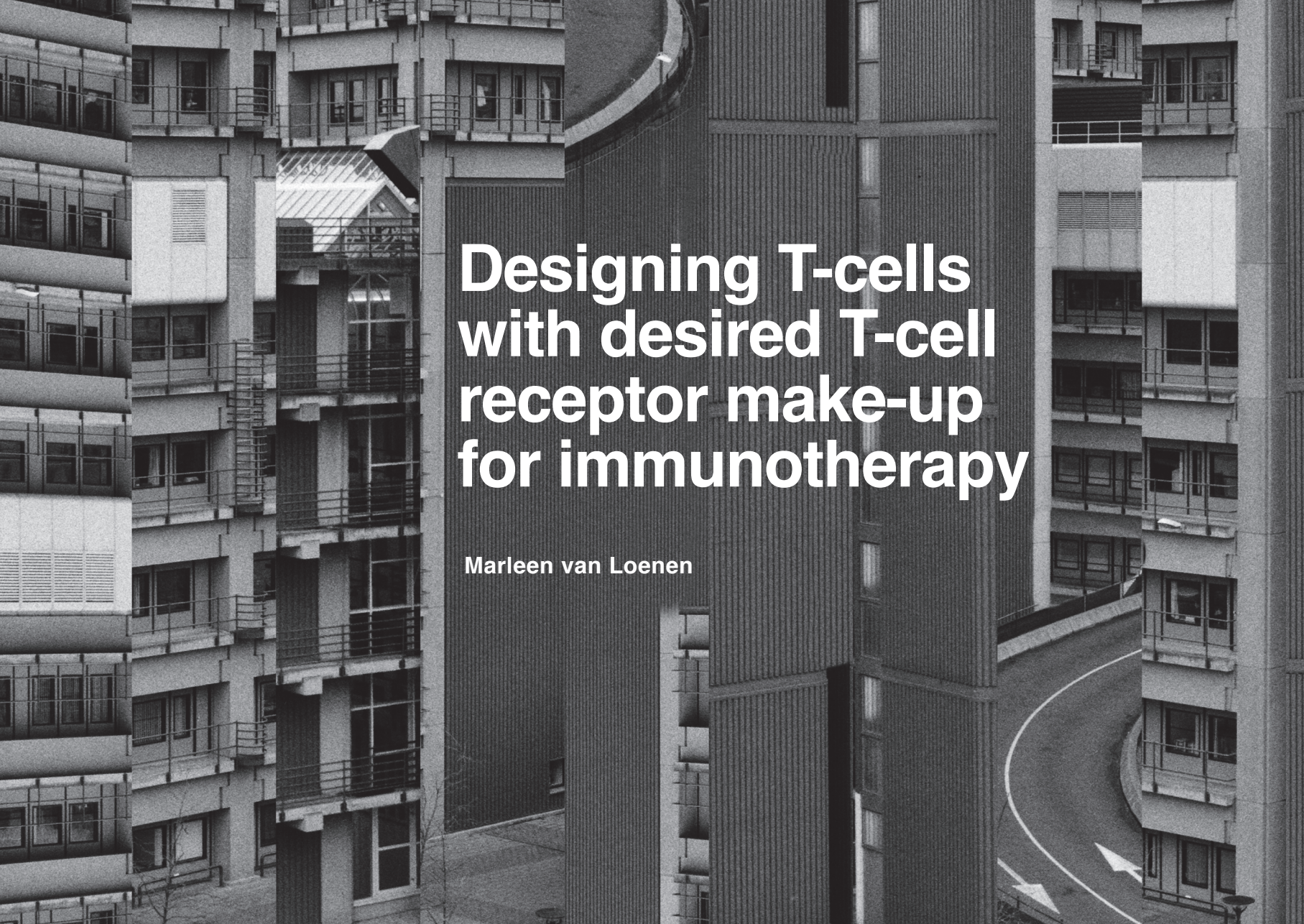

Designing T-cells with desired T-cell receptor make-up for immunotherapy

Marleen van Loenen

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

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Introduction

1

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

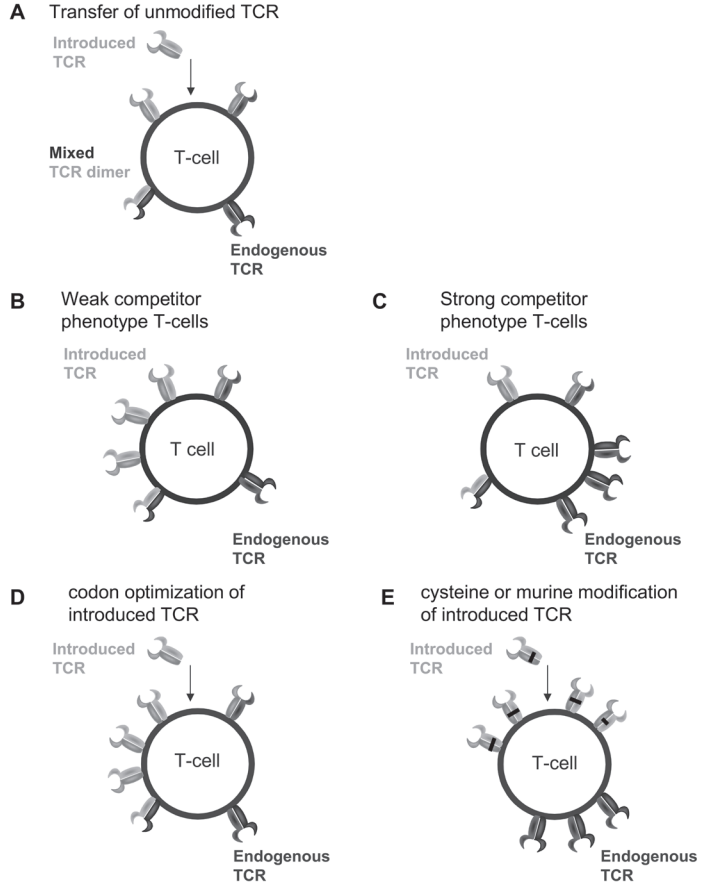

Figure 1. Simplified representation of TCR cell surface make up after TCR gene transfer in different T-cells using different strategies to improve cell surface expression of the introduced TCR.

*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.

Rapid re-expression of retrovirally introduced versus endogenous TCRs in engineered T-cells after antigen-specific stimulation

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ABSTRACT

To broaden the applicability of cellular immunotherapy, adoptive transfer of T-cell receptor (TCR) transferred T-cells may be an attractive strategy. Using this approach, high numbers of defined antigen-specific T-cells can be engineered. Since the introduced TCR has 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. We examined whether this difference in regulation would result in differences in TCR internalization and re-expression of the introduced and endogenous TCR on dual TCR engineered T-cells as well as the antigen-responsiveness of both TCRs. We demonstrated comparable TCR downregulation of TCRs expressed under regulation of a retroviral promotor or the endogenous promotor. However, the introduced TCRs were rapidly re-expressed on the cell surface after TCR stimulation. Despite rapid re-expression of the introduced TCR, T-cells exerted similar antigen-sensitivity compared to control T-cells, illustrating that cell mechanisms other than TCR cell surface expression are involved in antigen-sensitivity directly after antigen-specific stimulation. These results demonstrate that TCR transduced T-cells are functionally not different from non-transduced T-cells and can potentially be used as an effective treatment strategy.

INTRODUCTION

Adoptive transfer of TCR transduced (td) T-cells may be an attractive strategy to obtain high numbers of defined antigen-specific T-cells for cellular immunotherapy without complicated isolation strategies and labour intensive culturing procedures[1]. Different studies have shown the effectiveness of TCR transfer, both in vitro[2-7] and in vivo[8-11], and recently the feasibility of this approach was demonstrated in clinical trials[8,10].

In TCR td T-cells, the introduced TCR has to compete for cell surface expression with the endogenous TCR. For optimal efficacy of TCR modified T-cells in vivo, the cell surface expression of the introduced TCR has to be high, allowing the TCR td T-cells to recognize clinically relevant target cells expressing endogenously processed antigen. One of the strategies to acquire high TCR cell surface expression on TCR gene modified T-cells, is to use a strong retroviral promotor to regulate the introduced TCR. However, retroviral promotor regions are constitutively active, and in addition, it has been described that viral promotor activity will increase by T-cell activation[12-14]. In contrast, the endogenous promotor regions regulating the endogenous TCR expression have been demonstrated to be transiently inactivated after TCR triggering. TCRαβ mRNA expression decreases within 4-7h after TCR triggering, followed by normalization of mRNA levels 24h after activation[15,16]. In addition, T-cell activation induced by TCR triggering has been demonstrated to induce internalization of the TCR-CD3-complexes. It has been suggested that internalization of TCR-CD3-complexes and transient inactivation of the promotor regions regulating the endogenous TCR result in a refractory period of activation in which all effector-target interactions are terminated[17-20]. This latter effect is supported by the observation that TCR-CD3 downregulation results in a loss of cellular sensitivity to subsequent stimulation for 72 hours or longer[18,20], and vice versa, the inhibition of receptor downregulation leads to enhanced signaling[17,21,22]. Thus, the control of TCR expression by internalization of TCR-CD3 complexes and degradation of all its subunits[23-25] is speculated to result in a refractory period important to prevent harmful hyperstimulation resulting in activation induced cell death (AICD).

Since the regulation of the endogenous and introduced TCRs differ, TCR transfer may induce differences in the refractory period of TCR engineered T-cells, rendering these cells more sensitive for AICD. In this study we therefore examined TCR internalization and re-expression of the introduced and endogenous TCR on TCR td T-cells and antigen responsiveness via both TCRs. Our results demonstrate that TCR downregulation of the endogenous and introduced TCRs shortly after TCR triggering is identical. However, 24h after antigen-specific triggering the retrovirally introduced TCR-CD3 complexes are rapidly re-expressed at the cell surface, in contrast to the endogenous TCR which is still downregulated. Despite rapid re-expression of the introduced TCR-CD3 complexes, the T-cells remained physiologically non-responsive to antigen, illustrating that cell mechanisms other than TCR-CD3 cell surface expression are involved in providing a protective refractory period.

RESULTS

Rapid re-expression of the introduced TCR-CD3 complex on TCR td T-cells

To ensure high and stable expression of the introduced TCR, most TCR gene transfer studies to date use retroviral vectors for transgene delivery. Expression of the introduced TCRs in these studies will be regulated by the retroviral long terminal repeats (LTRs), whereas endogenous promotor regions will regulate the endogenous TCR expression. We assessed whether TCR-triggering of TCR td virus-specific T-cells resulted in increased protein levels under regulation of the retroviral LTR by analyzing eGFP expression as a marker using flow cytometric analyses. As shown in Figure 1, eGFP expression was increased at 24h after antigen-specific TCR triggering, and showed further increase up till 48h after TCR triggering, confirming previous observations that protein levels under regulation of a viral promotor increase upon TCR stimulation[12-14]. To determine whether antigen-specific stimulation would result in changed TCR modulation between the introduced TCR under regulation of a viral promotor and the endogenous TCR, we sorted TCR td virus-specific T-cells based on double positivity for eGFP and truncated nerve growth factor receptor (NGF-R). These TCR engineered T-cells with dual-specificity were stimulated antigen-specifically via their endogenous or introduced TCR, and analyzed for TCR cell surface expression. We analyzed three different TCR td T-cells; HA-2-TCR td or CMV-TCR td EBNA3A-specific T-cells, and HA-2-TCR td pp65-specific T-cells. T-cells were stained at different time points

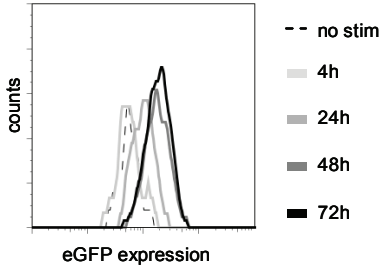

Figure 1. Protein levels under regulation of a viral promotor increase after stimulation.

after antigen-specific stimulation with TCRαβ-, CD3- or TCRβ-specific mAbs to determine the TCR-CD3 cell surface expression and to dissect between the endogenous and introduced TCRβ chains. Unfortunately no mAbs are available to stain for the endogenous or introduced TCRα chains. TCR downregulation of the different TCR-CD3 complexes in TCR td virus-specific T-cells was compared to TCR downregulation of mock td virus-specific T-cells. In Figure 2A a representative example of the kinetics of TCR cell surface expression after antigen-specific stimulation is depicted. The HA-2-TCR td pp65 T-cells demonstrate downregulation of the cell surface expressed TCRαβ complexes after 4h of stimulation similar to mock td pp65 T-cells. With mAbs specific for the endogenous and introduced TCRβ chain we observed after antigen-specific stimulation via the endogenous TCR (pp65 pep 1 μM) downregulation of both the endogenous as well as the introduced TCRβ chains. Likewise, we observed after antigen-specific stimulation via the introduced TCR (HA-2 pep 1 μM) downregulation of both the introduced as well as the

Figure 1: Sorted virus-specific T-cells transduced with vectors containing TCRα chains in combination with the marker gene eGFP and TCRβ chains in combination with the marker gene NGF-R were stimulated via their endogenous TCR using peptide pulsed target cells and eGFP expression was measured using FACS as an indication of viral promotor activity. eGFP expression of T-cells without stimulation (black dotted line), 4h after stimulation (light grey line), 24h after stimulation (grey line), 48h after stimulation (dark grey line) and 72h after stimulation (black line) is shown. Data is representative for several TCR td as well as mock td T-cells in six independent experiments.

Figure 2: ***(A)*** *As an example, the kinetics of total TCR re-expression (TCRαβ; black diamonds) or re-expression of the endogenous TCR-β (CMV-TCR BV2; grey triangles) or introduced TCR-β (HA-2-TCR BV18; white circles) of mock and HA-2 TCR td pp65 T-cells after stimulation with LCLs pulsed with either 1 μM of pp65 or HA-2 peptide is depicted. The cell surface expression of T-cells stimulated with unpulsed LCL (control) was set at 100%. Per timepoint the percentage cell surface expression was calculated as follows: [MFI of T-cells with peptide pulsed LCL / MFI of control T-cells] * 100. The average MFI of control stimulated mock td T-cells stained with anti-TCRαβ = 673; anti-TCR-BV2 = 736. The average MFI of control stimulated HA-2-TCR td T-cells stained with anti-TCRαβ = 744; anti-TCR-BV2 = 296; anti-TCR-BV18 = 29.*

(B)/(C)/(D) *Mock td pp65 and mock td EBNA3A T-cells (mock), HA-2-TCR td pp65 and HA-2-TCR td EBNA3A T-cells and CMV-TCR td EBNA3A T-cells (TCR td) were stimulated with LCL-Z (control stim; white bars) or with either LCL-Z pulsed with pp65 peptide or HLA-B7 td LCL-Z pulsed with EBNA3A peptide, respectively (stim endogenous TCR; grey bars) or with LCL-Z pulsed with either HA-2 or pp65 peptide (stim introduced TCR; black bars) and analyzed at the indicated time points for B) TCRαβ expression, C) CD3 expression, and D) endogenous and introduced TCRβ expression. The cell surface expression of T-cells stimulated with unpulsed LCL (control) was set at 100%. Per timepoint the percentage cell surface expression was calculated as follows: [MFI of T-cells with peptide pulsed LCL / MFI of control T-cells] * 100. Reported P values were considered statistically different if <0.01 and values statistically different are indicated with an asterisk. Data from six independent experiments were combined.*

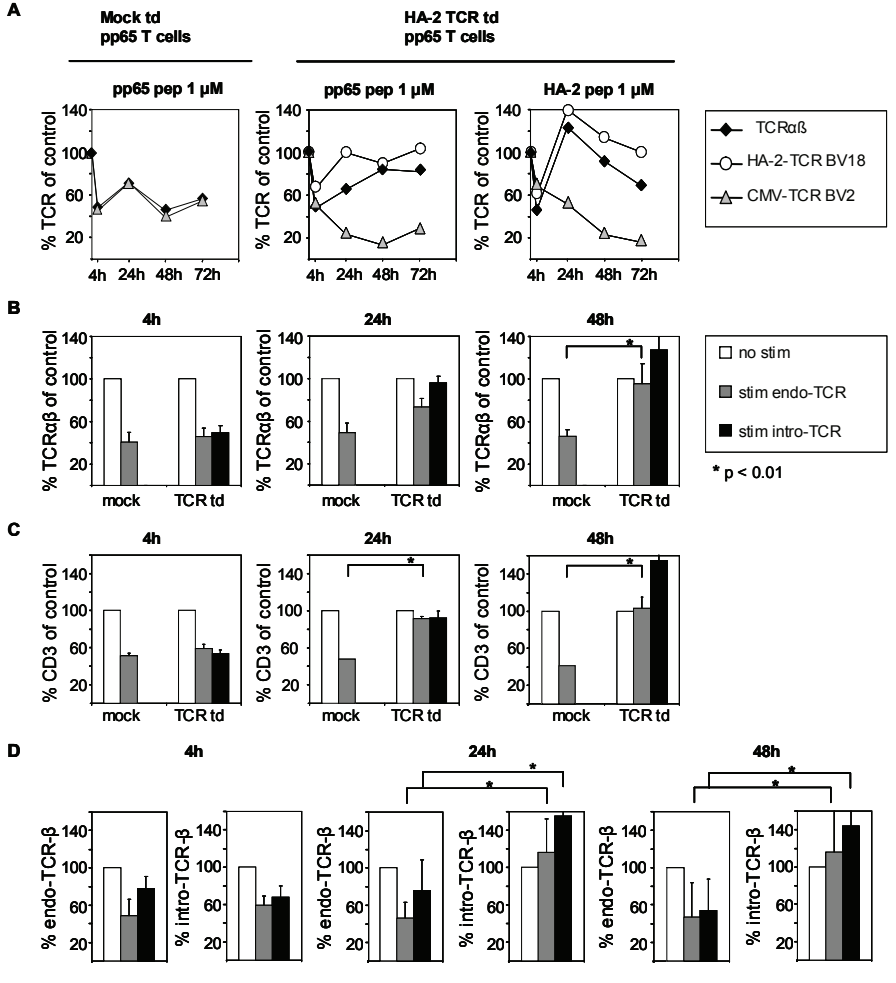

Figure 2. TCR td T-cells demonstrate fast TCR re-expression in comparison with mock td T-cells due to fast introduced TCR re-expression.

endogenous TCRβ chains. HA-2-TCR td pp65 T-cells, however, re-expressed TCRαβ complexes faster at their cell surface compared to mock td T-cells, both when stimulated via their endogenous or introduced TCR. Already 24h after stimulation TCRαβ complexes were re-expressed on TCR td pp65 T-cells, while TCRαβ expression of mock td pp65 T-cells was still decreased 72h after TCR stimulation. Also TCRαβ expression of the parental HA-2-specific T-cell clone was still decreased 72h after TCR stimulation (data not shown). Using mAbs specific for the endogenous and introduced TCRβ chain we demonstrated that re-expression of TCRαβ complexes 24h and 48h after TCR triggering correlated with recovery of the introduced TCRβ chain, whereas the endogenous TCRβ chain was still downregulated 72h after stimulation. Moreover, recovery of the endogenous TCRβ chain appeared to be even slower compared to mock transduced T-cells, indicating that the abundance of introduced TCR chains may compete for cell surface expression with the endogenous TCR chains. Since the introduced TCRα and TCRβ chain are both regulated via a similar retroviral LTR, it seems plausible that the recovered TCR complexes that stained with the mAb specific for the introduced TCRβ early after stimulation are primarily composed of the introduced TCRα and TCRβ chains. To demonstrate that the kinetics of TCR expression in dual TCR engineered T-cells is not influenced by the specificity of the virus-specific T-cells or the transferred TCR, we performed similar experiments with HA-2-TCR or CMV-TCR td EBNA3A T-cells. Figure 2B, 2C and 2D demonstrate that the TCR td pp65 and TCR td EBNA3A T-cells showed similar TCR expression kinetics using mAbs against TCRαβ, CD3 and against the endogenous and introduced TCRβ chains, respectively. Eventually, TCR make up as expressed by both mock and TCR td T-cells before stimulation was re-established 7 days after stimulation.

Based on these results, we conclude that although the retrovirally introduced and endogenous TCRs demonstrate a similar rapid downregulation after antigen-specific stimulation, the introduced TCR is re-expressed significantly faster at the T-cell surface compared to the endogenous TCR.

Fast re-expression of introduced TCR is not reflected in restored tetramer binding

Since the introduced TCR chains were re-expressed significantly faster at the cell surface compared to endogenous TCR chains, tetramers were used to stain the introduced as well as the endogenous TCRs at 4h and 48h after stimulation (Figure 3A and B). Consistent with the downregulation of TCR-CD3 complexes 4h after antigen-specific stimulation, we observed a marked reduction in the tetramer binding to both the introduced TCR as well as the endogenous TCR (Figure 3A, B), and this reduction in tetramer binding resembled tetramer staining of mock td virus-specific T-cells after TCR-triggering (Figure 3A, B). In contrast, whereas the cell surface expression of the introduced TCR was restored 48h after antigen-specific stimulation, no restored tetramer staining of the introduced TCR was observed. Since tetramer binding can be CD8 dependent, CD8 expression was analyzed at different time points after stimulation of TCR td T-cells (Figure 3A and C). Consistent with the downregulation

Figure 3: The cell surface expression of CD3, CD8 and the endogenous and exogenous TCR using tetramers was analyzed 4h and 48h after stimulation via either the endogenous or introduced TCR using specific peptide pulsed LCLs. ***(A)*** *As a representative example histograms of CD3, CD8 and tetramer stainings are depicted for mock td pp65 T-cells and HA-2-TCR td pp65 T-cells 4h and 48h after no stimulation (filled histograms), or stimulation via either the endogenous CMV-TCR (thick grey line) or introduced HA-2-TCR (dotted black line) by LCL-Z pulsed with pp65 or HA-2 peptide, respectively. In addition, the experiments were repeated with the same cells as well as mock td, HA-2-TCR td, and CMV-TCR td EBNA3A T-cells stimulated via their endogenous or introduced TCR using specific peptide pulsed LCLs. At the indicated time points mock and TCR td T-cells were stained for* ***(B)*** *the endogenous or introduced TCR using tetramers or* ***(C)*** *CD8. The cell surface expression of T-cells stimulated with unpulsed LCL (no stim) was set at 100%. Per timepoint the percentage cell surface expression was calculated as follows: [MFI of T-cells with peptide pulsed LCL / MFI of T-cells with unpulsed LCL] * 100. Data from 4 independent experiments were combined.*

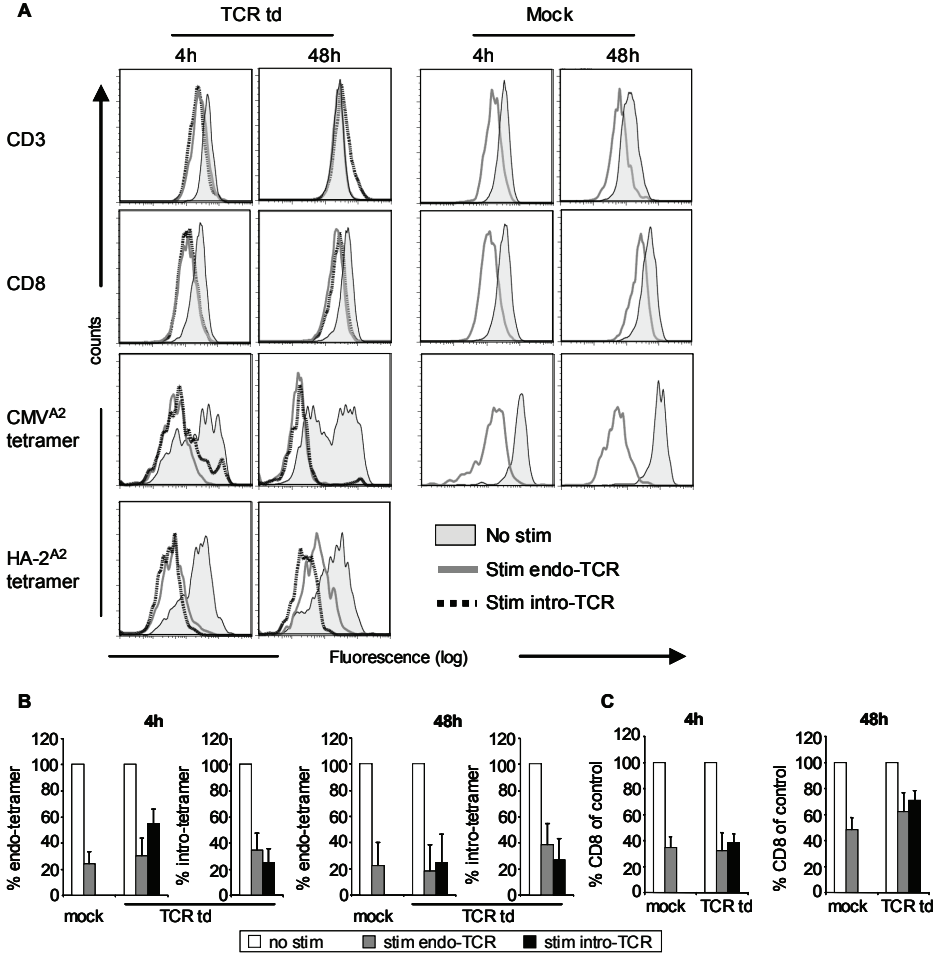

Figure 3. Restored introduced TCR expression is not coincided with restored tetramer staining, possibly due to low CD8 expression.

of TCR-CD3 complexes 4h after antigen-specific stimulation a marked downregulation of the CD8 complex on mock td and TCR td T-cells was observed. The expression of CD8 increased gradually, however, 48h after stimulation, CD8 expression was still diminished 30-40% compared to non-stimulated TCR td T-cells. This reduced CD8 expression was comparable with the CD8 expression of mock td T-cells 48h after TCR triggering(Figure 3A and C). These data indicate that although the introduced TCR is rapidly re-expressed at the cell surface this TCR-CD3 complex is not able to bind the specific tetramer, probably due to downregulated CD8 expression.

Rapid TCR re-expression does not result in restored T-cell functionality

Tetramer binding after T-cell activation is described to reflect the functional activity of the T-cells[26]. Therefore, we investigated whether high TCR expression but low tetramer binding reflected reduced or restored effector functions. For this purpose, HA-2-TCR td pp65 T-cells were stimulated via the endogenous or introduced TCR and tested at different time points for functionality via both TCRs in a cytotoxicity assay (Figure 4). Early after TCR stimulation (4h) with pp65 peptide, mock and HA-2-TCR td pp65 T-cells were not able to recognize EBV transformed lymphoblastoid cell lines (LCLs) expressing endogenously processed pp65 antigen (Zpp65), as well as LCLs expressing endogenously processed HA-2 (LCL-RZ). Likewise, 4h after TCR stimulation with HA-2 peptide TCR td T-cells were unable to recognize HA-2 positive LCL-RZ, as well as LCL-Zpp65. Although the HA-2-TCR

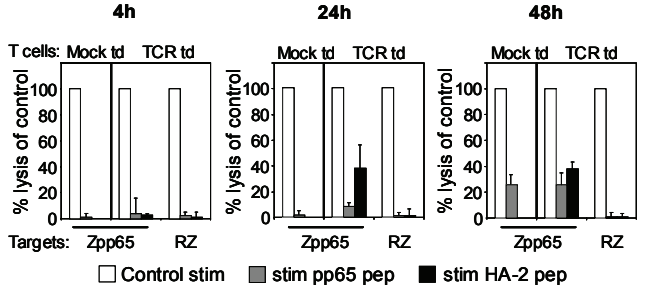

Figure 4. Despite rapid re-expression of the introduced TCR, T-cell functionality is not restored

cell surface expression at 24h after stimulation with either pp65 peptide or HA-2 peptide was restored to almost normal levels on the TCR td T-cells, no increase in cytotoxicity directed against LCL-RZ was observed, whereas some increased cytotoxic activity against LCL-Zpp65 was observed when T-cells were stimulated with HA-2 peptide. Analyses of the cytotoxic activity after 48h of stimulation demonstrated that in contrast to what could be expected from the cell surface expression, the cytotoxic activity against LCL-RZ was still not restored. Comparable cytotoxix activity of TCR td T-cells and mock td T-cells was observed against LCL-Zpp65. Eventually, when 7 days after stimulation the TCR make-up re-established to a comparable TCR make-up as expressed before stimulation, also functionality via both TCRs was restored (data not shown).

In conclusion, these results demonstrate that TCR cell surface expression is not accurately reflected in functionality and restored expression of the introduced TCR on the cell surface does not necessarily result in restored T-cell functionality.

*Figure 4: To determine the cytolytic activity after stimulation, mock (Mock td) and HA-2-TCR td pp65 T-cells (TCR td) were either control stimulated with LCL-Z (control stim; white bars), or stimulated via the endogenous TCR with LCL-Z pulsed with pp65 peptide (stim pp65 pep; grey bars) or via the introduced TCR with LCL-Z pulsed with HA-2 peptide (stim HA-2 pep; black bars) and tested for cytotoxic reactivity at 4h, 24h or 48h after stimulation as indicated above the panels. Targets used were pp65 positive LCL-Zpp65 and HA-2 positive LCL-RZ as indicated on the x-axis. Cytotoxic reactivity exerted by control and peptide stimulated mock and HA-2-TCR td T-cells after 4h of co-incubation with either chromium labeled LCL-Zpp65 or LCL-RZ as target cells is depicted. The cytolytic reactivity of mock td and TCR td T-cells stimulated with unpulsed LCL (control stim) was set at 100%. Per timepoint the percentage cell surface expression was calculated as follows: [% lysis of T-cells with peptide pulsed LCL / % lysis of control T-cells] * 100. The average % lysis of control stimulated mock td T-cells directed against LCL-Zpp65 = 61%. The average % lysis of control stimulated TCR td T-cells directed against LCL-Zpp65 = 55% and directed against LCL-RZ = 25%.Data presented are representative for 3 independent experiments.*

Figure 5: HA-2-TCR td pp65 T-cells were stimulated with unpulsed LCL-Z (no stim; filled histograms) or pulsed with pp65 and HA-2 peptide, and analyzed 4h (stim 4h; thick grey line) and 48h (stim 48h; thick black line) thereafter for surface expression of CD8 coreceptor, adhesion molecules CD2 and CD11a and for intracellular amount of granzyme B. As a control, cell surface expression of the CD58 molecule which is the ligand for CD2 and isotype controls (black hairline) are depicted. Data depicted are representative for 2 independent experiments.

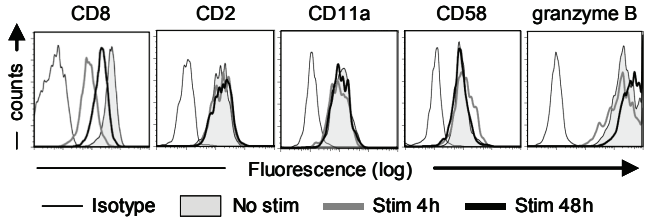

Figure 5. Reduced lytic activity of introduced TCRs is not associated with loss of granzyme or expression of adhesion molecules

No restored T-cell functionality despite sufficient expression of adhesion molecules and lytic granules

For restored functionality of effector T-cells, adhesion molecules at the cell surface like LFA-1 (CD11a) and LFA-2 (CD2) are needed for optimal target cell interaction as well as cytotoxic capacity[27-29]. To test whether low expression of adhesion molecules could have hampered restored lytic activity, HA-2-TCR td pp65 T-cells were stimulated and analyzed for expression of these molecules 4h and 48h after stimulation using mAbs directed against CD11a and CD2 and as a control for staining artefacts resulting from T-cell activation also the corresponding ligand for CD2, namely CD58 (ICAM-3), that is normally functioning on antigen presenting cells and isotype controls were measured. Expression of these molecules was compared with expression of CD8 (Figure 5A). In contrast to clear downregulation of CD8 after stimulation, no significant decrease in cell surface expression of adhesion molecules is observed 4h as well as 48h after stimulation (Figure 5A). To test whether reduced intracellular amounts of cytolytic granules was hampering restored lytic activity, the level of intracellular granzyme B was measured 4h and 48h after stimulation. Although 4h after stimulation the amount of cytolytic granules stored was slightly decreased, 48h after stimulation even higher amounts of granzyme B were present in the TCR td pp65 T-cells. These results demonstrate that restored T-cell functionality is not hampered due to low expression of adhesion molecules or low intracellular amount of lytic granules.

DISCUSSION

In this study, we demonstrated that early after antigen-specific stimulation of TCR td T-cells both the endogenous and introduced TCR complexes were downregulated irrespective of which TCR was triggered. The introduced and endogenous TCR were downregulated upon stimulation via the introduced TCR and likewise, the endogenous and introduced TCR were downregulated upon endogenous TCR stimulation. In agreement with this, we demonstrated that the functional activity both via the stimulated as well as the non-stimulated TCR was markedly reduced, indicating that stimulation via one TCR resulted in a period of non-responsiveness via both TCRs. Although TCR downregulation shortly after TCR triggering was similar in TCR td T-cells compared to mock td T-cells, we demonstrate that the introduced TCR under regulation of a retroviral promotor was rapidly re-expressed on the cell surface at 24h after TCR stimulation,

whereas the CD8 coreceptor as well as the endogenous TCR were still downregulated similar to mock td T-cells. Despite rapid re-expression of the introduced TCR, however, the T-cells were still physiologically non-responsive, similar to mock td T-cells. The T-cells exerted low cytolytic activity when stimulated via the endogenous or introduced TCR despite sufficient expression of adhesion molecules or intracellularly stored lytic granules. These results illustrate that cell mechanisms other than TCR cell surface expression are involved in providing a physiological period of non-responsiveness.

TCR cell surface expression is tightly controlled, and requires assembly of TCRαβ with all CD3 subunits[30-34]. Unassembled TCR subunits and incomplete complexes are either rapidly degraded or retained in the ER[35-37]. We therefore assume that the lack of restored functionality despite restored re-expression of the introduced TCR is unlikely to be due to cell surface expression of inappropriate assembled TCR-CD3 complexes. Our data on TCR downregulation of mock td T-cells, however, are in accordance with previous results showing that ligand-induced TCR degradation causes a prolonged reduction in the level of TCR expression and that over 72h were required for normalization of the TCR cell surface expression[20,38]. At the same time, the rapid re-expression of the introduced TCR chains after TCR-triggering of the TCR td T-cells indicates as proposed previously by others, that 24h after initial activation adequate levels of all CD3 subunits[23] are present and are not limiting TCR cell surface expression.

Although 24-48h after TCR stimulation the introduced TCR is re-expressed at the cell-surface, we could hardly detect the TCR complexes using tetrameric complexes and, in addition, the TCR td T-cells remained physiologically non-responsive comparable to mock td T-cells. Previously, it has been reported that after antigen-specific stimulation, tetramer staining is impaired despite almost completely restored TCR expression[26] and T-cells with redistributed TCRs but no or low tetramer staining were unable to completely exert their effector functions[26]. Our results demonstrate that the non-responsiveness of the TCR td T-cells could in part be due to reduced CD8 coreceptor expression, since 48h after specific stimulation the CD8 coreceptor expression was still downregulated. However, because a very small number of MHC-peptide complexes is sufficient to activate a T-cell[39-42], we hypothesize that restoration of CD8 expression levels 48h after stimulation to 60-70% of normal expression levels should be sufficient to result in restored functionality via the HA-2-TCR. It has been described previously that CTLs with low effector function and a low ability to bind tetramers despite having normal amounts of TCR and CD8 expressed on their cell surface lacked colocalization of TCR and CD8 molecules [43,44]. Besides inefficient colocalization of TCR and CD8, other cell mechanisms may have provided the TCR td T-cells with a protective refractory period as well.

An alternative explanation for the discordance in TCR expression and the absence of tetramer staining as well as functional activity could be the formation of mispaired TCR dimers. Theoretically, rapidly re-expressed TCR-complexes at the cell

surface of TCR transferred T-cells can consist of the introduced TCRβ chain pairing with either the introduced TCRα or endogenous TCRα chain. In this study we used mAbs against total TCRαβ-complexes and mAbs specific for the TCRβ chains of the endogenous or introduced TCRs. Unfortunatley, these mAbs do not allow analysis of mispaired TCR dimers. However, it is unlikely that restored TCRαβ-complexes consist of mixed TCR dimers. Restored total TCRαβ cell surface expression corresponded to the restored introduced TCRβ expression but not to the still decreased endogenous TCRβ expression. Both the endogenous TCRα and β chain are under control of an endogenous promotor and it seems plausible that the endogenous TCRα chain will still be downregulated 24h after stimulation, similar to the endogenous TCRβ chain. Furthermore, the introduced TCRα and TCRβ chain are both regulated via a retroviral LTR, which is activated upon TCR triggering. This is demonstrated in Figure 1 depicting increased eGFP expression after stimulation, which is in our studies linked to TCRα chain expression. Based on these arguments we assume that rapid re-expression of the introduced TCRβ chain is coincided with rapid re-expression of the introduced TCRα chain.

We confirmed comodulation of non-engaged TCRs as already observed by others[45-48], although other studies failed to demonstrate comodulation[49,50]. Furthermore, we demonstrated that the functional activity both via the stimulated as well as the non-stimulated TCR was markedly reduced, indicating that stimulation via one TCR resulted in a protective refractory period comprising of non-responsiveness via both TCRs.

Although the introduced and endogenous TCR are differently expressed directly after TCR stimulation, T-cells were completely functional again after 5-7 days, both via the endogenous and introduced TCR. At that time, the cell surface expression of both TCRs could be visualized with tetramers (data not shown).

In this study we confirmed that T-cell activation results in increased viral promotor activity and thus in increased introduced TCR cell surface expression[12-14]. It has been postulated that for sustained optimal cell surface expression of the introduced TCR, TCR td T-cells must be repetitively activated. This could be achieved using specific vaccination strategies encoding antigens recognized by the introduced TCR to reactivate the viral promotor regulating the introduced TCR[51]. However, if TCR td T-cells in a state of minimal residual disease encounter their antigen sporadically, cell surface expression of the introduced TCR will be low due the non-activated status of TCR td T-cells. Therefore, knowledge of the specificity of the endogenous TCR could provide a tool to induce proliferation and increased activity of TCR engineered T-cells[52]. To minimize the risk of loss of expression of the introduced TCR, usage of TCR td EBV- and CMV-specific T-cells can be an attractive strategy. These T-cells will frequently encounter viral antigens due to the latent presence of these viruses and this triggering of the endogenous TCR may result in increased introduced TCR expression in vivo. We have previously demonstrated that TCR td CMV-specific T-cells remained dual reactive via both the endogenous and introduced TCR, also after repetitive stimulation via the virus-specific TCR[53].

In conclusion, we have demonstrated that irrespective of whether the endogenous or introduced TCR is triggered, both the endogenous and the introduced TCRs are downregulated, resulting in a protective refractory period via both TCRs. Furthermore, we have shown that regulation of the introduced TCR by a viral promotor results in fast re-expression of TCR chains on the cell surface 24h after TCR triggering, but that this re-expression does not lead to immediate restored functionality, indicating a preserved protective refractory period. On bases of these results we conclude that TCR transduced T-cells can potentially be an effective treatment strategy due to maintenance of a normal physiological protective refractory period via both the endogenous and introduced TCR despite rapid re-expression of the introduced TCR.

MATERIALS AND METHODS

Construction of retroviral vectors and production of retroviral supernatant

The construction of retroviral vectors encoding for the TCR chains of the HA-2-specific T-cell clones HA-2.6 and HA-2.5, and the TCR-AV18 chain and TCR-BV13 chain of the CMV pp65-specific T-cell clone have been described previously[5]. Briefly, the HA-2.6-TCR-AV23, HA-2.5-TCR-AV15 and CMV-TCR-AV18 were cloned into bicistronic retroviral vectors encoding for the marker gene eGFP. The HA-2.6-TCR-BV18, HA-2.5-TCR-BV18, and CMV-TCR-BV13 were cloned in combination with the truncated nerve growth factor receptor (NGF-R)[54]. Retroviral vectors encoding eGFP or NGF-R only were used as control vectors. The Moloney murine leukemia virus-based retroviral vector LZRS and packaging cells φ-NX-A were used to obtain viral supernatant[55].

HLA Class I tetrameric complexes and flow cytometric analyses

PE- or APC-conjugated tetrameric complexes were constructed as previously described[56] with minor modifications. The following tetramers were used; tetrameric HLA-A2 molecules in complex with CMV-pp65 derived peptide NLVPMVATV (CMV tetramer) or HA-2 peptide YIGEVLVSV (HA-2 tetramer), and tetrameric HLA-B7 molecules in complex with EBV EBNA3A derived RPPIFIRRL (EBV tetramer). For flow cytometric analyses cells were labeled with tetramers for 1 h at 4°C in RPMI without phenol, supplemented with 2% FBS, and washed two times or labeled with monoclonal antibodies (mAb) directed against various cell surface molecules for 30 minutes at 4°C. The mAbs used were anti-BV13, anti-BV7, anti-BV18 and anti-BV2, all PE-conjugated (Beckman Coulter, Mijdrecht, The Netherlands), anti-TCRαβ PE-Cy5-conjugated and CD3 APC- or PE-conjugated (BD Pharmingen, San Diego, CA, USA) and CD8 APC- or PE-conjugated (Invitrogen, Paisley, UK). For detection of ΔNGF-R, anti-human NGF-R mAbs were used either PE- (BD Pharmingen, San Diego, CA, USA) or APC-conjugated (Cedarlane Laboratories, Hornby, Ontario, Canada). For the different adhesion and costimulatory molecules PE-conjugated anti-CD54 (CLB, Amsterdam, The Netherlands), anti-CD58 (Southern Biotechnology Associates, Birmingham, AL, USA), anti-CD2 ,

anti-CD11a, and anti-CD50 (BD Pharmingen, San Diego, CA, USA) were used. For intracellular stainings, T-cells were stained with mAbs for 20 minutes at 4°C and were subsequently fixated for 10 minutes using paraformaldehyde. T-cells were permeabilized by washing 2 times using PBS containing saponine, stained for another 20 minutes at 4°C, washed and analyzed using FACS.

Isolation, transduction and culture of T-cells and LCLs

Purified (>95% pure) HLA-A2-restricted CMV pp65 NLV-specific T-cells (pp65 T-cells) and HLA-B7-restricted EBV EBNA3A RPP-specific T-cells (EBNA3A T-cells) were isolated from peripheral blood of EBV and CMV seropositive healthy individuals using tetramers. After informed consent, peripheral blood mononuclear cells (PBMCs) were harvested and labeled with tetramers for 1 h at 4 °C in RPMI without phenol supplemented with 2% FBS, washed, and sorted at 4 °C using the FACS Vantage™ (Becton Dickinson, San Jose, CA, USA). Tetramer-positive T-cells were stimulated with PHA (Murex Biotec Limited, Dartford, UK) and irradiated feeder cells (30 Gy), and after 2 d of culture, the T-cells were transduced with retroviral supernatant. The transduction procedure used for the peripheral blood T-cells has been described previously[57]. In brief, $1x10^6$ T-cells were cultured on CH-296-coated 24-well non-tissue culture-treated plates (Falcon) together with 1 ml thawed retroviral supernatant overnight at 37°C, washed, and transferred to 24-well tissue culture plates. TCR transduced (td) virus-specific T-cells were sorted on bases of marker gene expression and cultured in IMDM supplemented with 5% FBS, 5% human serum and IL-2 (100 IU/ml) (Chiron, Amsterdam, The Netherlands). T-cells were nonspecifically stimulated every 2 wk with feeder cell mixtures containing $1x10^6$/ml irradiated allogeneic PBMCs (30Gy), $1x10^5$/ml irradiated EBV-transformed B cells (LCLs; 50 Gy), and PHA (800 ng/ml).

For TCR internalization and re-expression assays different LCLs were used. LCL-Z and LCL-RZ originate from an HLA-identical sibling pair with HA-2 disparity. LCL-Z is HLA-A2 positive but HA-2 negative, while LCL-RZ is HLA-A2 and HA-2 positive. Both LCLs were transduced with HLA-B7, and pulsed with the EBNA3A peptide in particular experiments. To obtain LCLs presenting endogenously processed pp65, LCL-Z was transduced with the lower matrix protein pp65 of HCMV AD169 (Zpp65). LCLs were maintained in IMDM supplemented with 10% FBS. This study was approved by the Leiden University Medical Center institutional review board.

TCR internalization assay

$2x10^4$ TCR td and mock td T-cells were plated in 96-well U-bottomed microtiter plates. LCL-Z was used as stimulator cell, either unpulsed, or pulsed with 1 µM of pp65 or HA-2 peptide for 1 h at 37°C. In some assays, HLA-B7 td LCL-Z was pulsed with 1 µM of EBNA3A peptide. At different time points, $2x10^4$ LCLs were added to the T-cells in a final volume of 200 µl in medium containing 30 IU/ml of IL-2. After a stimulation period of maximal 72 h at 37°C and 5% CO_2, 96-well U-bottomed microtiter plates were spinned down, and T-cells were either tested functionally in a chromium release assay or cells were stained with different mAbs and analyzed for cell surface expression by flow cytometry. Cell

surface expression was calculated as follows: [MFI of T-cells with stimulator / MFI of T-cells without stimulator] * 100.

Cytotoxicity assay

$1x10^4$ TCR td and mock td T-cells were plated in 96-well U-bottomed microtiter plates and stimulated using LCL-Z either unpulsed or pulsed with 1 µM of pp65 or HA-2 peptide. Target cells were labeled with 70 µCi $Na2^{51}CrO4$ for 1 h at 37°C, washed three times, and added at different time points after antigen-specific stimulation to the effector cells in a final volume of 150 µl IMDM supplemented with 10% FBS in 96-well U-bottomed microtiter plates, resulting in a 10:1 effector-to-target-ratio. Target cells were pulsed with EBNA3A, pp65 or HA-2 peptide (1 µM) during $Na2^{51}CrO4$ labeling. Targets incubated in medium or 1% Triton X-100 were used for determination of the spontaneous and maximum release, respectively. The tests were done in triplicate. After 4 h of incubation at 37°C and 5% $CO2$, 25 µl of the supernatant was harvested and measured in a luminescence counter (Topcount-NXT, Packard Instrument Company, Meriden, CT, USA). The percentage of specific lysis was defined as [(experimental release – spontaneous release) / (maximum release – spontaneous release)] * 100.

Statistics

Cell surface expression of the TCR chains and complexes, CD3 and CD8 of stimulated TCR and mock transduced virus-specific T-cells were evaluated in a paired fashion by use of the students' T-test at each time interval (4h, 24h and 48h). Reported P values are 2-sided and were considered statistically different if <0.01.

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Kinetic preservation of dual-specificity of coprogrammed minor histocompatibility antigen-reactive virus-specific T-cells

3

Cancer Res. 2009 Mar 1;69(5):2034-41. Epub 2009 Feb 17. Reprinted with permission.
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ABSTRACT

Adoptive transfer of antigen specific T-cells is an attractive strategy for the treatment of hematological malignancies. It has been demonstrated that T-cells recognizing minor histocompatibility antigens (mHags) selectively expressed on hematopoietic cells mediate anti-leukemic reactivity after allogeneic stem cell transplantation (allo-SCT). However, large numbers of T-cells with defined specificity are difficult to attain. An attractive strategy to obtain large numbers of leukemia-reactive T-cells is retroviral transfer of mHag-specific T-cell receptors (TCRs). TCR transfer into T-cells specific for persistent viruses may enable these T-cells to proliferate both after encountering viral antigens as well as mHags, increasing the possibility of in vivo survival. We analyzed whether the dual-specificity of the TCR transferred T-cells after repetitive stimulation via either the introduced anti-leukemic HA-2-TCR or the endogenous CMV-TCR was preserved. We demonstrate that after repetitive stimulation, T-cells skew to a population predominantly expressing the triggered TCR. However, HA-2-TCR transferred CMV-specific T-cells with high anti-leukemic HA-2-TCR expression but low CMV-TCR expression were able to persist and proliferate after repetitive stimulation with pp65. Moreover, HA-2-TCR transferred CMV-specific T-cells remained dual-specific after repetitive stimulation and TCR expression could be reverted after additional stimulation via the previously non stimulated TCR, restoring high avidity interactions. These data imply persistence of TCR transferred virus-specific T-cells with both anti-leukemic and anti-virus reactivity in vivo.

INTRODUCTION

Patients with relapsed hematological malignancies after HLA-matched allo-SCT can be succesfully treated with donor lymphocyte infusion (DLI)[1,2]. However, the beneficial graft versus leukemia (GVL) effect of donor lymphocytes is frequently accompanied by graft versus host disease (GVHD). GVL as well as GVHD appear to be caused by T-cells that are capable of recognizing mHags on patient cells[3-5]. mHags are immunogenic peptides derived from polymorphic proteins presented in the context of HLA molecules which are disparate between donor and recipient. T-cell responses against ubiquitously expressed mHags may be responsible for both GVL and GVHD. T-cells reactive with mHags selectively expressed on cells of the hematopoietic lineage may solely mediate GVL reactivity. HA-2 and HA-1 are examples of mHags selectively expressed in cells of the hematopoietic system and are presented in an HLA-class I-restricted fashion[6,7].

To separate the beneficial GVL from GVHD, adoptive transfer of T-cells recognizing mHags selectively expressed on cells of the hematopoietic system is a promising strategy. However, therapeutic cell numbers of mHag-specific T-cells are difficult to attain. An attractive alternative would be to equip T-cells with mHag-specific TCRs via retroviral gene transfer. Different studies have shown the effectiveness of TCR transferred T-cells in vitro[8-12] and in vivo[13-15]. Redirected T-cells were able to produce cytokines and exhibited Ag-specific cytolytic activity when triggered via the introduced TCR. Moreover, in mouse models it was shown that redirected T-cells could be activated via their introduced TCR, home to effector sites, and eradicate tumors. Recently, Rosenberg and colleagues demonstrated in a clinical trial the feasibility of adoptive transfer of TCR engineered T-cells in melanoma patients[14].

In most TCR gene transfer studies unselected peripheral blood T-cells were used as recipient T-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 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. Therefore, we previously proposed TCR transfer into virus-specific T-cells[16], since selection of these Ag-specific CD8+ T-cells leads to exclusion of regulatory T-cells. In addition, virus-specific memory T-cells generally consist of an oligoclonal population with restricted TCRαβ usage[17-21], minimizing the number of different mixed TCR dimers that can be formed. 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[22-24] in patients after SCT has proven to be a therapeutic strategy without toxicity or GVHD. 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[25].

We previously demonstrated that CMV-specific T-cells could be redirected into anti-leukemic T-cells by transfer of TCRs directed against the mHag HA-2 without the loss of their original specificity. T-cells were capable of exerting effector functions via their endogenous virus-specific TCR as well as via their introduced HA-2-specific TCR[16]. The TCR cell surface make up of HA-2-TCR transferred CMV-specific T-cells, however, varied. T-cells either highly expressed the endogenous TCR with a low expression of the introduced TCR, or highly expressed the introduced TCR with a low expression of the endogenous TCR, or expressed both TCRs intermediately at the cell surface. Different studies have shown that there is a threshold in expression of TCR complexes and costimulatory molecules needed for TCR signaling leading to proliferation[26,27]. Although both the HA-2-TCR and CMV-TCR used in this study are high-affinity TCRs, it is likely that differential TCR expression leads to differences in avidity and thus in proliferation. For long-term protection, we hypothesize that proliferative capacity via both TCRs will be important. When patients relapse, mHags will be abundantly present. However, when there is only minimal residual disease (MRD) it may be expected that HA-2-TCR transferred virus-specific T-cells will primarily encounter viral antigens latently present in the recipient, as the HA-2 antigen is only expressed by recipient hematopoietic cells. When frequent encounter of viral antigens would lead to selective survival of HA-2-TCR transferred CMV T-cells predominantly expressing the CMV-TCR incapable of proliferating via the HA-2-TCR, persistence in vivo of HA-2-TCR transferred CMV T-cells capable of controlling MRD may fall short.

In this study we analyzed the TCR expression, cytolytic potential and proliferation of HA-2-TCR transferred CMV-specific T-cells after repetitive stimulation with the CMV-pp65 antigen or the HA-2 antigen. We demonstrate that TCR-transferred virus-specific T-cells repetitively stimulated skewed to T-cells predominantly expressing one TCR. However, HA-2-TCR transferred CMV-specific T-cells with high anti-leukemic HA-2-TCR expression but low CMV-TCR expression were able to persist after repetitive stimulation with pp65. Moreover, HA-2-TCR transferred CMV-specific T-cells preserved their functional activity via both TCRs after repetitive stimulation, and TCR expression could be reverted after additional stimulation, restoring high avidity functionality of both the endogenous CMV-TCR and the introduced anti-leukemic HA-2-TCR.

MATERIALS AND METHODS

Construction of retroviral vectors and production of retroviral supernatant

The construction of retroviral vectors encoding for pp65 of HCMV AD169 and the TCR chains of the HA-2 reactive T-cell clone HA2.5 has been described previously[16]. Briefly, the

HA-2-TCR AV15 and HA-2-TCR BV18 chains were cloned into bicistronic retroviral vectors encoding the marker genes eGFP and ΔNGF-R[28], respectively. As control vectors, retroviral vectors were used containing eGFP or ΔNGF-R only.

HLA Class I tetrameric complexes and sorting by flow cytometry

Tetrameric HLA-A2 molecules in complex with CMV pp65 derived peptide NLVPMVATV (CMV tetramer) and the HA-2 derived peptide YIGEVLVSV (HA-2 tetramer) either PE- or APC-conjugated were constructed as previously described[29] with minor modifications. For flow cytometric analyses as well as FACS sorting, cells were labeled with tetramers for 1 hour at 4°C in RPMI without phenol, supplemented with 2% FBS, and washed two times or labeled with either anti-BV2 PE (Immunotech, Marseille, France), or anti ΔNGF-R either PE- (PharMingen, San Diego, California, USA) or APC-conjugated (Cedarlane Laboratories, Hornby, Ontario, Canada) for 30 minutes at 4°C.

Cells

For all stimulations and functional experiments EBV-transformed lymphoblastoid cell lines (EBV-LCLs) were used of an HLA-identical sibling pair with HA-2 disparity. EBV-LCL Z is HLA-A2 positive but HA-2 negative (EBV-Z), while EBV-LCL RZ is HLA-A2 and HA-2 positive (EBV-RZ HA-2). To obtain EBV-LCLs presenting endogenously processed pp65, EBV-Z was transduced with the lower matrix protein pp65 of CMV (EBV-Z pp65). EBV LCLs were maintained in IMDM supplemented with 10% FBS.

Malignant cells used in this study were chronic myeloid leukemia mononuclear cells (CML) CML-Z which is HLA A2 positive but HA-2 negative and CML-T which is HLA A2 and HA-2 positive. CML cells were thawed 1 day prior to testing and cultured in IMDM supplemented with 10% FBS.

Virus specific T-cells were isolated from peripheral blood of healthy individuals using CMV tetramers, as previously described[16], and expanded in T-cell medium containing IMDM supplemented with 5% FBS, 5% human serum and 100 IU/ml IL-2. T-cells were non specifically stimulated using 800 ng/ml PHA (Murex Biotec Limited, Dartford, UK) and irradiated autologous feeder cells. The CMV-specific T-cells were subsequently sorted using anti-BV2 PE, and non specifically restimulated, followed by retroviral transduction at day 2. For the transduction procedure recombinant human fibronectin fragments CH-296[30,31] were used. HA-2-TCR transduced BV2 positive T-cells were FACS sorted based on eGFP and NGF-R positivity, and the cells were expanded in bulk. T-cells were cultured in T-cell medium and either stimulated non specifically every 2 weeks with feeder cell mixtures containing $1x10^6$/ml irradiated allogeneic PBMCs (20Gy) and $1x10^5$/ml irradiated EBV-LCLs (50 Gy), or were repetitively stimulated with $1x10^6$/ml irradiated HLA-A2 negative allogeneic PBMCs and $1x10^5$/ml irradiated EBV-RZ HA-2 or EBV-Z pp65. Subsets of HA-2-TCR transduced virus-specific T-cells with various levels of TCR cell surface expression were sorted based on either high CMV-TCR and low HA-2-TCR expression using a combination of anti-BV2 mAb and HA-2 tetramer, or based on low CMV-TCR expression using only anti-BV2 mAb. No tetramers were used for

positive selection, since tetramer binding to the TCR can lead to specific stimulation[32]. Subsequently, sorted T-cells were tested functionally either directly after sorting, or after 7 days of stimulation with pp65 or HA-2. This study was approved by the Leiden University Medical Center institutional review board.

Cytotoxicity assay and PKH-26 based proliferation assay

Cytotoxicity assay was performed as previously described[16] using a standard 4 hours ^{51}Cr release assay at 10:1 effector-to-target ratios. The tests were done in duplicate. To test the capacity of T-cells to specifically proliferate in response to antigen, T-cells were labeled with PKH-26 (St. Louis, Missouri, USA) according to manufacturer's instructions, and stimulated with different feeder cell mixtures containing $1x10^6$/ml irradiated allogeneic HLA-A2 negative PBMCs in combination with $1x10^5$/ml target cells. The following targets were used: EBV-Z either unpulsed or pulsed with 1 µg/ml CMV-NLV or HA-2 YIG peptide, EBV-Z pp65 and EBV-RZ HA-2. Alternatively, T-cells were stimulated non-specifically by adding PHA. PKH dilution was analyzed at day 4 after stimulation using flow cytometry.

RESULTS

Skewing of TCR cell surface make-up upon specific TCR triggering

We hypothesize that for long-term protection the capacity of TCR transferred virus-specific T-cells to proliferate and exert effector functions in response to triggering via each TCR is important. Therefore, we studied whether repetitive Ag-specific stimulation of these dual-specific T-cells resulted in skewing of T-cells to a population predominantly expressing one TCR, incapable of exerting effector functions via the other TCR. For this purpose, T-cells recognizing the pp65 protein of CMV in the context of HLA-A2 (pp65-NLV) were isolated from PBMCs of healthy CMV seropositive individuals, transduced with the mHag-specific HA-2-TCR, and sorted on basis of marker gene expression. These TCR transferred virus-specific T-cells showed differences in TCR cell surface make up, which was stable for months after repetitive non-specific TCR triggering. The T-cells expressed either both TCRs at intermediate levels at the cell surface, or the endogenous TCR was highly expressed with a low expression of the introduced TCR, or the introduced TCR was highly expressed with a low expression of the endogenous TCR (Figure 1A). These HA-2-TCR transferred CMV T-cells exerted cytolytic activity directed against HA-2 expressing EBV-LCLs, as well as HA-2 expressing mononuclear CML cells (Figure 1B). To test whether all different T-cell subpopulations were able to persist after repetitive stimulation with either pp65 or HA-2, the dual-specific T-cells were stimulated with EBV-LCLs expressing either endogenously pp65 or HA-2. Differences in TCR expression were measured at day 7 after stimulation using CMV and HA-2 tetramers (Figure 1C). A gradual decrease in HA-2-TCR expression was observed after repetitive stimulation with pp65. Likewise, a gradual decrease in CMV-TCR expression was observed after repetitive stimulation with HA-2, while HA-2-TCR expression increased. To study whether changes in TCR expression could be

*Figure 1.: **(A)** CMV and HA-2 tetramer staining was analyzed for mock and HA-2-TCR transferred purified CMV T-cells previously sorted on eGFP en NGF-R marker gene positivity. Numbers indicate % T-cells per quadrant. **(B)** Mock CMV T-cells and HA-2-TCR transferred CMV T-cells were tested for anti-pp65 and anti-leukemic reactivity in an ^{51}Cr release assay. Target cells were EBV-Z, EBV-Z pp65, EBV-RZ HA-2, HLA-A2+ HA-2- CML-Z (CML-Z) and HLA-A2+ HA-2+ CML-T (CML-T). **(C)** CMV and HA-2 tetramer staining was analyzed for HA-2-TCR transferred CMV T-cells after every stimulation. T-cells were either stimulated three times with pp65 (black squares), or twice with pp65 and additionally with HA-2 (grey squares), or T-cells were stimulated three times with HA-2 (black circles) or twice with HA-2 and additionally with pp65 (grey circles). The percentage of tetramer positive T-cells is shown. **(D)** The dot plots of CMV and HA-2 tetramer staining of HA-2-TCR transferred CMV T-cells after three rounds of stimulation as indicated in **(C)** are depicted. Per quadrant % T-cells are indicated, numbers in brackets indicate MFI of the tetramer positive T-cell population. **(E)** HA-2-TCR transferred CMV T-cells stimulated three times with either pp65 or HA-2, and mock CMV T-cells were tested for cytotoxic activity against HA-2 and pp65 positive targets. The T-cells were tested at an E:T ratio of 10:1 against EBV-Z, pp65 peptide pulsed EBV-Z (EBV-Z + pp65 peptide), EBV-Z pp65, HA-2 peptide pulsed EBV-Z (EBV-Z + HA-2 peptide), and EBV-RZ HA-2. Data shown is representative for two independent experiments.*

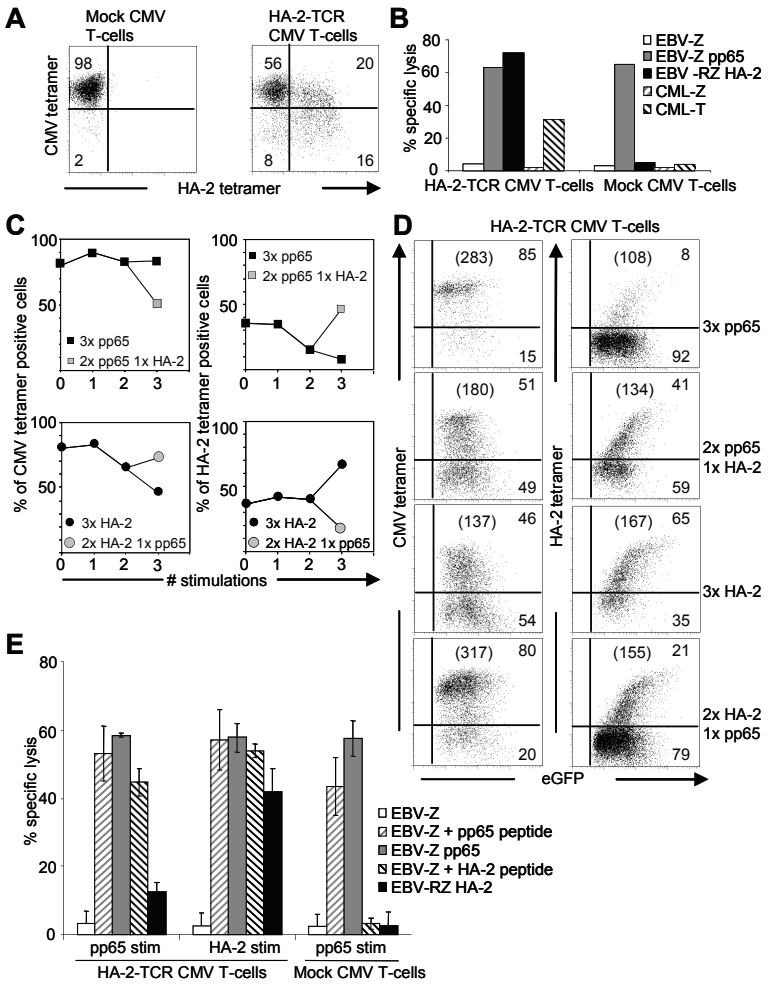

Figure 1. Cell surface expression and functional activity of the introduced and endogenous TCR after repetitive antigen-specific stimulation.

reversed by changing the stimulation, T-cells that were stimulated twice with pp65 were stimulated alternatively with HA-2. Likewise, T-cells that were stimulated twice with HA-2 were stimulated alternatively with pp65. The results demonstrate that by Ag-specific triggering of the previously non triggered TCR the TCR expression rapidly reverted (Figure 1C). On bases of the changed TCR make up (Figure 1D) we tested the HA-2 TCR transferred virus-specific T-cells stimulated repetitively with either only pp65 or only HA-2 for Ag-specific cytotoxic capacity (Figure 1E). Both T-cell populations were capable of killing HA-2 peptide loaded target cells, but the cytolytic activity of HA-2-TCR transduced T-cells repetitively stimulated with pp65 directed against target cells endogenously expressing HA-2 (EBV-RZ HA-2) was reduced. This was in accordance with the TCR expression, since in the T-cell population repetitively stimulated with pp65, only low numbers of HA-2 tetramer positive T-cells were present, while in the T-cell population repetitively stimulated with HA-2 still significant numbers of CMV tetramer positive T-cells were present.

These data illustrate that repetitive stimulation of HA-2-TCR transferred

CMV-specific T-cells with either HA-2 or CMV pp65 antigen resulted in preferential TCR expression of the triggered TCR, whereas expression of the non-triggered TCR gradually decreased. However, the T-cells with either predominant CMV or HA-2-TCR expression preserved their dual-specificity, although the level of reactivity in response to activation of the triggered TCR was higher than via the non-triggered TCR. In addition, changes in TCR expression could rapidly be reverted by Ag-specific triggering of the previously non-triggered TCR.

Generation and functionality of opposing T-cell subsets

To be able to dissect whether the difference in TCR make up after reverting the stimulation of T-cells predominantly expressing one TCR was due to selective outgrowth or due to differential TCR distribution, these T-cells were sorted into opposing subsets with either high CMV-TCR expression based on high CMV-TCR BV2 mAb staining and low HA-2 tetramer staining (Figure 2A; CMV-TCR^{hi}), or low CMV-TCR expression based on low CMV-TCR BV2 mAb staining (figure 2A; HA-2-TCR^{hi}). No tetramers were used for positive selection of the T-cells, since binding of the tetramers to the TCR would result in Ag-specific triggering via either the CMV or HA-2-TCR[32]. Directly after sorting, TCR expression of the sorted T-cell populations was analyzed using HA-2 tetramer and CMV-TCR BV2 mAb staining (Figure 2A). Both sorted T-cell subsets were positive for the marker genes eGFP and NGF-R (Figure 2B). To investigate whether T-cells almost exclusively expressing one TCR were still able to exert both HA-2 and pp65-specific cytolytic activity, the T-cells were tested in a

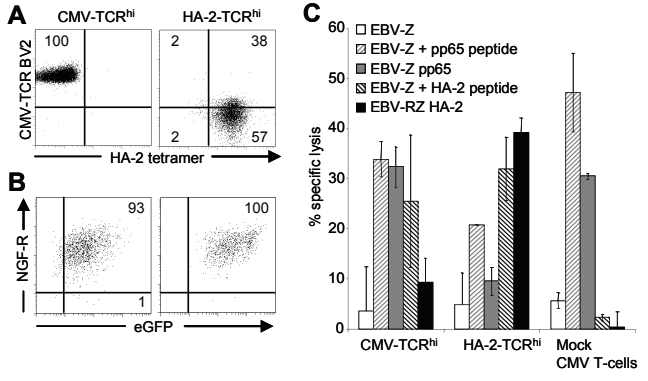

Figure 2. TCR cell surface expression and functional activity of opposing CMV-TCR^{hi} or HA-2-TCR^{hi} sorted T-cell populations.

cytotoxicity assay directly after sorting (Figure 2C). The CMV-TCR^{hi} T-cells exerted efficient cytotoxic activity against both pp65-peptide pulsed target cells and target cells endogenously expressing pp65, that was comparable to the mock transduced CMV T-cells. Although no HA-2-TCR expression could be measured on these T-cells using HA-2 tetramers (Figure 2A), the cells were still cytotoxic against HA-2 peptide pulsed target cells, but demonstrated marginal cytotoxic activity against target cells endogenously expressing HA-2, indicating that these T-cells only exhibit low avidity HA-2 reactivity (Figure 2C). The HA-2-TCR^{hi} T-cells efficiently lysed both HA-2 peptide pulsed target cells as well as the endogenous HA-2 positive target cells. Only low CMV-TCR expression could be measured on these T-cells, and the T-cells still demonstrated low cytotoxicity against pp65 peptide

*Figure 2: **(A)** HA-2-TCR transferred CMV T-cells were sorted on basis of CMV-TCR BV2^{high} and HA-2 tetramer^{low} (CMV-TCR^{hi}) or BV2^{low} staining (HA-2-TCR^{hi}), respectively. Directly after sorting, TCR expression of the sorted T-cell populations was analyzed using HA-2 tetramer and CMV-TCR BV2 mAb staining. **(B)** Both sorted T-cell subsets were analyzed for marker gene expression (eGFP and NGF-R). Numbers in **(A)** and **(B)** indicate % of cells per quadrant. **(C)** Mock CMV T-cells, CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cell populations were tested for cytotoxic activity against HA-2 and pp65 positive target cells. One representative experiment out of four is shown.*

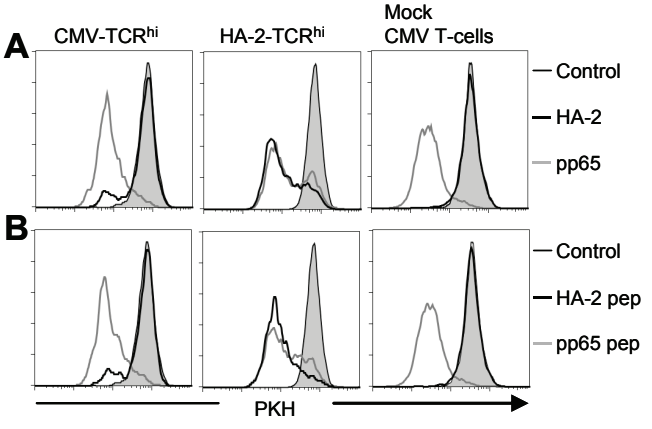

*Figure 3: **(A)** and **(B)** CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cell subsets and mock CMV T-cells were labeled with PKH-26, and stimulated Ag-specifically with HLA-A2 negative allogeneic feeders in combination with EBV-Z (Control), EBV-RZ HA-2 (A; HA-2) or HA-2 peptide pulsed EBV-Z (B; HA-2 pep) or EBV-Z pp65 (A: pp65) or pp65 peptide pulsed EBV-Z (B; pp65 pep). Only a small subset of CMV-TCR^{hi} sorted T-cells proliferate after HA-2-specific stimulation. One representative experiment out of four is shown.*

Figure 3. Proliferation of CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cells after pp65 and HA-2-specific stimulation.

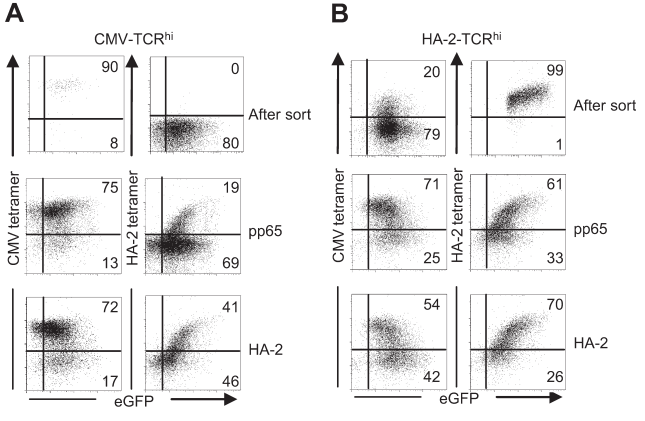

*Figure 4: CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cell subsets were stimulated pp65 or HA-2-specifically, and TCR expression was analyzed using CMV and HA-2 tetramers. **(A)** TCR expression of CMV-TCR^{hi} and **(B)** TCR expression of HA-2-TCR^{hi} sorted T-cells directly after sorting (after sort) or 7 days after stimulation with either EBV-Z pp65 (middle panel; pp65) or EBV-RZ HA-2 (lower panel; HA-2). Numbers indicate % of cells per quadrant. One representative experiment out of four is shown.*

Figure 4. TCR re-expression after restimulation of the CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cell subsets.

pulsed target cells, but only marginal cytotoxicity against target cells endogenously expressing pp65.

To study whether the sorted opposing T-cell subsets were able to proliferate upon HA-2 and pp65-specific stimulation, the T-cells were labeled with PKH-26 and analyzed using FACS at day 4. Both the CMV-TCR^{hi} and HA-2-TCR^{hi} T-cells were able to proliferate after stimulation with pp65 peptide pulsed EBV-LCLs or EBV-LCLs endogenously expressing pp65 (Figure 3A and B). Only a small percentage of the CMV-TCR^{hi} T-cells were capable of proliferating after stimulation with HA-2 peptide pulsed EBV-LCLS or EBV-LCLs endogenously expressing HA-2 (Figure 3A and B). The proliferation of HA-2-TCR^{hi} T-cells stimulated with HA-2 positive EBV-LCLs was similar to the proliferation induced by pp65 positive EBV-LCLs. Since the HA-2-TCR^{hi} T-cells were capable of proliferating both after HA-2 and pp65-specific stimulation, specific outgrowth as the main cause of reverting TCR make up was less plausible.

These results demonstrate that low CMV-TCR cell surface expression on HA-2-TCR^{hi} T-cells was sufficient for these cells to exert pp65-specific cytotoxic activity against pp65 peptide pulsed target cells, as well as pp65-specific proliferation. The low HA-2-TCR expression on CMV-TCR^{hi} T-cells was sufficient for these cells to exert specific cytotoxic activity against HA-2 peptide pulsed target cells, but was not enough for HA-2-specific proliferation. Therefore, it appears that the threshold of the endogenous TCR to induce proliferation and cytotoxic reactivity is more easily reached than the threshold of the introduced TCR, underlining the importance of targeting T-cells which will encounter antigens

that trigger their endogenous TCR in vivo to ensure persistence of TCR transferred T-cells.

Opposing T-cell subsets redistribute TCR expression on their cell surface after additional stimulation

To test whether also the CMV-TCR^{hi} and the HA-2-TCR^{hi} sorted T-cells were able to change their TCR make up after different specific stimulations, CMV-TCR^{hi} sorted T-cells (Figure 4A) and HA-2-TCR^{hi} sorted T-cells (Figure 4B) stimulated with EBV-LCLs presenting either endogenously processed pp65 or HA-2 were analyzed for TCR expression at day 7. After 7 days of Ag-specific stimulation CMV-TCR^{hi} sorted T-cells re-expressed the HA-2-TCR after stimulation with pp65 or HA-2 (Figure 4A). Although stimulation with HA-2 was not robust enough to induce proliferation of the CMV-TCR^{hi} sorted T-cells (Figure 3), it resulted in restored HA-2-TCR expression. Similarly, HA-2-TCR^{hi} sorted T-cells re-expressed the CMV-TCR after stimulation with pp65 or HA-2 (Figure 4B). These results demonstrate that also in the sorted T-cell subsets with predominant CMV or HA-2-TCR expression, T-cells are still capable of upregulating their TCR expression after stimulation via either the endogenous or introduced TCR.

Since we observed redistribution of TCR cell surface expression one week after stimulation (Figure 4), we investigated in a cytotoxicity assay whether cytolytic activity of CMV-TCR^{hi} or HA-2-TCR^{hi} sorted T-cells was improved (Figure 5A). After additional stimulation, only marginal differences in cytotoxic activity against HA-2 and pp65 positive target cells were observed between the CMV-TCR^{hi} and HA-2-TCR^{hi} T-cells (Figure 5A),

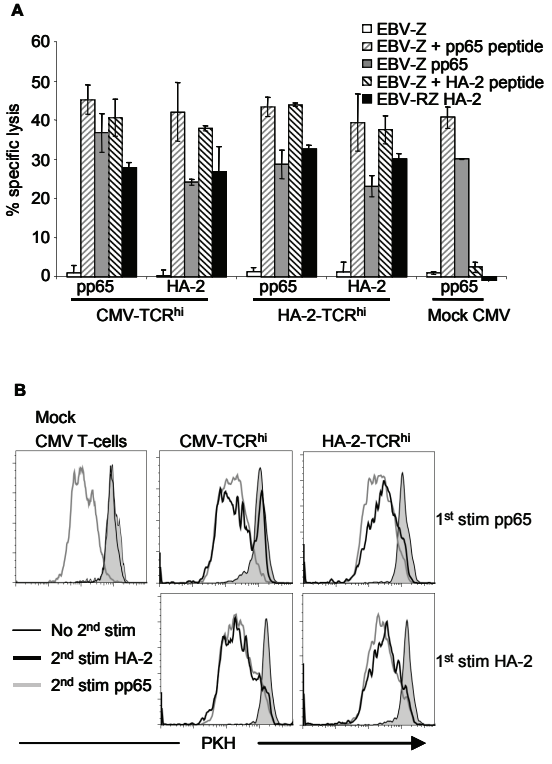

Figure 5. Comparable HA-2 and pp65 reactivity of CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cells stimulated once after sorting.

corresponding with the restored co-expression of the HA-2- and CMV-TCR (Figure 4). All subsets displayed high cytotoxic activity against HA-2 positive and pp65 positive target cells, illustrating restored high avidity interactions.

*Figure 5: **(A)** CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cell subsets stimulated either pp65 or HA-2-specifically and mock CMV T-cells were analyzed for their pp65 and HA-2 reactivity 7 days after stimulation in a cytotoxicity assay. **(B)** Concurrently, the proliferative capacity of mock CMV T-cells, CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cells was analyzed 7 days after their first specific stimulation. CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cells stimulated directly after sorting with EBV-Z pp65 (upper panel; 1st stim pp65) or EBV-RZ HA-2 (lower panel; 1st stim HA-2) were either stimulated additionally with negative control EBV-Z (No 2nd stim; shadowed line), or with EBV-RZ HA-2 (2nd stim HA-2; black line), or with EBV-Z pp65 (2nd stim pp65; grey line). One representative experiment out of two is shown.*

To test whether the restored HA-2 and CMV-TCR co-expression also led to improved proliferation after stimulation via either endogenous or introduced TCR, the CMV-TCR^{hi} and the HA-2-TCR^{hi} T-cells were labeled with PKH-26, stimulated again with HA-2 or pp65 and analyzed using FACS at day 4 (Figure 5B). All CMV-TCR^{hi} T-cells and HA-2-TCR^{hi} T-cells stimulated with pp65 or HA-2 were able to proliferate vigorously after a second stimulation with pp65 positive EBV-LCLs. In contrast to the minimal amount of proliferation after HA-2-specific stimulation directly after sorting (Figure 3), most CMV-TCR^{hi} T-cells stimulated once with HA-2 were capable of proliferating after a second HA-2-specific stimulation. A small part of the CMV-TCR^{hi} T-cells stimulated once with pp65 was not able to proliferate upon HA-2-specific stimulation.

In conclusion, opposing T-cell populations are able to redistribute their TCRs at the cell surface after an additional Ag-specific stimulation, leading to restored functionality via both TCRs. These data imply that no loss of dual-specificity is likely to occur due to skewing of T-cells to a population predominantly expressing one TCR.

DISCUSSION

In this study, we demonstrate that TCR transferred virus-specific T-cells repetitively stimulated via one TCR remained dual reactive in response to triggering via both the endogenous and the introduced TCR. After repetitive stimulation of one TCR, TCR transferred T-cells preferentially expressed the triggered TCR, losing high avidity interaction via the previously non-triggered TCR. However, after a single stimulation via the previously non-triggered TCR, TCR expression reverted within one week. When the dual-specific T-cells were sorted in opposing CMV-TCR^{hi} T-cells and HA-2-TCR^{hi} T-cells, both subsets still demonstrated cytotoxic activity against HA-2 peptide pulsed target cells and CMV peptide pulsed target cells, respectively, but limited cytotoxic activity against targets presenting endogenously processed antigen, indicating loss of high avidity interactions. After additional stimulation, both subsets were able to re-express the HA-2 and CMV-TCR, respectively. When TCR expression was redistributed on the T-cells, high avidity functionality via both the endogenous and the introduced TCR was restored. Therefore, we speculate that also HA-2-TCR^{hi} T-cells are capable of persisting during MRD when HA-2-TCR transferred CMV T-cells will predominantly encounter viral antigens. Furthermore, we anticipate that HA-2-TCR transferred CMV T-cells after a long period of MRD are still able to gain anti-leukemic effector functions when the patient would relapse.

Directly after sorting T-cells predominantly expressed the CMV-TCR (CMV-TCR^{hi}) or the HA-2-TCR (HA-2-TCR^{hi}). However, after an additional stimulation TCR re-expression was observed. Surprisingly, HA-2-TCR re-expression was observed on CMV-TCR^{hi} TCR T-cells both after stimulation with HA-2 as well as with pp65, and CMV-TCR re-expression was observed on HA-2-TCR^{hi} T-cells both after stimulation with pp65 as well as with HA-2. TCR make up on transduced T-cells appears to be activation

dependent, however, a trend of preferential redistribution of the TCR being triggered was observed. It has been described that upon activation, T-cells enlarge and increase TCR expression[33-36] which is accompanied with restructuring compartmentalization of plasma membrane molecules[27,37,38]. Possibly, because of both increased TCR expression and localized high TCR density, HA-2-TCR on CMV-TCR^{hi} T-cells could be visualized using HA-2 tetramer staining after an additional stimulation, whereas this is not possible when HA-2-TCR is equally distributed along the cell membrane. Another possibility is that initial downregulation of the triggered TCR enabled surface expression of intracellular TCRs consisting of both the endogenous and introduced TCRs, whereas later on TCR expression will be dominated by newly synthesized previously triggered TCR. This would result in the preferential but not exclusive re-expression of the triggered TCR. It is evident that despite low CMV-TCR expression, HA-2-TCR^{hi} T-cells are capable of persisting during repetitive stimulation with pp65, although they do not proliferate as vigorously after stimulation with pp65 as CMV-TCR^{hi} T-cells. Even in a stringent selection of T-cells with predominant expression of either the introduced or the endogenous TCR, re-expression of the other TCR was observed, implying that TCR expression on these T-cells is dynamic rather than static.

Our results indicate that the threshold of the endogenous TCR to induce proliferation and cytotoxic reactivity is more easily reached than the threshold of the introduced TCR, underlining the importance of targeting T-cells which will encounter antigens that trigger their endogenous TCR in vivo to ensure persistence of TCR transferred T-cells. EBV and CMV are viruses which latently persist after initial infection and have to be continuously controlled by the immune system. Both in immunocompetent and immunocompromised hosts, immune responses result in viral containment in latent stage rather than virus eradication[39-41]. Therefore, we propose to use EBV or CMV-specific T-cells as host cells for TCR transfer. When there is only minimal residual disease it may be expected that HA-2-TCR transferred virus-specific T-cells will primarily encounter viral antigens latently present in the recipient, as the HA-2 antigen is only expressed by recipient hematopoietic cells. We hypothesize that low dose triggering of the endogenous TCR due to the persistence of the virus will also boost the anti-leukemic immune response mediated via the HA-2-TCR.

To ensure persistence and correct homing of transduced virus-specific T-cells it is discussed that different memory subsets should be used[42,43], or virus-specific T-cells responsible for the immunodominant response in the donor should be selected[21,44]. Recent studies demonstrated that distinct memory subsets are raised in different viral infections[45,46]. Even within one virus-specific memory response distinct subsets of virus-specific CD8+ T-cells can be found. For example, the CD8+ memory T-cells specific for EBV lytic antigens predominantly have a more differentiated effector memory phenotype, whereas CD8+ memory T-cells specific for EBV latent antigens predominantly have a central memory phenotype[40]. Therefore it is hypothesized that phenotype of CD8+ memory T-cells could well be dictated by different routes of antigen exposure. Based

on phenotypic characteristics CD8+ memory T-cells specific for CMV are mainly effector-type or late memory T-cells[41]. However, studies have demonstrated that CD8+ memory T-cells specific for CMV are able to respond with renewed clonal expansion upon viral reactivation[23,47], suggesting that phenotypic classification alone is not indicative for functional characteristics. We therefore would like to use for clinical application a pool of CMV or EBV-specific T-cells with distinct phenotypic characteristics, resulting in virus-specific T-cells with different functional characteristics and homing capacities.

In conclusion, although after repetitive stimulation HA-2-TCR transferred CMV-specific T-cells skew to populations predominantly expressing one TCR, all subsets are able to persist and repopulate after stimulation via the previously non-triggered TCR. Therefore we conclude that TCR transduced virus-specific T-cells behave favorably in view of future clinical applications.

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Mixed TCR dimers harbor potentially harmful neoreactivity

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ABSTRACT

Adoptive transfer of TCR transduced T-cells may be an attractive strategy to target both hematological malignancies and solid tumors. By introducing a TCR, large numbers of T-cells with defined antigen (Ag) specificity can be obtained. However, by introduction of a TCR, mixed TCR dimers can be formed. Besides the decrease in TCR expression of the introduced and endogenous TCR, these mixed TCR dimers could harbor potentially harmful specificities. In this study, we demonstrate that introduction of TCRs resulted in formation of neoreactive mixed TCR dimers, composed of the introduced TCR chains pairing with either the endogenous TCRα or β chain. Neoreactivities observed were HLA class I or class II restricted. Most neoreactive mixed TCR dimers were allo-HLA reactive, however, neoreactive mixed TCR dimers with autoreactive activity were also observed. We demonstrate that inclusion of an extra disulfide bond between the constant domains of the introduced TCR markedly reduced neoreactivity, whereas enhanced effectiveness of the introduced TCR was observed. In conclusion, TCR transfer results in the formation of neoreactive mixed TCR dimers with the potential to generate off-target effects, underlining the importance of searching for techniques to facilitate preferential pairing.

INTRODUCTION

Adoptive transfer of T-cells is a strategy used to target both solid tumors and leukemia. Patients with relapsed hematological malignancies after allogeneic stem cell transplantation can be successfully treated with donor lymphocyte infusion (DLI) [1,2], and patients with solid tumors can be effectively treated with tumor infiltrating lymphocytes (TILs) cultured from tumor tissue[3]. The beneficial graft-versus-leukemia effect of DLI mediated by the recognition of minor histocompatibility antigens (mHags) is, however, often accompanied by graft-versus-host disease. Furthermore, isolation and expansion of TILs is feasible only for a fraction of patients with solid tumors. The adoptive transfer of T-cells transduced with TCRs recognizing tumor associated antigens or mHags may be an attractive alternative strategy to target hematological malignancies and solid tumors. By introducing a TCR, large numbers of T-cells with defined antigen (Ag) specificity can be obtained without long in vitro culture periods. Different studies have shown the effectiveness of TCR transfer, both in vitro[4-8] and in vivo[9-11]. Recently, the in vivo efficacy of adoptively transferred TCR transduced (td) T-cells was demonstrated in melanoma patients[10,12].

The introduction of an exogenous TCR into T-cells has several consequences for the TCR make-up of the cell. The introduced TCR has to compete for cell surface expression with the endogenous TCR, and with mixed TCR dimers consisting of an endogenous TCR chain pairing with an introduced TCR chain[13]. Because of competition of these different TCR complexes for binding with CD3, the frequency of TCRs at the cell surface will be lower in TCR td T-cells than in parental T-cells. Therefore, a prerequisite of the introduced TCR is that it exhibits high affinity for its antigen, and is able to efficiently compete with the endogenous TCR for cell surface expression[13]. Different studies have attempted to improve TCR surface expression and subsequently biological activity, by facilitating matched pairing of the introduced TCR chains. Exchange of the human constant regions for murine constant regions was described to improve TCR expression and functionality[14,15]. Another strategy that resulted in preferential pairing of the introduced TCR chains and increased TCR surface expression is the introduction of a disulfide bond in the extracellular constant domain[16,17].

Not only the decrease in TCR expression of the introduced Ag-specific TCR, but also the formation of mixed TCR dimers with unknown specificity is an additional potential drawback of clinical application of TCR gene transfer[13,18,19]. Because the specificity of mixed TCR dimers is unpredictable, hazardous specificities may be formed. In this study, 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 7 different well characterized Ag-specific TCRs and tested these for newly acquired reactivities against an HLA-typed LCL panel covering all prevalent HLA class I and II molecules.

Our results demonstrate that pairing of endogenous TCR chains with introduced TCR chains can result in the formation of mixed TCR dimers with new potentially hazardous specificities recognizing allo-antigens as well as auto-antigens, both HLA class I and class II restricted.

RESULTS

Introduction of different TCRs into several virus-specific T-cells elicits neoreactivity mediated via mixed TCR dimers

To study whether TCR transfer can lead to mixed TCR dimers with new detrimental reactivities, we transduced various virus-specific T-cell lines from 4 healthy donors with different Ag-specific TCRs. HLA-A1 restricted pp50- or pp65-specific T-cells and HLA-B8 restricted IE-1- or BZLF-1-specific T-cells were sorted, resulting in 5 different virus-specific T-cell lines (Table S1). These T-cell lines were transduced with 7 different TCRs, consisting of 4 different HA-2-specific TCRs (HA2.5-TCR, HA2.6-TCR, HA2.19-TCR, and HA.2.20-TCR), 2 different HA-1-specific TCRs (HA1.M2-TCR, HA1.M7-TCR) and the CMV-TCR. The transduced virus-specific T-cells were sorted based on high eGPF and NGF-R positivity, and tested for neoreactivity against the LCL panel (Table S2) covering all prevalent HLA class I and class II molecules. Introduction of different TCRs resulted in newly acquired reactivities against different LCLs, of which representative examples are shown in Figure 1. Some LCLs were excluded from analysis, as the non td virus-specific T-cells already recognized the LCLs,

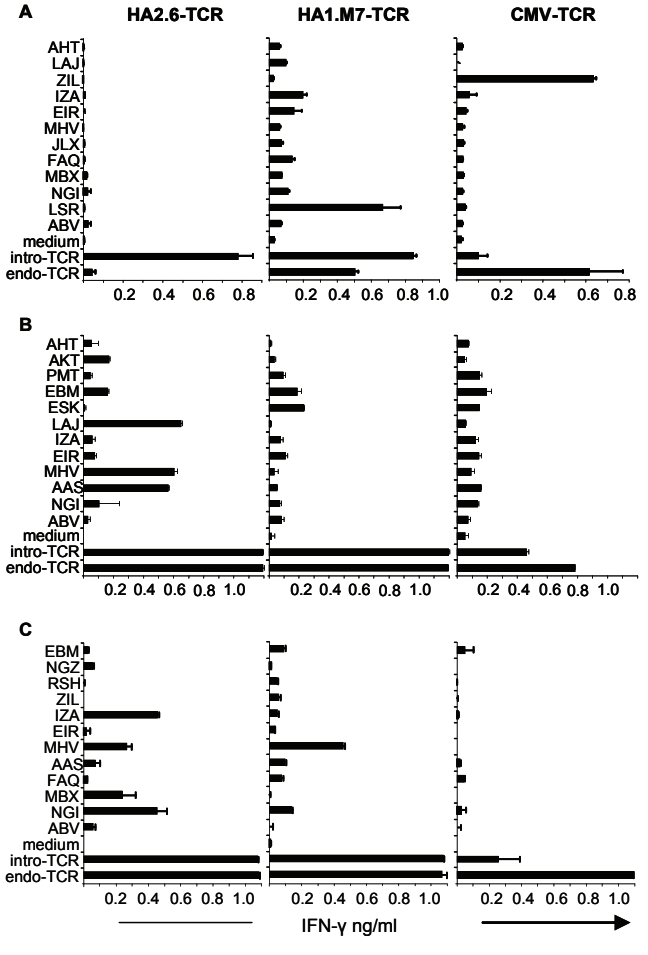

Figure 1. TCR td virus-specific T-cells demonstrate neoreactivity.

*Figure 1: T cells were tested against a broad LCL panel. IFN-γ production of 3 of the 5 different virus-specific T-cell populations transduced with 3 different TCRs is depicted, namely of **(A)** pp50-specific T-cells isolated from donor CVO, **(B)** pp50-specific T-cells of donor UKL and **(C)** IE-1-specific T-cells isolated from donor MBX. As a control for the reactivity of the endogenous and introduced TCR, LCLs with the restricting HLA molecules were pulsed with the relevant peptides (endo-TCR and intro-TCR, respectively). IFN-γ production depicted is representative of 3 separate experiments performed in duplo.*

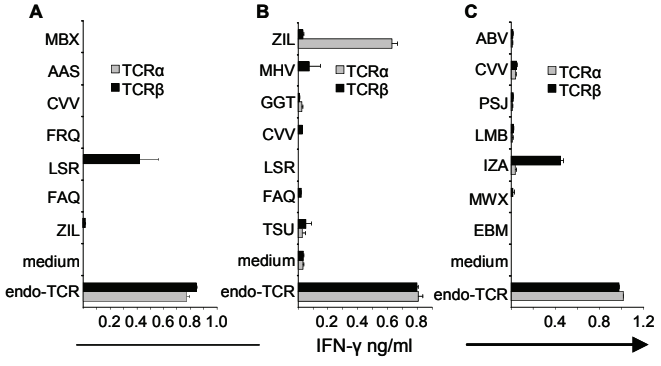

Figure 2. Neoreactivities caused by mixed TCR dimers.

*Figure 2: pp50 T-cells from CVO were transduced with **(A)** HA1.M7-TCRα or β chains or with **(B)** CMV-TCRα or β chains, and IE-1 T-cells from MBX were transduced with **(C)** HA2.6-TCRα or β chains and tested against the LCL panel for neoreactivity. As a control for the reactivity of the endogenous TCR, LCLs with the restricting HLA molecules of the endogenous virus-specific TCR were pulsed with the viral peptides (A, B, C; endo-TCR). The IFN-γ production depicted is representative of 2 separate experiments.*

indicative for alloreactivity of the virus-specific T-cells rather than neoreactivity via mixed TCR dimers (Table S1). We could exclude alloreactivity of the introduced TCRs, as the parental T-cell clones of which these TCRs were derived were not reactive against the LCLs present in the panel. In each of the 5 different virus-specific T-cell lines, transfer of at least 2 out of 7 TCRs induced neoreactivity (Table S1). As illustrated in Figure 1A, pp50-specific T-cells of donor CVO (CVO pp50 T-cells) transferred with the CMV-TCR exhibited strong reactivity particularly against ZIL. This reactivity was not seen with HA2.6-TCR td or HA1.M7-TCR td CVO pp50 T-cells. HA1.M7-TCR transfer resulted in strong reactivity directed against LSR, which was not observed with HA2.6-TCR td or CMV-TCR td CVO pp50 T-cells. Introduction of the HA2.6-TCR into pp50-specific T-cells of donor UKL (UKL pp50 T-cells) resulted in clear neoreactivity (Figure 1B), whereas low neoreactivity was observed after introduction of the HA1.M7-TCR or the CMV-TCR into these T-cells. Introduction of the HA2.6-TCR and HA1.M7-TCR into IE-1-specific T-cells of healthy individual MBX (MBX IE-1 T-cells) resulted in neoreactivity against different LCLs (Figure 1C). Strikingly, some neoreactivities were as robust as reactivity via the introduced or endogenous TCR against peptide pulsed target cells. To determine whether the observed neoreactivities against LSR after HA1.M7-TCR transfer and against ZIL after CMV-TCR transfer (Figure 1A) were mediated via mixed TCR dimers, we transduced CVO pp50 T-cells with either the HA1.M7-TCRα or β chain (Figure 2A), or either the CMV-TCRα or β chain (Figure 2B). Transduction of only the HA1.M7-TCRβ and not α chain (Figure 2A) resulted in neoreactivity directed against LSR. Transduction of only the CMV-TCRα chain and not β chain (Figure 2B) into these T-cells resulted in neoreactivity directed against ZIL. In addition, to test whether the observed neoreactivities of MBX IE-1 T-cells after HA2.6-TCR transfer (Figure 1C) were mediated via mixed TCR dimers, we transduced these T-cells with either only the HA2.6-TCRα or β chain. As shown in Figure 2C only HA-2.6-TCRβ td T-cells demonstrated neoreactivity directed against IZA. Furthermore, we deliberately created mixed TCR dimers by recombining HA-2-specific TCRα and TCRβ chains of 4 different HA-2-TCRs, namely the HA2.5-TCR, the HA2.6-TCR, the HA2.19-TCR and the HA2.20-TCR and transducing all possible combinations into monoclonal CVO pp50 T-cells. Taking into account that also the introduced HA-2-TCR chains can pair with the endogenous TCR of the pp50 T-cells, this resulted in potentially 20 mixed TCR dimers. Of these 20 mixed TCR dimers, the recombination of HA2.19-TCRα and HA2.6-TCRβ chain (Figure

S1A; mixed TCR dimer) resulted in significant IFN-γ production against DMD, whereas the parental HA2.19-TCR and HA2.6-TCR demonstrated only HA-2-specific reactivity against HA-2 peptide pulsed target cells. These results indicate that each recombination of TCR chains after TCR transfer can potentially result in a harmful new reactivity.

These results demonstrate that neoreactivities can occur in multiple virus-specific T-cells after transfer of different TCRs. The neoreactive mixed TCR dimers can be composed of introduced TCR chains pairing with either the endogenous TCRα chain or the endogenous TCRβ chain.

Mixed TCR dimers can acquire both HLA class I and class II restricted allo- and autoreactivities

To study whether neoreactivities of the mixed TCR dimers were HLA restricted, blocking experiments were performed. Because in oligoclonal virus-specific T-cell lines theoretically different mixed TCR dimers can be formed and this can potentially hinder analysis of HLA-restriction, as well as functional activity of the individual-specificities, monoclonal CVO pp50 T-cells and MBX IE-1 T-cells were sorted on bases of TCR usage. By transfer of the different TCRs we could confirm that the most prominent TCR-BV1 positive population present in CVO pp50 T-cells was responsible for the neoreactivity against LSR after HA1.M7-TCR transfer, and the neoreactivity against ZIL after HA2.6-TCR transfer. Likewise, of the oligoclonal populations of MBX IE-1 T-cells only the TCR-BV1 positive T-cells transduced with HA2.6-TCR demonstrated neoreactivity against IZA. Neoreactivity of

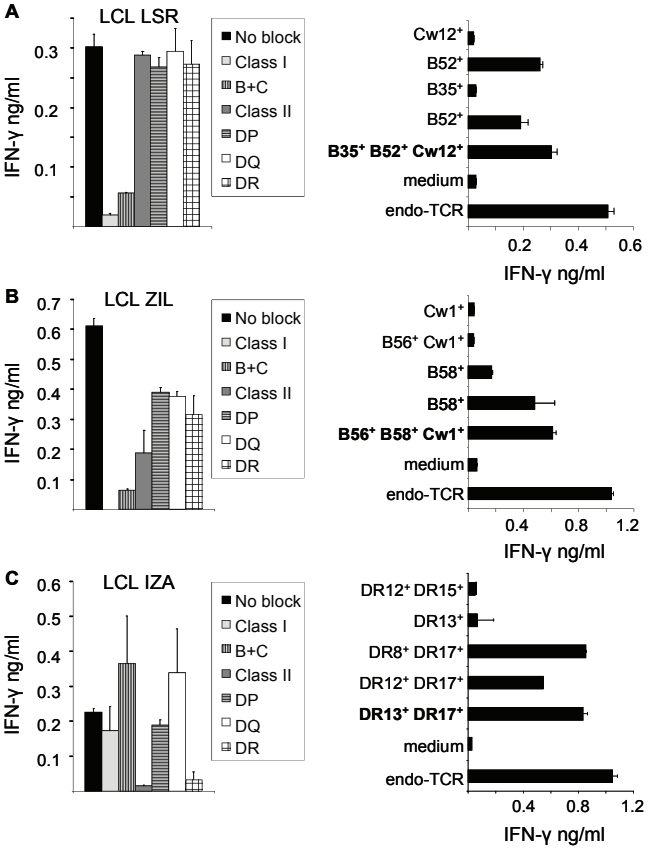

Figure 3. Neoreactivity of mixed TCR dimers is both HLA class I and II restricted.

*Figure 3: HLA restriction of neoreactive mixed TCR dimers of **(A)** BV1+ HA1.M7-TCRβ td CVO pp50 T-cells, **(B)** BV1+ CMV-TCRα td CVO pp50 T-cells, or **(C)** BV1+ HA2.6-TCRβ td MBX IE-1 T-cells was tested in IFN-γ production assay using blocking antibodies. Relevant HLA typing of the LCLs is indicated, with the HLA typing of the blocked LCL in bold. IFN-γ production depicted is representative of 3 separate experiments performed in triplo.*

*Figure 4: **(A)** CMV-TCRα td and non td CVO pp50 T-cells were tested against HLA-B58+ LCL ZIL and HLA-B58- LCL from CVO and CD14+, CD19+ and CD4+ MACS-isolated cell subsets derived from HLA-B58+ healthy individual IGN. HA-2.6-TCRβ td and non td MBX IE-1 T-cells were tested against LCLs and CD14+, CD19+ and CD4+ MACS-isolated cell subsets derived from **(B)** HLA-DR17+ MBX or **(C)** HLA-DR17+ NGI. HLA-DR17- LCL EBM was included in the experiment as a control. T-cells were tested against resting cell subsets (ex vivo) or activated cell subsets. CD14+ cells were either activated into immature DCs (iDC) or mature DCs (mDCs) using activating cytokines. CD19+ cells were activated using activating cytokines and CD40L (B act). CD4+ cells were activated using PHA (T act). IFN-γ production depicted is representative of 2 separate experiments.*

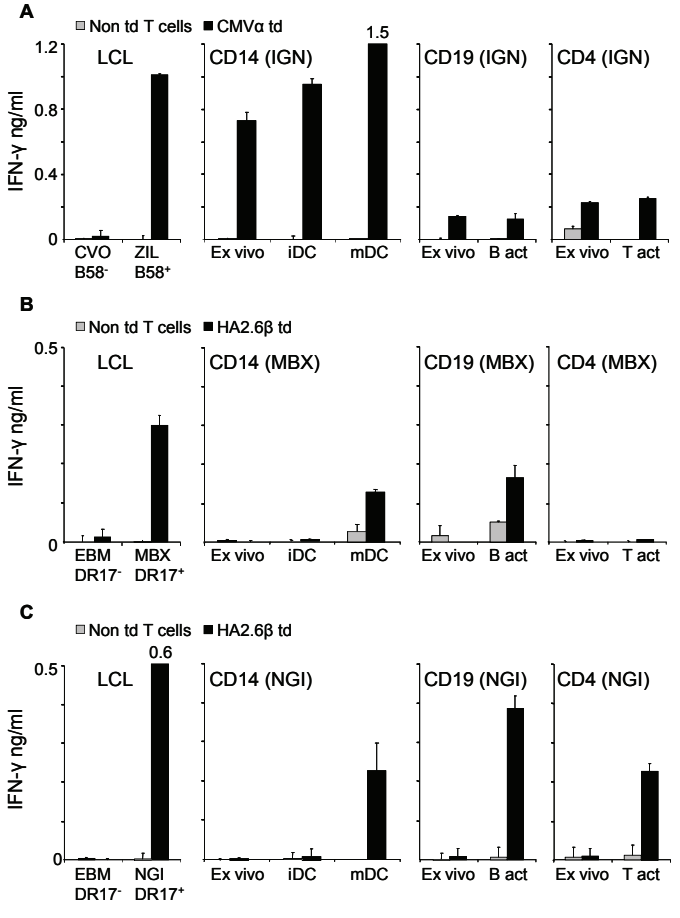

Figure 4. Neoreactivity also directed against normal human cell subsets.

HA1.M7-TCRβ td BV1+ CVO pp50 T-cells against LSR (Figure 1A, 2A) could be blocked by HLA class I and HLA-B/C blocking antibodies, indicating HLA-B or HLA-C restricted recognition, as demonstrated in Figure 3A. LSR expressed HLA-B35, B52 and Cw12. Additional experiments using various LCLs expressing one of these HLA restriction molecules demonstrated that this neoreactivity was HLA-B52 mediated (Figure 3A). In addition, the neoreactivity of CMV-TCRα td BV1+ CVO pp50 T-cells against ZIL (Figure 1A, 2B) was also HLA-B or HLA-C restricted, as the reactivity could be blocked with HLA class I and HLA-B/C antibodies (Figure 3B). ZIL expressed HLA-B56, B58, and Cw1, and additional testing against LCLs covering these different HLA restriction molecules demonstrated this neoreactivity to be HLA-B58 restricted (Figure 3B). The neoreactivity of the HA2.6-TCRβ td MBX IE-1 T-cells (Figure 1C, 2C) was demonstrated to be HLA-DR17 restricted, based on blocking with HLA class II and HLA DR mAbs and testing with an additional LCL panel (Figure 3C). The neoreactivity of the HA2.19-TCRα and HA2.6-TCRβ mixed TCR dimer could be blocked with HLA class II and HLA-DQ mAbs, and testing on an additional LCL panel demonstrated that this neoreactivity was HLA-DQ3(8/9) restricted (Figure S1B). In conclusion, mixed TCR dimers derived from HLA class I restricted T-cells can acquire neoreactivities that can be both HLA class I and HLA class II restricted.

In Figure 3C we demonstrate that the HA2.6-TCRβ chain in combination with the TCRα chain of the endogenous TCR from MBX IE-1 T-cells resulted in a HLA-DR17 restricted

neoreactivity. Because MBX was also HLA-DR17 positive, we tested these neoreactive T-cells for recognition of autologous LCLs derived from MBX. Mixed TCR dimers produced IFN-γ (Figure 4B left panel, Figure S2A) and were cytolytic (Figure S2B) against HLA-DR17+ LCLs including MBX, and reactivity against all LCLs could be blocked using HLA class II and HLA-DR mAbs (Figure S2C).

These results indicate that mixed TCR dimers may lead not only to newly acquired alloreactivity, but also to autoreactivity.

Mixed TCR dimers are neoreactive against normal human cell subsets

To study whether the observed neoreactivities directed against LCLs were predictive for reactivity against normal human cell subsets, we tested both HLA class I and class II restricted neoreactive mixed TCR dimers against different MACS isolated cell subsets. Neoreactive HLA-B58 restricted CMV-TCRα td CVO pp50 T-cells and HLA-DR17 restricted HA2.6-TCRβ td MBX IE-1 T-cells were tested against freshly isolated and in vitro activated CD4+, CD19+ and CD14+ cell subsets isolated from PBMCs of an HLA-B58+ or an HLA-B58- individual (Figure 4A, Figure S3A) or HLA-DR17+ individuals (Figure 4B and C, Figure S3B). The HLA-B58 restricted neoreactive T-cells were able to recognize all different cell subsets directly ex vivo (Figure 4A). The HLA-DR17 restricted neoreactive T-cells did not recognize the cell subsets directly ex vivo but recognized the autologous activated CD19+ and CD14+ cell subsets of MBX as well as the allogeneic activated CD19+, CD14+ and CD4+ cell subsets of NGI (Figure 4B and C). The absence of IFN-γ production against autologous activated CD4+ T-cells derived from MBX was not surprising, as no signs of self-reactivity of the HA2.6-TCRβ td MBX IE-1 T-cells were observed, and these T-cells could be easily expanded using feeder cells and PHA. Cytolytic capacity of CMV-TCRα td CVO pp50 T-cells (Figure S3A) corresponded with the IFN-γ production against these cell subsets (Figure 4A). The HA2.6-TCRβ td MBX IE-1 T-cells, however, exerted cytolytic activity against allogeneic nonactivated CD19+ and CD4+ cell subsets derived from NGI, whereas no IFN-γ production was observed after stimulation with these cell subsets, indicating that the threshold for

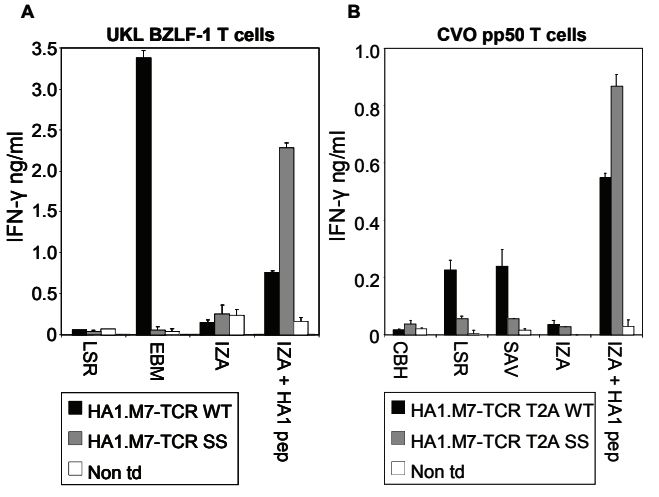

Figure 5. Neoreactivities are markedly diminished using cysteine modified TCRs.

*Figure 5: **(A)** BZLF-1 T-cells from healthy individual UKL were transduced with two separate retroviral vectors encoding either the unmodified HA1.M7-TCRα and β chains (HA1.M7-TCR WT; black bars) or cysteine modified HA1.M7-TCRα and β chains (HA1.M7-TCR SS; grey bars), sorted on bases of high eGFP and ΔNGF-R expression and tested against several LCLs for neoreactivity in duplo. **(B)** CVO pp50 T-cells were transduced with retroviral vectors containing T2A linked unmodified HA1.M7-TCRα and β chain (HA1.M7-TCR 2A WT; black bars) or T2A linked cysteine modified HA1.M7-TCRα and β chain (HA1.M7-TCR 2A SS; grey bars), sorted on high ΔNGF-R expression and tested in duplicate against several LCLs for neoreactivity. As a control, non td UKL BZLF-1 T-cells and CVO pp50 T-cells (non td; white bars) were tested against the same LCL panel. IFN-γ production depicted is representative of 3 separate experiments.*

cytolytic activity is easier reached than the threshold for cytokine production. The T-cells did not exert cytolytic activity against the nonactivated autologous cell subsets from MBX (Figure S3B) corresponding with the IFN-γ production (Figure 4B). These results demonstrate that the observed neoreactivities against the LCL panel are predictive for reactivity against normal human cell subsets.

In conclusion, T-cells expressing neoreactive mixed TCR dimers can recognize normal cell subsets, and are capable of both producing cytokines and demonstrating cytolytic activity.

Transfer of cysteine modified TCRs reduces neoreactivity

To determine whether strategies facilitating matched pairing could reduce potentially harmful neoreactivities, we modified the HA1.M7-TCR by inclusion of extra cysteine residues in the constant domains of the TCR chains. UKL BZLF-1 T-cells that exhibited HLA-DR4 restricted neoreactivity after transduction with the HA1.M7-TCR (Figure S4) were either transduced with retroviral vectors encoding the unmodified HA1.M7-TCRα and β chains (HA1.M7-TCR WT) or with cysteine modified HA1.M7-TCRα and β chains (HA1.M7-TCR SS), sorted on bases of high eGFP and ΔNGF-R expression and tested against the LCL panel for neoreactivity. Whereas the HA1.M7-TCR WT td T-cells exhibited neoreactivity against the HLA-DR4+ EBM, the HA1.M7-TCR SS td T-cells showed limited neoreactivity (Figure 5A). In contrast to reduced neoreactivity, the HA1.M7-TCR SS td T-cells exhibited increased HA-1-specificity (Figure 5A). In addition, we studied whether HLA-B52 restricted neoreactivity of HA1.M7-TCR td BV1+ CVO pp50 T-cells (Figure 1A, 2A, 3A) could be reduced by inclusion of cysteine residues in the HA1.M7-TCR. CVO pp50 T-cells were transduced with retroviral vectors encoding both the HA-1-TCRα and β chain linked with a self-cleaving 2A sequence (T2A) that were either unmodified (HA1.M7-TCR T2A WT) or cysteine modified (HA1.M7-TCR T2A SS), sorted on bases of high ΔNGF-R expression and tested against the LCL panel for neoreactivity. As can be observed in Figure 5B, CVO pp50 T-cells transduced with the HA1.M7-TCR T2A WT demonstrated neoreactivity directed against HLA-B52+ LSR and SAV. However, also this HLA-B52 restricted neoreactivity was markedly reduced by cysteine modification of the HA1.M7-TCR (Figure 5B), whereas the reactivity against HA-1+ target cells increased.

The results indicate that inclusion of an additional disulfide bond between the introduced TCR chains markedly decreased neoreactivity and, in addition, increased the effectiveness of the introduced TCRs.

DISCUSSION

In this study, we investigated whether TCR gene transfer can lead to the generation of new detrimental reactivities by creating T-cells that express mixed TCR dimers. For this purpose, we introduced 7 different TCRs into 5 virus-specific T-cell populations derived from healthy donors, and tested these transduced T-cell populations against an LCL panel covering the most prevalent HLA class I and II molecules. Per virus-specific T-cell line, at least

2 out of 7 TCR-transductants demonstrated neoreactivities. We could demonstrate that introduction of only TCRα or TCRβ chains resulted in neoreactivity, and that this neoreactivity could be HLA class I or class II mediated. Furthermore, we not only observed neoreactive mixed TCR dimers harbouring alloreactivity, but also autoreactivity. Therefore, we conclude that mixed TCR dimers formed will frequently harbour new, potentially harmful specificities.

Relatively high frequencies of neoreactive mixed TCR dimers were found. Normally, during development, T-cells undergo thymic selection resulting in a T-cell repertoire consisting of T-cells capable of binding to self-peptide-self-MHC complexes with adequate affinity. Potentially autoimmune T-cells that have high affinity for self-peptide-self-MHC complexes are deleted. Alloreactivity refers to the ability of T-cells to recognize peptide-allogeneic-MHC complexes that were not encountered during thymic development, and we have recently described that alloreactivity by virus-specific T-cells is frequently observed[20]. In the case of the mixed TCR dimers no thymic selection has occurred at all, and by chance both allo- and autoreactive mixed TCR dimers can be engineered. TCR td T-cells harbouring autoreactive mixed TCR dimers will only be able to survive when the peptide recognized is not expressed on the T-cells themselves, since this may lead to fratricide of these T-cells.

In our model we measured T-cell reactivity against an LCL panel covering a large spectrum of different HLA molecules expressing different peptides. Theoretically, by using this model it is more likely that we pick up neoreactive mixed TCR dimers recognizing either a peptide in the context of allo-HLA than in the context of self-HLA, since a maximum of 12 self-HLA alleles will be shared with the LCLs in the panel, whereas up to 77 HLA molecules will be foreign to the T-cells. However, we also identified a neoreactive mixed TCR dimer recognizing peptides bound to self-HLA, namely the HLA-DR17 reactive HA2.6-TCRβ td MBX IE-1 T-cells. These selfreactive T-cells were capable of recognizing only autologous DCs and activated B cells, and not activated autologous CD4+ T-cells, whereas both activated and resting allogeneic cell subsets (NGI) were recognized. This lack of reactivity against activated autologous CD4+ T-cells was not surprising, as these HLA-DR17 neoreactive MBX IE-1 T-cells could be easily expanded using feeder cells and PHA. Furthermore, NGI derived target cells were always better recognized (Figure 4B/C, S2, S3), indicating that the target antigen might be higher expressed in NGI derived CD4+ T-cells compared with MBX derived CD4+ T-cells. In addition, HLA-DR expression on activated T-cells is lower than on LCLs and DCs, and, in combination with lower antigen expression on MBX derived target cells, possibly the threshold for activation of the autoreactive T-cells by MBX derived CD4+ T-cells is not reached. We cannot conclude from these data whether the mixed TCR dimers recognize different antigens expressed by MBX and NGI, although IFN-γ production against both LCL MBX and LCL NGI could be blocked using HLA class II and HLA-DR blocking mAbs, or whether they recognize possibly differentially expressed antigens by these two LCLs.

In a pp50-specific T-cell clone, we observed neoreactivity in 3 out of 7 TCR transductions. Theoretically, the introduction

of 7 TCRs into a monoclonal virus-specific T-cell population will result in 14 mixed TCR dimers. Of these 14 mixed TCR dimers, 3 were demonstrated to be neoreactive, indicating that approximately 1 out of 5 mixed TCR dimers will harbour a new specificity. Furthermore, deliberately creating mixed TCR dimers by recombining 4 different HA-2-TCRs into BV1+ CVO pp50 T-cell clone resulted in 1 neoreactive mixed TCR dimer out of 20. On average, we conclude that approximately 1 out of 10 mixed TCR dimers will harbor potentially hazardous neoreactivity. The results demonstrate that selecting strong competitor TCRs could not avoid occurence of neoreactive mixed TCR dimers, as has been proposed previously as a strategy to acquire single TCR expression on transduced T-cells[21]. For example, introduction of a strong competitor CMV-TCR into weak competitor pp50-specific T-cells resulted in HLA-B58 restricted neoreactive mixed TCR dimers. Also, the introduction of a strong competitor HA2.6-TCR into strong competitor IE1-specific T-cells resulted in HLA-DR17 restricted neoreactive mixed TCR dimers. These results imply that TCR transfer will frequently result in the formation of neoreactive mixed TCR dimers.

To date however, no off-target toxicity has been observed in clinical trials treating in total 51 patients with either MART-1-TCR td or gp100-TCR td T-cells[10,12]. While no evidence of mixed TCR dimer induced autoimmunity was observed in earlier murine experiments and these first clinical trials, in a recent set of experiments an often lethal autoimmune pathology was observed under conditions that promote the expansion of adoptively transferred T-cells more strongly, and this pathology appeared dependent on the action of mixed TCR dimers[22].

There are different techniques described that facilitate matched pairing of the introduced TCR chains. Exchange of the human constant regions for murine constant regions was described to improve TCR expression and functionality[14,15]. However, murine constant regions can be potentially immunogenic in vivo. Another strategy that facilitated matched pairing and increased TCR surface expression is the introduction of an extra disulfide bond in the constant domains of the introduced TCR chains[16,17]. In this study, we demonstrate that cysteine modification of the potentially clinical useful HA1.M7-TCR considerably reduced the neoreactivity of two TCR td virus-specific T-cell populations tested. Potentially, the stochiometric production of TCRα and β chains, when linked with a self-cleaving 2A peptide[23], could also result in increased preferential pairing of the TCR chains and lower expression of mixed TCR dimers. However, CVO pp50 T-cells transduced with HA1.M7-TCR chains linked with a T2A sequence still demonstrated marked neoreactivity (Figure 5B), indicating that stochastic expression of the TCRα and β chain does not rule out the generation of mixed TCR dimers. Next to decreased neoreactivity using cysteine modified TCRs, increased HA-1-specificity was observed, making the cysteine modified HA-1.M7-TCR more attractive than the unmodified HA1.M7-TCR for future clinical trials. Whether the results obtained with the cysteine modified HA1.M7-TCR are predictive for other TCRs potentially useful for clinical therapy has yet to be tested.

To completely rule out formation of harmful mixed TCR-dimers, another option would be to transduce γδ-T-cells, as the γδ-TCR chains are not able to pair with αβ-TCR chains[19]. Human γδ-T-cells redirected with αβ-TCRs were fully functional in vitro[19] and in vivo[24]. 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. We therefore propose to limit the diversity of the TCR repertoire of the recipient T-cells by transducing virus-specific T-cell populations. Because virus-specific T-cell populations consist of a restricted TCR repertoire[25,26], the amount of different mixed TCR dimers formed will be limited. In addition, the reactivity of these T-cells is known, allowing detection of harmful neoreactivities by introducing into these virus-specific T-cells as controls only the TCRα or TCRβ chain of interest and subsequent testing against different patient-derived cell types. By this procedure TCR td virus-specific T-cells can be selected that show no off-target toxicity.

In conclusion, in this study we demonstrated that TCR transfer results in neoreactive mixed TCR dimer formation. This formation of neoreactive mixed TCR dimers is not a feature of a specific TCR, because we observed this in all virus-specific T-cells tested, with different introduced TCRα or β chains. We therefore underline the importance of facilitating matched pairing of introduced TCR chains, and diminishing the chance of formation of harmful neoreactive mixed TCR dimers by using T-cell populations with restricted TCR repertoire as host cells for TCR transfer.

MATERIALS AND METHODS

Retroviral vector construction and production of retroviral supernatant

TCRAV and TCRBV gene usage of the different Ag-specific T-cell clones was determined as previously described[7]. All TCR AV and BV chains derived from different high affinity mHAg- (HA-2 and HA-1) and virus-specific T-cell clones (CMV) were cloned separately into the Moloney murine leukemia virus-based LZRS retroviral vector and are described in detail in the supporting information. In addition, cysteine modified HA-1.M7-TCR chains were constructed as previously described by introducing cysteine residues at positions 48 of the TCRα and position 57 of the TCRβ constant domains[16,17]. TCR-AV chains were always combined via the IRES sequence with the marker eGFP, and the TCR-BV chains with the truncated nerve growth factor receptor (ΔNGF-R). The retroviral vectors used in Figure 5B contained either the unmodified or cysteine modified HA1.M7 TCRα and β chains linked with picornavirus-derived self-cleaving 2A sequence (T2A)[23] and were combined via the IRES sequence with the marker ΔNGF-R. Retroviral supernatant was generated using φ-NX-A as previously described[27].

HLA Class I tetrameric complexes, flow cytometric analyses and cell sorting

PE-or APC-conjugated tetrameric complexes were constructed as previously described[28] with minor modifications. The following tetrameric complexes were constructed: tetrameric HLA-A1

complexes in combination with CMV-pp50 VTE (pp50) or CMV-pp65 YSE (pp65) peptide, and tetrameric HLA-B8 complexes in combination with CMV-IE-1 ELR (IE-1) or EBV-BZLF-1 RAK (BZLF-1). For flow cytometric analyses as well as flow cytometry-based sorting, cells were labeled with tetramers for 1 hour at 4°C and during the last 30 mins, mAbs directed against the various cell surface molecules were added. Sorting was performed at 4°C. mAbs used are described in the supporting information.

Cells

All studies were conducted with approval of the institutional review board at Leiden University Medical Center. After informed consent, virus-specific T-cells were isolated from different healthy individuals (UKL, MBX, CVO, UGW) using different virus-specific tetramers (>95% purity). Tetramer positive T-cells were restimulated every two weeks as described previously[13] and expanded. Retroviral transduction was performed as described previously[8] using recombinant human fibronectin fragments CH-296[27]. TCR transduced (td) virus-specific T-cells were sorted based on eGFP and ΔNGF-R positivity (>99% purity), and the cells were expanded in bulk. To analyze the reactivity of TCR td T-cells, a panel of HLA typed EBV-transformed lymphoblastoid cell lines (LCLs) was used (Table S2). LCLs were maintained in Iscoves modified dulbecco's medium (IMDM) supplemented with 10% FBS.

Analysis of Ag-specific IFN-γ production

TCR td virus-specific T-cells were tested for IFN-γ production against the HLA typed LCL panel. To determine IFN-γ production, 5.000 T-cells were cocultured with 20.000 LCLs, and after overnight incubation supernatant was harvested and tested in a standard ELISA (CLB, Amsterdam, The Netherlands). As positive control for the activity of the endogenous and introduced TCRs, the T-cells were stimulated with LCLs pulsed for one hour at 37°C with the different viral and mHag peptides at a final concentration of 1 μg/ml. To determine the HLA restriction molecules essential for recognition of the mixed TCR dimers, blocking studies were performed and antibodies used are described in the supporting information.

TCR td virus-specific T-cells were tested for IFN-γ production against normal human cell subsets and for this purpose CD4+, CD19+ and CD14+ cell subsets were MACS-isolated from peripheral blood mononuclear cells (PBMC) as described in the supporting information. TCR td virus-specific T-cells were tested against these different purified (>90%) CD4+, CD19+ and CD14+ cell subsets directly ex vivo, and after in vitro activation of these cell subsets, as described in the supporting information.

Chromium release assay and CFSE based cytotoxicity assay

To test the capacity of T-cells to specifically lyse Ag positive target cells, a standard 4 h chromium release assay using different effector-to-target ratios was performed as previously described[8]. Furthermore, to be able to analyse cytotoxicity after several days, we used a CFSE based cytotoxicity assay[29] as described in the supporting information.

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Supporting information

MATERIALS AND METHODS

Construction of retroviral vectors and production of retroviral supernatant

The TCR AV and BV chains used in this study are: AV15S1; BV18S1 (HA2.5-TCR), AV23S1; BV18S1 (HA2.6-TCR), AV30S1; BV18S1 (HA2.19-TCR), and AV23S1; BV6S2A1 (HA2.20-TCR) derived from 4 different T cell clones recognizing the HA2 YIGVEVLVSV peptide in the context of HLA-A2[1], AV8S1; BV6S4 (HA1.M2-TCR), AV32S1; BV6S4 (HA1.M7-TCR) derived from 2 different T cell clones recognizing the HA1 VLHDDLLEA peptide in the context of HLA-A2 and AV18S1; BV13S1 (CMV-TCR)[2] derived from a T cell clone specific for the CMV-pp65 derived NLVPMVATV peptide presented in the context of HLA-A2.

mAbs used in this study

To obtain more oligoclonal or monoclonal cell subsets, cells were labeled with tetramers and with either anti-TCR-BV1 or anti-TCR-BV14 PE (Immunotech, Marseille, France). To obtain the transduced cells, cells were labeled with anti ΔNGF-R either PE- (PharMingen, San Diego, CA, USA) or APC-conjugated (Cedarlane Laboratories, Hornby, Ontario, Canada) for 30 minutes at 4°C and were subsequently sorted.

To determine the HLA restriction molecules essential for recognition of the mixed TCR dimers, blocking studies were performed using W6.32 (anti-HLA class I), B1.23.2 (anti-HLA-B/C), PdV5.2 (anti-HLA class II), B8.11.2 (anti-HLA-DR), SPV-L3 (anti-HLA-DQ) or B7.21 (anti-HLA-DP) mAbs (kindly provided by A. Mulder from the LUMC). LCLs were preincubated with saturating concentrations of mAbs for 1 hour at RT before addition of T cells.

mAbs used in the CFSE based cytotoxicity assay are anti-CD19 and anti-HLA-DR or anti-CD4 (Beckman Coulter, Fullerton, CA, USA) and anti-HLA-DR mAbs (PharMingen, San Diego, CA, USA).

MACS-enrichement and activation of isolated cell subsets

PBMC of healthy donors were thawed, incubated with DNAse for 15 minutes at 37 °C, washed and stained with either anti-CD4, anti-CD19 or anti-CD14 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and isolated according to manufacturer's instructions. The $CD4^+$ cell subset was activated for a week (T act) using PHA (800 ng/ml), the $CD19^+$ fraction was activated

ID	specificity	restriction element	excluded EBV-LCLs due to alloreactivity	HA2.5 TCR	HA2.6 TCR	HA2.19 TCR	HA2.20 TCR	HA1.M2 TCR	HA1.M7 TCR	CMV TCR
UKL	pp50	A1	FAQ, FRQ, GGT, JLX, RSH, LSR	-‡	+#	+	+	+	-	-
UKL	BZLF1	B8	MHV	+	+	+	+	+	+	+
UGW	pp65	A1	AAS, ALY, MWX	-	+	-	-	-	+	-
MBX	IE1	B8	AHT, GGT, JLX, LAJ, LSR	-	+	+	-	+	+	-
CVO	pp50	A1	-	-	-	-	-	+	+	+

‡ - indicates that no neoreactivity of the TCR td virus-specific T cell lines against any of the LCLs present in the panel was observed.
# + indicates that neoreactivity of the TCR td virus-specific T cell lines against one of the LCLs present in the panel was observed.

Table S1. Used virus-specific T-cell lines and allo- and neoreactivity exerted against LCL panel.

for 3 days (B act) by culturing them on CD40L transduced murine fibroblasts[3] in medium containing CpG (10 µg/ml) and IL-4 (500 IU/ml) (Schering-Plough, Innishammon, Cork, Ireland). The $CD14^+$ fraction was activated into immature DCs (iDC) by culturing in medium containing GM-CSF (100 ng/ml) (Novartis, Basel, Switzerland) and IL-4 (500 IU/ml). After 3 days of culturing, immature DCs were activated for 3 days into mature DCs[4] (mDC) by culturing them in medium containing GM-CSF (100 ng/ml), TNF-α (10 ng/ml), IL-1ß (10 ng/ml), IL-6 (10 ng/ml) (Cellgenix, Freiburg, Germany), PGE-2 (1 µg/ml) (Sigma-Aldrich, St Louis, MO, USA) and IFN-γ (500 IU/ml) (Immukine, Boehringer Ingelheim, Alkmaar, The Netherlands).

Figure S1: ***(A)*** *To gain more insight into the frequency of neoreactive mixed TCR dimers and to analyze whether mixed TCR dimers consisting of conserved TCRs with the same specificity could acquire new harmful reactivity, we deliberately created mixed TCR dimers by recombining HA-2-specific TCRα and TCRβ chains of 4 different HA-2-TCRs, namely the HA2.5-TCR, the HA2.6-TCR, the HA2.19-TCR and the HA2.20-TCR. For this purpose, we sorted pp50-specific T cells derived from healthy individual CVO using tetramers and BV1-staining, confirmed monoclonality, and transduced all possible combinations into these T cells. Taking into account that also the introduced HA-2-TCR chains can pair with the endogenous TCR of the pp50 T cells, this resulted in potentially 20 mixed TCR dimers. BV1+ CVO pp50 T cells consisting of these deliberately created mixed TCR dimers were also tested against the LCL panel for neoreactivity; here, reactivity of CVO pp50 T cells transduced with the HA2.19-TCRα and HA2.6-TCRβ chains is depicted (A; mixed TCR-dimer). As a control, the parental HA2.19-TCR (TCR HA2.19) and the parental HA2.6-TCR (TCR HA2.6) combinations were included in the experiment. In addition, as a control for the reactivity of the introduced TCR, IZA was pulsed with HA-2 peptide (A; intro-TCR). The experiments were performed in duplicate. IFN-γ production depicted is a representative experiment out of 2 experiments.* ***(B)*** *To elucidate HLA restriction of the neoreactive mixed TCR dimer, blocking experiments were performed. Neoreactivity directed against DMD could be blocked by HLA class II and HLA-DQ blocking antibodies. DMD expressed HLA-DQ5, and DQ9, as indicated in bold. Additional experiments using various LCLs expressing one of these HLA restriction molecules demonstrated that this neoreactivity was DQ3(8/9) mediated. Blocking experiments were performed in triplicate. IFN-γ production depicted is a representative experiment out of 3 separate experiments.*

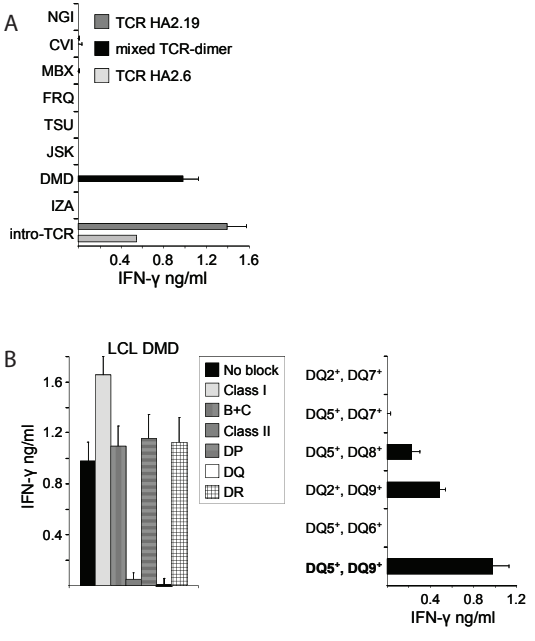

Figure S1. Each recombination of TCR chains after TCR transfer can potentially result in a HLA-restricted harmful new reactivity.

Figure S2: ***(A)*** *HA2.6-TCRα, HA2.6-TCRβ and HA2.6-TCRαβ td MBX IE-1 T cells were tested for IFN-γ production against HLA-DR17+ LCLs IZA, NGI and MBX and against HLA-DR17- EBM.* ***(B)*** *In a 4h cytotoxicity assay, HA2.6-TCRα (white symbols), HA2.6-TCRβ (black symbols) and HA2.6-TCRαβ td MBX IE-1 T cells (grey symbols) were tested against HLA-DR17+ LCLs IZA (diamonds), MBX (triangles) and NGI (squares) in several effector-to-target ratios in triplo. As a negative control, HA2.6-TCRα, HA2.6-TCRβ and HA2.6-TCRαβ td T cells were tested against HLA-DR17- EBM (white, black and grey circles, respectively). Cytotoxicity depicted representative for 2 separate experiments.* ***(C)*** *To confirm that neoreactivity against MBX and NGI cells was also HLA-DR restricted, blocking experiments were performed. Neoreactivity directed against MBX and NGI cells could be blocked using class II and HLA-DR blocking antibodies, indicating that neoreactivity directed against these LCLs was also HLA-DR17 restricted. The experiments were performed in triplo and IFN-γ depicted is representative for 3 separate experiments.*

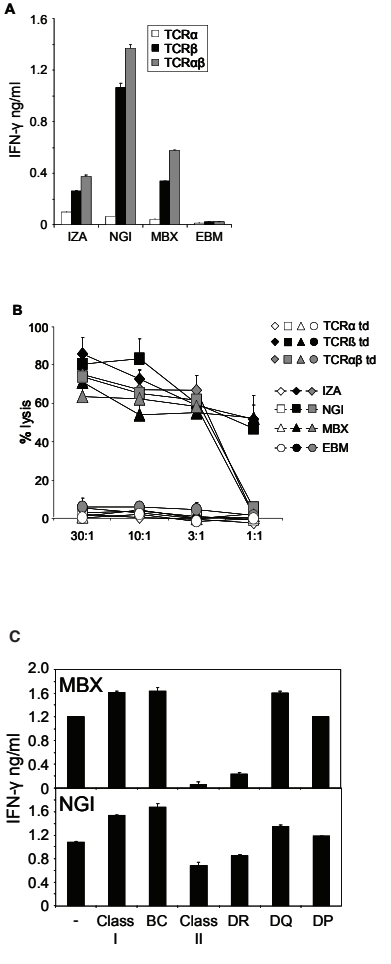

Figure S2. Cytokine production and cytotoxic activity of HLA-DR restricted autoreactive mixed TCR dimer.

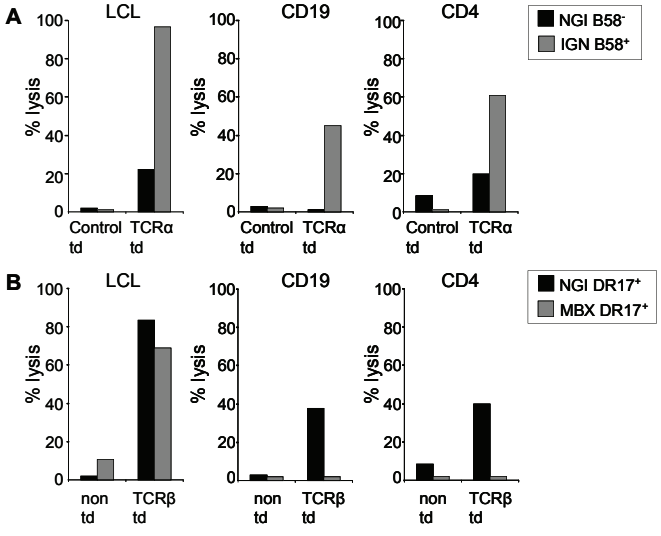

Figure S3. Mixed TCR dimers are able to exhibit cytolytic activity against normal human cell subsets.

Figure S3: To analyze whether TCR td T cells were able to lyse the target cell subsets directly ex vivo, ***(A)*** *HLA-B58 restricted neoreactive CMV-TCRα td CVO pp50 T cells and*

(B) *HLA-DR17 restricted neoreactive HA2.6-TCRβ td MBX IE-1 T cells were tested using a CFSE cytotoxicity assay. T cells were labeled with 5 µM of CFSE (Molecular Probes Europe, Leiden, the Netherlands), and coincubated with either HLA-B58- (NGI) or HLA-B58+ (IGN) or HLA-DR17+ (NGI, MBX) PBMCs or LCLs. Control cultures with T cells only or target cells only were included. Cultures were stained after 24h of coincubation with a combination of either anti-CD19 and anti-HLA-DR or anti-CD4 and anti-HLA-DR mAbs, and the different samples were analyzed using flow cytometry. Propidium iodide (PI) (1 µg/ml) was added to exclude dead cells. Percentage of lysis per cell subset (CD19+ or CD4+) was calculated as follows: [(cell counts of PIneg cell subset with effector cells) / (cell counts of PIneg cell subset without effector cells)] * 100%. Percentage of lysis of LCLs, CD19+ and CD4+ cells is depicted. The experiment was performed in duplicate, and a representative experiment out of 2 is depicted.*

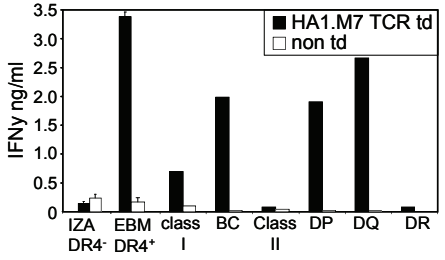

Figure S4. Neoreactivity of HA1.M7-TCR td UKL BZLF1 T cells directed against LCL EBM is HLA-DR restricted.

Figure S4: HA1.M7-TCR td but not non td UKL BZLF1 T cells demonstrated reactivity directed against EBM. This reactivity could be blocked using class II and HLA-DR blocking antibodies. Because EBM is homozygous HLA-DR4 positive, this indicated that this neoreactivity was HLA-DR4 restricted. HLA-DR4 negative IZA was not recognized. The experiment was performed in duplicate, and representative IFN-γ production for 2 independent experiments is depicted.

Optimization of the HA-1-specific T-cell receptor for gene therapy of hematological malignancies

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ABSTRACT

Background: To increase the efficacy of adoptive immunotherapy with TCR-modified T-cells, high expression of the introduced TCR is necessary. TCRs directed against the minor histocompatibility antigen (MiHA) HA-1 are good candidates for TCR gene transfer to treat hematological malignancies because of the hematopoiesis-restricted expression of HA-1. Previously it has been demonstrated, however, that gene transferred HA-1-TCRs are poorly expressed at the cell surface. In this study several strategies were explored to improve expression of transferred HA-1-TCRs.

Design and Methods: To investigate the underlying problem of low HA-1-TCR cell surface expression, TCR-deficient jurkat cells were used to analyze pairing properties of the HA-1-TCR chains and HA-1-TCR mRNA and cell surface expression levels were determined in parental HA-1-specific T-cell clones. To improve HA-1-TCR expression, HA-1-TCR chains were modified using sequence specific modifications, codon optimization or inclusion of cysteine residues and analyzed.

Results: Low HA-1-TCR expression was already apparent in parental HA-1-specific T-cells, and was demonstrated not to be due to impaired pairing properties of the specific HA-1-TCRα and ß chains but due to intrinsic properties of the HA-1-TCRß chain. Of different strategies explored, the most marked improvement in HA-1-TCR expression and functionality was observed after TCR transfer of a codon optimized and cysteine modified HA-1-TCR.

Conclusions: T-cells transduced with a codon optimized and cysteine modified HA-1-TCR efficiently recognized target cells that endogenously process and present HA-1, independent of whether the recipient T-cells were strong or weak competitor T-cells. Based on these results, these modified HA-1-TCRs will be used for an HA-1-TCR gene therapy trial in patients with leukemia.

INTRODUCTION

Patients with hematological malignancies can be successfully treated with allogeneic stem cell transplantation (allo-SCT). After allo-SCT relapse of the hematological malignancy can occur that can be successfully treated with donor lymphocyte infusion (DLI) from the original stem cell donor inducing complete remissions[1,2]. It has been demonstrated that T-cells recognizing minor histocompatibility antigens (MiHAs) selectively expressed on hematopoietic cells mediate anti-leukemic reactivity after allo-SCT without causing graft versus host disease (GvHD)[3,4]. MiHAs are derived from genetically polymorphic proteins that can be differentially expressed between donor and recipient[5]. The MiHA HA-1 is exlusively expressed on hematopoietic[6] and carcinoma cells[7], making it an attractive target antigen to treat hematological malignancies relapsing after allo-SCT when the patient is HA-1+ and the donor is HA-1-. The emergence of CD8+ T-cells recognizing the hematopoiesis-restricted MiHA HA-1 was observed to be associated with anti-leukemic responses in combination with no or only mild GvHD[4]. HA-1 is presented in the context of HLA-A*0201[8] and has a favorable population frequency[9], thus the chance that donor and patient are disparate for HA-1 expression is relatively high. Therefore, adoptive transfer of donor T-cells directed against HA-1 is an attractive strategy to induce anti-leukemic responses without GvHD. However, large numbers of T-cells with defined specificity are difficult to attain. To obtain large numbers of leukemia-reactive T-cells without long culture periods MiHA-specific T-cell receptors (TCRs) can be retrovirally transferred. From patients with anti-leukemic responses without GvHD high-affinity HA-1- and HA-2-specific T-cells have been isolated[10] and their TCRs have been characterized. Functional T-cells with redirected anti-leukemic reactivity have been generated by HA-1-TCR or HA-2-TCR gene transfer to donor lymphocytes[11,12].

To broaden the applicability of adoptive T-cell therapy in hematological malignancies, we aim to start a clinical study using HA-1-TCR transferred virus-specific T-cells. For optimal anti-leukemic reactivity, high cell surface expression of the introduced TCR and persistence of the gene modified T-cells are important. However, HA-1-TCR modified T-cells expressed the HA-1-TCR at low levels at the cell surface[12], requiring optimization of the strategy. A strategy to promote expression of the introduced TCR could be the selection of host T-cells with weak competitor phenotype. Recently, we have described that weak and strong competitor phenotype of virus-specific T-cells is, to some extent, correlated with specificity[13]. Based on the specificity of the virus-specific T-cells, selectively the weak competitor phenotype virus-specific T-cells may be isolated and used for TCR gene transfer. However, this selection would also reduce the pool of host T-cells useful for TCR gene transfer. Therefore, we set out to optimize the HA-1-TCR to the extent that also TCR modified strong competitor phenotype virus-specific T-cells expressed the introduced HA-1-TCR on their cell surface. In previous studies we observed low HA-1-TCR expression due to low HA-1-TCRß cell surface expression[12]. Since there is exclusive TCRBV chain usage of HA-1-specific T-cells[14,15], possibly because parts of the variable region of the ß chain are crucial for HA-1-specificity, this ruled out

the possibility to select for other HA-1-TCRs for use in clinical studies, and led to the hypothesis that sequence specific properties resulted in low HA-1-TCRß cell surface expression. There are several possible explanations for sequence specific low HA-1-TCRß cell surface. Potentially, the HA-1-TCRβ chain is not able to be efficiently expressed on the cell surface in combination with the HA-1-TCRα chain. In addition, low mRNA levels due to low promotor activity, instable mRNA, or instability of the protein could be the underlying problem of low HA-1-TCRß cell surface expression.

In this study, we demonstrate using TCR-deficient J76 cells that the HA-1-TCRβ chain is not able to be efficiently expressed on the cell surface with any TCRα chain. In addition, on the parental HA-1-specific T-cell clones the HA-1-TCR complex is also relatively lowly expressed despite normal TCRα and ß chain mRNA levels. Sequence specific modification to improve HA-1-TCRß expression by exchange of the CDR1 region did not result in improved HA-1-TCRß expression, but completely abolished HA-1-specific reactivity. Modification of the HA-1-TCR using a combination of codon optimization described to enhance translation of the introduced TCR chains[16] and inclusion of cysteine residues described to facilitate matched pairing of the introduced TCR chains[17-19] led to improved TCR cell surface expression. Moreover, using this modified HA-1-TCR for TCR transfer, even virus-specific T-cells exhibiting a strong competitor phenotype expressed the introduced HA-1-TCRs efficiently and transduced T-cells exerted robust HA-1-specific functionality.

DESIGN AND METHODS

Construction of HA-1-TCR encoding retroviral vectors

TCRAV and TCRBV gene usage of the HA-1-specific, the HA-2-specific and the CMV-specific T-cell clones was determined as previously described[20]. TCRα and TCRβ chains were cloned separately into the retroviral vector LZRS. The HA-1-TCRβ chain was also cloned into the retroviral vector MP71. The TCRα chains were always linked via IRES with the marker eGFP[21], and the TCRβ chains were always linked via IRES with the truncated nerve growth factor receptor (NGF-R)[22], except for the HA-1-TCR chains linked with a T2A sequence[23] which were either expressed in the pLZRS vector combined with the NGF-R marker-gene or in the MP71 vector without markergenes. The HA-1-TCRβ chain comprising of the HA-2.20-TCR CDR1 region (construct A), and the HA-2.20-TCRβ chain comprising of either only the HA-1-TCRβ CDR1 region (construct B) or both the HA-1-TCRβ CDR1 and CDR3 region (construct C) were obtained by two-step polymerase chain reactions (PCR). For construct C, the plasmid cDNA of construct B was used as template. Constructs were inserted in the pLZRS vector using restriction sites EcoR1 and XhoI. Primers used are depicted in Table 1. Codon-modified TCR genes were designed and produced by GENEART (Regensburg, Germany). In addition, cysteine modified HA-1-TCR chains were constructed as previously described by introducing cysteine residues at positions 48 and 57 of the TCRα and TCRß constant domains, respectively[17-19]. HA-1-TCR chains with incorporated cysteine residues linked with a self-cleaving 2A sequence of porcine

teschovirus (T2A) sequence with or without codon optimization were produced by GENEART. Using the retroviral vectors LZRS or MP71[24,25] and packaging cells φ-NX-A[26] viral supernatant was generated as previously described[13,27].

Tetrameric HLA class I-peptide complexes, flow cytometric analyses

PE- or APC-conjugated tetrameric complexes were constructed as described with minor modifications[28]. Tetrameric HLA-A2 molecules in complex with HA-1 peptide VLHDDLLEA (HA-1 tetramer), HLA-A1 molecules in complex with CMV-pp50 peptide VTEHDTLLY (pp50 VTE), HLA-B7 molecules in complex with CMV-pp65 TPRVTGGAM (pp65 TPR) or CMV-pp65 RPHERNGFTVL (pp65 RPH) and tetrameric HLA-B8 molecules in complex with EBV-EBNA3A FLRGRAYGL (EBNA3A FLR) were constructed. For flow cytometric analyses or cell sorting experiments, cells were labeled with tetramers for 1 h at 4°C. During the last 30 min mAbs directed against CD4 FITC-conjugated (Beckton Dickinson [BD], San Diego, CA, USA), CD40 FITC-conjugated (Bio-connect, Huissen, The Netherlands) or NGF-R PE-conjugated [BD] or APC-conjugated (Cedarlane Laboratories, Hornby, Ontario, Canada) were added. Cell surface staining with anti-TCRαβ PE-Cy5-conjugated (Beckman Coulter, Mijdrecht, The Netherlands) or anti-CD3 APC-conjugated (BD Pharmingen, San Diego, CA, USA) was performed for 30 minutes at 4°C.

qRT-PCR

A qRT-PCR was performed to measure the mRNA level of TCRα chain and TCRß chain. Total RNA was isolated from 0.5 to 1x10^6 T-cells, including 5 different HA-1-T-cell clones, 3 different CMV^{B7} T-cell clones, 6 different HA-2 T-cell clones, 2 different CMV^{A2} T-cell clones, 2 different PHA blasts and as a negative control Mesenchymal stem cells (MSCs), using the RNeasy mini kit (Qiagen). First strand cDNA synthesis was performed with oligo dT primers using M-MLV reverse transcriptase (Invitrogen). PCRs were started with a hotstart 10 min 95°C followed by 50 cyles of 30 sec 95°C, 30 sec 60°C and 30 sec 60°C. Samples were run on a 7900HT Fast Real-Time PCR System of Applied Biosystems. Primers used are depicted in Table 1. Probes used TET as a dye and TAMRA as a quencher and were chosen over an intron/exon boundary. Each sample was run in duplo with 1 and 10 ng cDNA from 2 µg of total RNA and normalized to the internal Porphobilinogen Deaminase (PBGD) gene. The normalized Ct value of PHA blasts was set at 1 and expression of other samples was shown referenced to that sample using the following formula [(Ca or CbTCR Ct sample – PBGD Ct sample)/ (Ca or CbTCR Ct pha – PBGD Ct pha)].

Isolation of T-cell clones, selection of virus-specific T-cells using flow cytometry-based cell sorting and retroviral transduction

All studies were conducted with approval of the institutional review board at Leiden University Medical Center. The following T-cell clones were used in this study; HLA-A2 restricted HA2.1, HA2.5, HA2.6, HA2.19, HA2.20 and HA2.27 T-cell clones specific for the MiHA HA-2 [4,10], HLA-A2 restricted T-cell clones HA-1.M2, HA-1.M7, HA-1.7, HA-1.12 and HA-1.83 specific for the MiHA HA-1[10], HLA-B7 restricted T-cell clones CMV^{B7}.90, CMV^{B7}.108 en

CMV^{B7}.113 specific for pp65 TPR[29], and HLA-A2 restricted CMV^{A2}.AV2 and CMV^{A2}.AV13 T-cell clones specific for pp65 NLV[30]. Virus-specific T-cells were isolated from PBMCs of CMV and EBV seropositive persons. After informed consent, PBMCs were harvested and labeled with the relevant tetramers for 1 h at 4°C in RPMI without phenol, supplemented with 2% FBS, washed 2 times, and sorted at 4°C using the FACS Vantage (BD) into weak competitor phenotype pp50 VTE or pp65 RPH specific T-cells and strong competitor phenotype EBNA3A FLR or pp65 TPR specific T-cells with >95% purity. Virus-specific T-cells were stimulated with $1x10^6$ cells/ml irradiated allogeneic PBMCs (30 Gy), 800 ng/ml PHA, and 100 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands). After 2 days of culture T-cells were transduced with retroviral supernatant using recombinant human fibronectin fragments CH-296[31] and this procedure has been described previously[32]. Markergene eGFP and NGF-R double positive T-cells were subsequently sorted. TCRαβ-/- Jurkat clone 76[11] (J76) needed no stimulation prior to transduction.

Cytokine secretion assay and cytotoxicity assay

To test HA-1-specific functionality, 5.000 purified TCR transduced or mock transduced T-cells were cocultured with 20.000 different target cells and after overnight incubation specific IFN-γ production was measured by standard ELISA[33]. In addition, 50.000 virus-specific T-cells were tested one week after transduction with the clinical vector against 20.000 target cells. In the cytotoxicity assay purified TCR transduced or mock transduced T-cells were cocultured with different target cells at an 10:1 effector-to-target ratio and cytotoxic reactivity was determined after 4h[13]. The tests were done in triplicate. Targets used were HLA-A2+ HA-1+ or HA-1- EBV-transformed lymphoblastoid cell lines (LCLs), and acute myeloid or lymphoblastoid leukemia (AML and ALL, respectively) primary cells.

RESULTS

Low HA-1-TCR cell surface expression due to intrinsic properties of HA-1-TCRβ chains

The HA-1-specific TCR would be a good candidate for TCR gene transfer to treat hematological malignancies after allo-SCT because of the hematopoiesis-restricted expression of this MiHA. Based on the low cell surface expression of HA-1-TCRs after gene transfer as described by us previously[12], we investigated whether this low expression was due to the inability of the TCR chains to pair efficiently with each other or due to intrinsic properties of the TCR chains. TCRαß-deficient jurkat J76 cells[11] were transduced (td) with individual HA-1-TCRα and HA-1-TCRβ chains in combination with different TCRα and TCRβ chains and TCR cell surface expression was measured using anti-TCRαβ mAbs. In Figure 1A, TCR cell surface expression is shown for HA-1-TCRαß, CMV^{B7}-TCRαß, HA-2-TCRαß, CMV^{A2}-TCRαß and mixed TCRα and ß chain combinations. HA-2-TCRαß td J76 cells (MFI 330) and CMV^{A2}-TCRαß td J76 cells (MFI 274) demonstrated high TCR expression. TCR expression of HA-1-TCRαß td J76 cells (MFI 129) was low compared to HA-2-TCRαß td J76 cells. Moreover,

no restored TCR cell surface expression was observed when J76 cells were transduced with combinations of the HA-1-TCRß with either the HA-2- or CMV^{A2}-TCRα (Figure 1A). In addition, no restored TCR expression could be observed in any of the transductions of the HA-1-TCRβ chain with one of the 14 other TCRα chains (data not shown). Also four other HA-1-TCRß chains derived from different HA-1-specific T-cell clones expressing a similar TCR BV6S4 variable domain (BV7S9 according to IMGT nomenclature) but different CDR3 regions exhibited a similar expression pattern (data not shown). In contrast, the HA-1-TCRα chain in combination with HA-2- or CMV^{A2}-TCRß chains resulted in comparable TCR cell surface expression as parental HA-2- and CMV^{A2}-TCR complexes, indicating that reduced HA-1-TCR cell surface expression was not due to the HA-1-TCRα chain but due to the HA-1-TCRß chain. Since the TCR cell surface expression of the HA-1-TCRß with all 14 other TCRα chains tested remained low we concluded that low HA-1-TCR cell surface expression was not due to inefficient pairing of specifically the HA-1-TCRα with the HA-1-TCRß chain. To exclude that the LZRS vector used to introduce the TCR chains caused selectively low expression of the HA-1-TCR, the HA-1-TCRβ gene was inserted into the MP71 vector which was described to mediate high transgene expression in T lymphocytes[34]. As can be seen in Figure 1A, HA-1-TCR cell surface expression was not improved using the MP71 vector encoding the HA-1-TCRβ chain, indicating that the low HA-1-TCR cell surface expression of td J76 cells was not due to vector specific properties. To investigate whether transfer of the HA-1-TCRβ chain resulted in low cell surface expression due to sequence specific properties of the always identical variable region of the HA-1-TCR BV6S4 chain, cell surface expression of the CMV^{B7}-TCRß with an identical variable BV6S4 region as the HA-1-TCRß chain but a completely different CDR3 region was analyzed. As shown in Figure 1A, the parental CMV^{B7}-TCR complex demonstrated comparably low cell surface expression as the parental HA-1-TCR complex. This low TCR expression was also not restored when the CMV^{B7}-TCRβ chain was combined with either the HA-2- or CMV^{A2}-TCRα chain, whereas CMV^{B7}-TCRα chains in combination with HA-2- or CMV^{A2}-TCRß chains resulted in high TCR cell surface expression that was comparable to the expression of the parental HA-2- or CMV^{A2}-TCRs. These results imply that low HA-1- and CMV^{B7}-TCRß chain expression was due to sequence specific properties of the variable region.

These data together indicate that low HA-1-TCR cell surface expression is due to intrinsic properties of the HA-1-TCRß chain.

Low HA-1-TCR cell surface expression already apparent in parental HA-1-specific T-cell clones

To confirm that the sub-optimal cell surface expression of the HA-1-TCR after gene transfer was due to intrinsic properties of the TCRβ chain, the HA-1-TCR cell surface expression and HA-1-TCRα and β chain mRNA levels of different parental HA-1-specific T-cell clones were determined. As demonstrated in Figure 1B, FACS analyses with antibodies directed against the TCRαβ and CD3 complex demonstrated that the HA-1-specific T-cell clones as well as the CMV^{B7}-specific T-cell clones expressed lower levels of

TCR-CD3 complexes at the cell surface compared to HA-2- and CMV^{A2}-specific T-cell clones. The HA-1-specific T-cell clones, however, stained with similar intensity with their respective tetramer compared to other T-cell clones (Figure 1C), and were on basis of cytokine production and cytotoxicity fully functional T-cells (data not shown). To exclude that the low TCRαß expression was due to lower transcriptional activity, TCRα and ß mRNA levels of the HA-1-specific T-cell clones were determined and compared to TCRα and ß mRNA levels of other T-cell clones. As demonstrated in Figure 1D, no significant differences in HA-1-TCRα or β mRNA expression levels compared to other T-cell clones could be detected. In addition, no differences between TCRα and β mRNA expression within individual HA-1-specific T-cell clones could be detected. In conclusion, the parental HA-1-specific T-cell clones demonstrate lower TCR cell surface expression despite normal TCRαß mRNA levels. These results indicate that the low HA-1-TCR expression observed in HA-1-TCR transferred T-cells is an intrinsique feature of the HA-1-TCR, since already TCR expression of the parental HA-1-specific T-cell clones is low.

CDR1 region of HA-1-TCRβ chain partly responsible for low cell surface expression, but indispensable for HA-1 specificity

To be able to improve HA-1-TCR expression after gene transfer, we investigated whether we could determine the specific region of the HA-1-TCRß responsible for this low TCR cell surface expression and improve HA-1-TCR expression by modification of this region. For this purpose, the sequences of several TCRß chains belonging to the BV6 variable domain family and

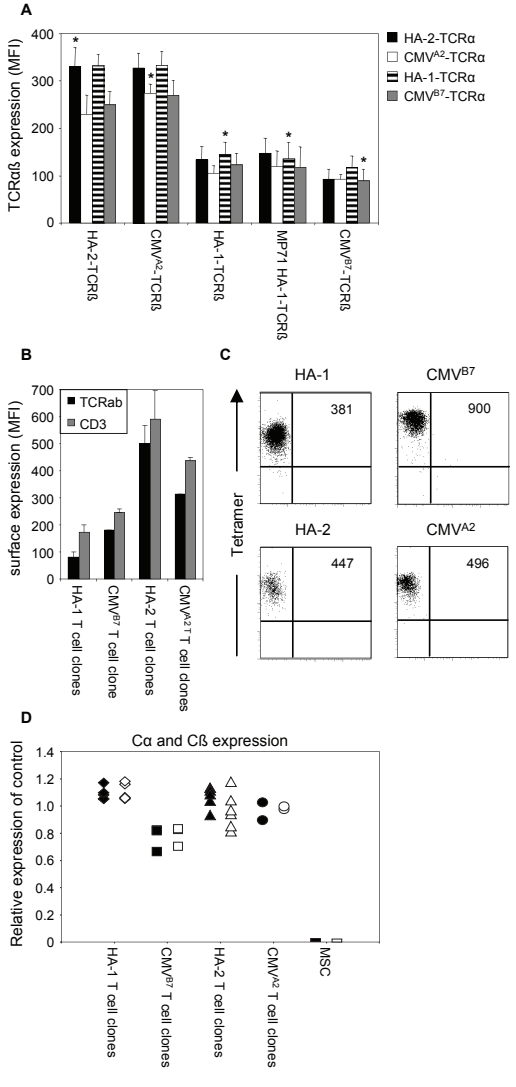

Figure 1. Low HA-1-TCR cell surface expression due to intrinsic properties of the HA-1-TCRß chain.

Figure 1: ***(A)*** *The pairing properties of HA-1-TCRα and ß chains were analyzed by transducing the J76 cells with combinations of the HA-1-TCRα or TCRβ chains with 14 other antigen-specific TCRα and TCRß chains. TCR cell surface expression of these different combinations was measured by staining with anti-TCRαß mAbs and analyzing eGFP/NGF-R double positive J76 cells using flow cytometry 5 days after transduction. Here depicted are the mean fluorescence intensity (MFI) of the TCRαß expression of all the TCRα chains of the HA-2-, HA-1-, CMV^{B7}, and CMV^{A2}-specific TCRs combined with all the TCRß chains of these TCRs. All TCR chains are encoded by pLZRS retroviral vectors with the exception of the HA-1-TCRß chain that is in addition also encoded, as indicated, by the MP71 retroviral vector. Non td J76 cells showed little background staining with anti-TCRαß mAbs (MFI = 16). Parental TCR combinations are indicated with an asterisk.* ***(B)*** *Several T-cell clones including 5 different HA-1-specific T-cell clones, 6 different HA-2-specific T-cell clones, and 2 different CMV-A2-specific T-cell clones were stained with anti-TCRαß and anti-CD3 mAbs and analyzed using flow cytometry. MFIs shown are means of the different T-cell clones.* ***(C)*** *The different T-cell clones were stained with their respective tetramers and MFI is depicted in the dot plots.* ***(D)*** *mRNA levels of TCRα (closed symbols) and TCRβ chains (open symbols) were analyzed for the different T-cell clones using q-RT-PCR. As a negative control, cDNA of MSCs was included. Stainings were performed in duplo, and data shown are representative for 2 independent experiments.*

*Figure 2: **(A)** Different TCR-BV6 chains that demonstrate high cell surface expression after TCR gene transfer and the HA-1-TCR BV6S4 chains that demonstrate low cell surface expression after TCR gene transfer were aligned and differences in nucleotide sequences were analyzed. 30 shared nucleotide differences were observed in the 309 aa long variable region between the highly expressed HA-2-TCR BV6S2, the JBBun-TCR BV6S3 and the 10G5-TCR BV6S7 and the marginally expressed HA-1-TCR BV6S4. Sequences shown are from amino acid 41 to 80 of the BV6 chains (total aa 309) containing the CDR1- and CDR2-region of the HA-1-TCRß and the HA-2-TCRß chain. The shared differences between all the other BV6 TCR chains and the HA-1-TCRß chain are indicated with arrows. **(B)** To test the role of the HA-1-TCRβ CDR1 region in low HA-1-TCR expression, J76 cells were transduced with combinations of the HA-1-TCRα or HA-2-TCRα with several constructs encoding for either the HA-1-TCRß chain unmodified or exchanged with the HA-2-TCRß CDR1 region, or the HA-2-TCRß chain unmodified or exchanged with the HA-1-TCRß CDR1 region only or exchanged with the HA-1-TCRß CDR1 and CDR3 region. Using flow cytometry TCR cell surface expression was analyzed for the eGFP/NGF-R double positive J76 cells. Non td J76 cells showed little background staining with anti-TCRαß mAbs (MFI = 16). Parental TCR combinations are indicated with an asterisk. Stainings were performed in duplo, and data shown are representative for 2 independent experiments **(C)** To test the role of the HA-1-TCRβ CDR1 region in HA-1-specificity, virus-specific T-cells were transduced with several constructs encoding unmodified HA-1-TCRα chains combined with either unmodified HA-1-TCRβ chains or exchanged with the HA-2-TCRß CDR1 region or the HA-2-TCRß chain exchanged with the HA-1-TCRß CDR1 region only or with the HA-1-TCRß CDR1 and CDR3 region. Cells were sorted based on eGFP and NGF-R double positivity and subsequently tested in a standard IFN-γ ELISA. Targets used were: HLA-A2^{pos} and HA-1neg LCL-IZA, HLA-A2^{pos} and HA-1^{pos} LCL-BDV and LCL-IZA pulsed with different HA-1-peptide concentrations, as indicated in the figure.*

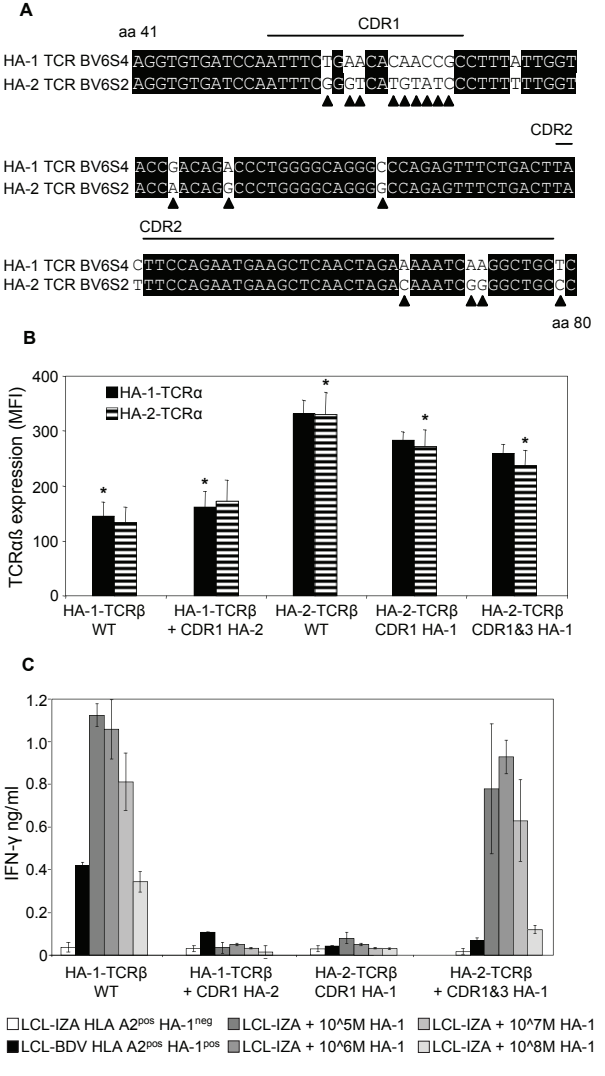

Figure 2. CDR1 region not responsible for low HA-1-TCR expression, but indispensable for HA-1-specificity.

known to exhibit high cell surface expression after gene transfer, namely the HA-2-TCR BV6S2, the JBBun-TCR BV6S3, and the 10G5-TCR BV6S7, were aligned with the sequences of the HA-1 and CMV^{B7}-TCR BV6S4. In total, 30 shared differences were scattered throughout the 309 amino acids (aa) long variable region, of which 9 nucleotide differences clustered in the 18 nucleotide-long CDR1 region, as depicted in Figure 2A. Based on these results, we hypothesized that primarily the CDR1 region of HA-1-TCR BV6S4 may be influencing cell surface expression of the HA-1-TCRβ chain. To study this, different constructs were made in which the HA-1-TCRβ CDR1 region was exchanged with the HA-2-TCRβ CDR1 region and vice versa. J76 cells transduced with modified HA-1- and HA-2-TCRs were analyzed for TCR cell surface expression using an anti-TCRαβ-specific mAb. As demonstrated in Figure 2B, exchange of the HA-1-TCRβ CDR1 region with the CDR1 region of the HA-2-TCRβ did not result in marked improvement of TCR cell surface expression on J76 cells. Likewise, the exchange of the HA-2-TCRβ CDR1 region with the HA-1-TCRβ CDR1 region did not result in significantly decreased TCR cell surface expression on J76 cells. These results indicate that the CDR1 region is not solely responsible for the low TCR cell surface expression. In addition, we demonstrate by the transduction of

virus-specific T-cells with the different modified TCR chains that exchange of the CDR1 region of the HA-1-TCRß with the CDR1 region of the HA-2-TCRß resulted in a complete abolishment of HA-1-specific IFN-γ production (Figure 2C), illustrating that the HA-1-TCRß CDR1 region is crucial for HA-1-specificity. However, exchange of the HA-2-TCRß CDR1 region with the HA-1-TCRß CDR1 region demonstrated that exchange of only this region was not enough to transfer HA-1-specificity. Exchange of both the HA-2-TCRß CDR1 and CDR3 region with the regions of the HA-1-TCRß resulted in HA-1-specificity (Figure 2C). However, these td T-cells were still less efficient compared to the parental HA-1-TCR td T-cells, since only low recognition of endogenously processed HA-1 (LCL-BDV) was observed (Figure 2C).

In conclusion, the HA-1-TCRβ CDR1 region seems to play a modest role in low TCR cell surface expression, but is crucial for HA-1-specificity.

Introduction of cysteine residues combined with HA-1-TCR codon optimization leads to highly improved HA-1-TCR expression after gene transfer

Since HA-1-TCR expression could not be improved by modification of specific sequences of the HA-1-TCRß chain, other strategies described to improve TCR cell surface expression of gene transferred TCRs were explored. We studied whether TCR codon optimization or inclusion of cysteine residues in the constant domains of both the HA-1-TCRα and ß chain resulted in potent HA-1-specific T-cells after gene transfer. We analyzed the HA-1-TCR cell surface expression after transfer of the different constructs into virus-specific T-cells known to possess endogenous TCRs which weakly compete for cell surface expression (weak competitor; pp50 VTE specific T-cells, Figure 3) and virus-specific T-cells known to possess endogenous TCRs which strongly compete for cell surface expression (strong competitor; EBNA3A FLR specific T-cells, Figure 3). As demonstrated in Figure 3A, transfer of the unmodified HA-1-TCR complex into weak competitor T-cells resulted in 40% of HA-1 tetramer positive T-cells, whereas after transfer of the unmodified HA-1-TCR complex into strong competitor T-cells no clear HA-1-TCR expression could be measured using tetramers after transfer of the unmodified HA-1-TCR complex. The inclusion of cysteine residues in both HA-1-TCR chains improved HA-1-TCR expression especially in the strong competitor virus-specific T-cells. As expected, inclusion of cysteine residues in only one of the two HA-1-TCR chains significantly diminished HA-1-TCR expression. Codon optimization, in addition, improved HA-1-TCR expression both in weak and strong competitor virus-specific T-cells. The increased HA-1-TCR expression, however, appeared not to be due to improved HA-1-TCRβ chain expression, but due to improved HA-1-TCRα chain expression, since T-cells transferred with the codon optimized HA-1-TCRα chain in combination with the wild type HA-1-TCRβ chain showed a similar improvement in percentage of HA-1-tetramer positive T-cells compared to T-cells transferred with both codon optimized HA-1-TCRα and ß chain. In both the weak and strong competitor virus-specific T-cells a combination of codon optimized and cysteine modified HA-1-TCRα chain with

Figure 3: The different modification strategies were tested for their potential to optimize HA-1-TCR expression and functionality. ***(A)*** *Weak (pp50 VTE T-cells) and strong competitor phenotype (EBNA3A FLR T-cells) virus-specific T-cells were transduced with either unmodified HA-1-TCRα chains (AV_{32} WT), cysteine modified HA-1-TCRα chains (AV_{32} SS), codon optimized HA-1-TCRα chains (AV_{32} opt) or codon optimized and cysteine modified HA-1-TCRα chains (AV_{32} opt SS) in combination with either unmodified (BV6 WT), cysteine modified (BV6 SS), or codon optimized (BV6 opt) HA-1-TCRß chains. Dot plots are depicted of eGFP and NGF-R double positive virus-specific T-cells.* ***(B)*** *All these modified HA-1-TCR td weak competitor and strong competitor virus-specific T-cells were tested in a standard IFN-γ ELISA. Numbers in the figures correspond with the numbers indicated in the dot plots of A. Targets used were HLA-$A2^{pos}$ HA-1neg LCL-IZA, HLA-$A2^{pos}$ HA-1^{pos} LCL-MRJ and LCL-IZA pulsed with different concentrations of HA-1-peptide. The experiment was performed in duplo. Data shown are representative for 3 independent experiments using virus-specific T-cells of 2 different healthy donors.*

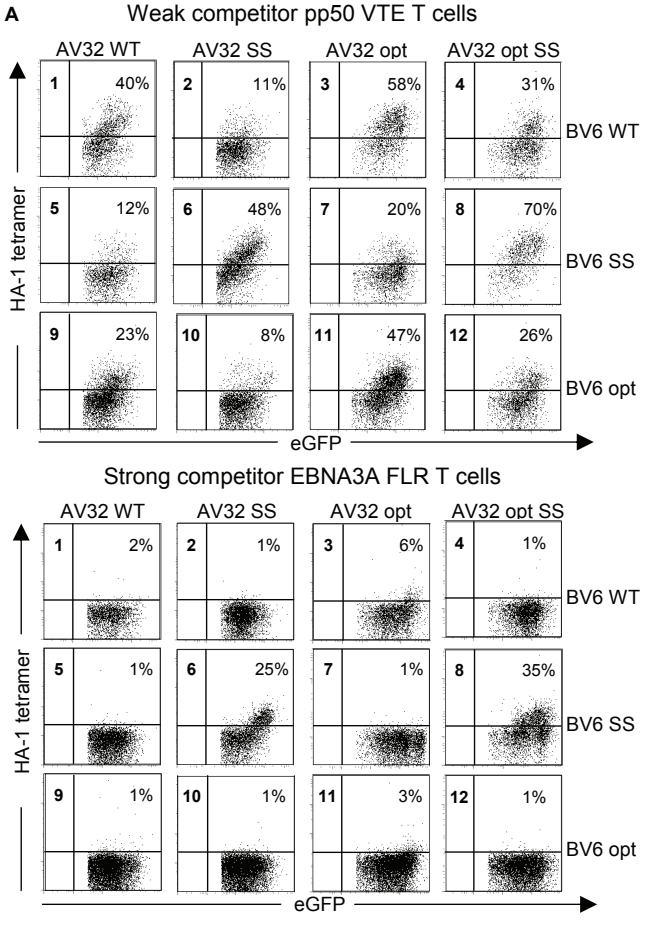

Figure 3A. Analysis of HA-1-TCR expression of virus-specific T-cells transduced with different modified HA-1-TCRs.

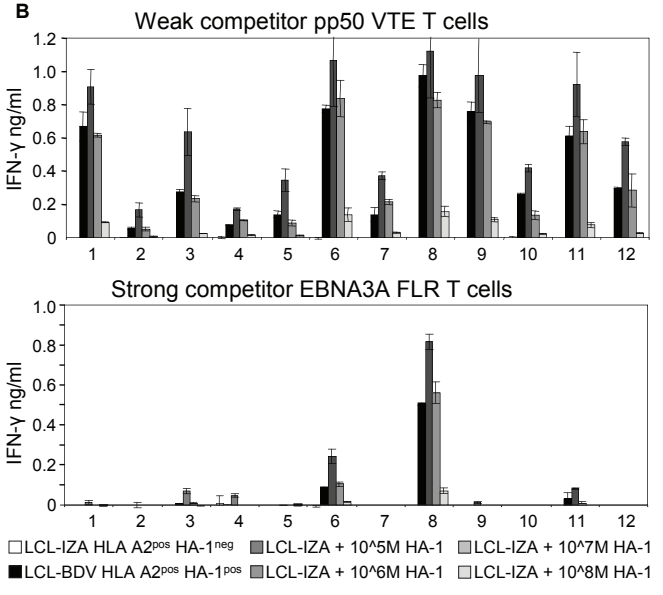

Figure 3B. Analysis of HA-1-specific functionality of virus-specific T-cells transduced with different modified HA-1-TCRs.

cysteine modified HA-1-TCRß chain improved HA-1-TCR expression most prominent (Figure 3A).

To test whether the improved HA-1-TCR expression resulted in improved HA-1-specific functionality, HA-1-TCR td weak and strong competitor virus-specific T-cells were tested against HA-1 peptide loaded target cells as well as target cells endogenously expressing the HA-1 antigen (Figure 3B). In weak competitor virus-specific T-cells, the combination of codon optimized and cysteine modified HA-1-TCRα chain with the cysteine modified

HA-1-TCRβ chain (combination #8) demonstrated highest IFN-γ production against peptide loaded target cells as well as against target cells presenting endogenously processed HA-1 antigen. Most evidently, in strong competitor T-cells, this TCR combination was the only one able to exert significant HA-1-specific reactivity.

In conclusion, the combination of cysteine modification of the HA-1-TCR chains with codon optimization of the HA-1-TCRα chain resulted in efficient HA-1-TCR expression after gene transfer, even in strong competitor T-cells, and resulted in robust HA-1-specific functionality.

T-cells transduced with codon optimized and cysteine modified HA-1-TCR recognize HA-1+ malignant cells

To confirm the generality of these data, polyclonal peripheral CD8+ T-cells, as well as other weak and strong competitor T-cells were transduced with single retroviral vectors encoding both the unmodified or codon optimized and cysteine modified HA-1-TCRα and ß chain linked with a picorna virus derived self-cleaving 2A sequence and tested for HA-1-TCR cell surface expression (Figure 4A). Also the HA-1-TCRß chain was codon optimized, although we did not observe improved cell surface expression of codon optimized HA-1-TCRß chains, to warrant that mRNA stability of the TCRß chain was not negatively influencing TCRα chain expression. Correspondingly, transduction with the modified HA-1-TCR resulted in most efficient cell-surface expression in both weak and strong competitor T-cells. The polyclonal CD8+ T-cells demonstrated similar to strong competitor T-cells

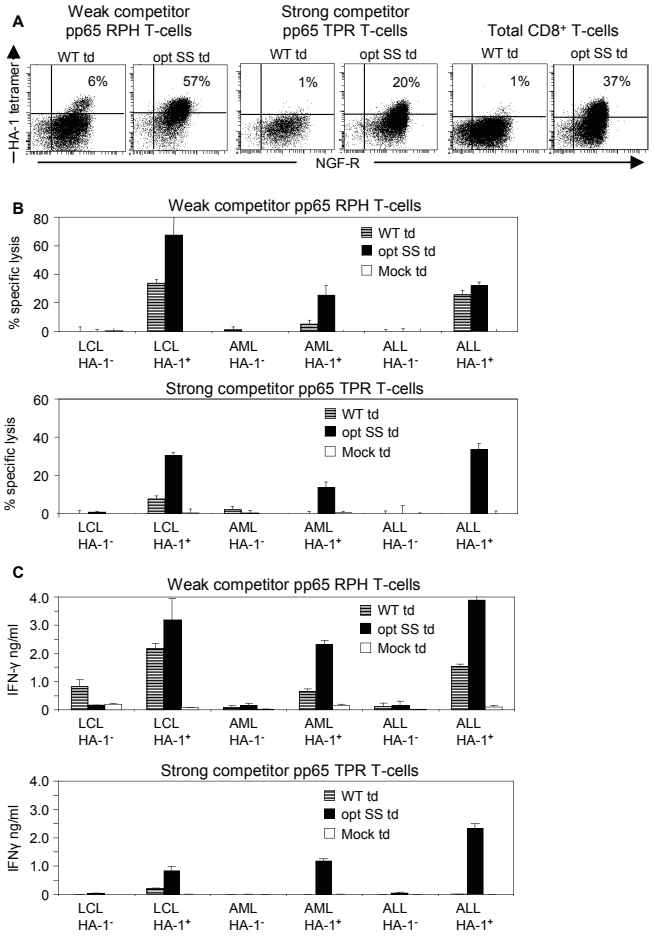

Figure 4. Introduction of codon optimized and cysteine modified HA-1-TCR generally results in efficient HA-1-TCR expression and robust HA-1-specific functionality.

*Figure 4: **(A)** Weak competitor phenotype (pp65 RPH) T-cells, strong competitor phenotype (pp65 TPR) T-cells and polyclonal peripheral CD8+ T-cells were transduced with either a single construct encoding the unmodified (WT td) or the codon optimized and cysteine modified HA-1-TCR chains (opt SS td) and HA-1 tetramer staining was analyzed. Dot plots depict HA-1 tetramer staining of NGF-R positive virus-specific T-cells and percentages of HA-1 tetramer positive T-cells are indicated. Dot plots depicted are representative for 2 independent experiments using T-cells of 3 different healthy individuals. (B/C) pp65 RPH and pp65 TPR T-cells transduced with a single construct encoding either HA-1-TCR WT or HA-1-TCR opt SS, or empty vectors were tested against different targets for HA-1-specific **(B)** cytotoxic reactivity in a chromium release assay and **(C)** IFN-γ production. Targets used were HLA-A2^{pos} LCLs, AML and ALL primary cells that were either positive or negative for HA-1. Data presented is representative for 2 independent experiments using T-cells of 3 different healthy individuals.*

Figure 5.: To improve the vector for clinical use, the modified HA-1-TCR chains linked with a T2A sequence were expressed in the MP71 vector without marker gene. pp50 VTE and EBNA3A FLR virus-specific T-cells were transduced with pLZRS vectors encoding unmodified HA-1-TCR chains linked with a T2A sequence and linked with an IRES sequence to a marker gene (WT TCR) or with MP71 vector without markergene encoding HA-1-TCR opt SS or empty vectors (mock td) and one week after transduction tested for HA-1-specific IFNγ production against different HLA-A2^{pos} AML and ALL primary cells that were either positive or negative for HA-1 as indicated in the figure. The experiment was performed in duplo and is representative for 2 independent experiments.

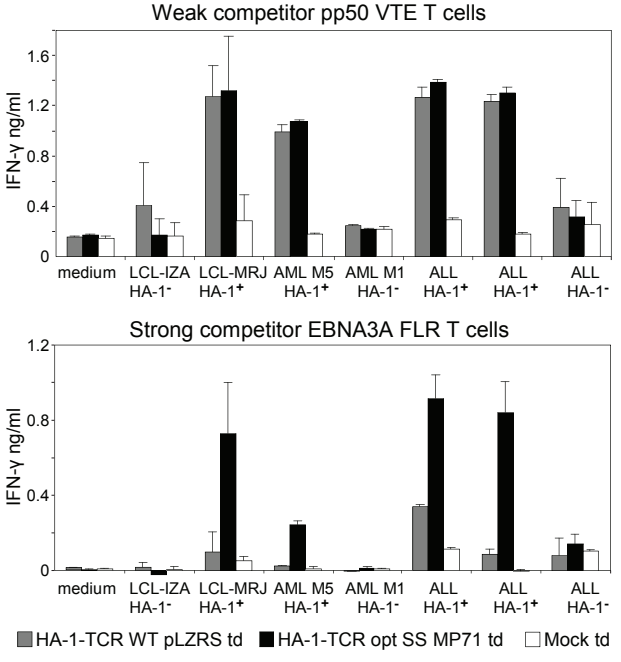

Figure 5. Strong competitor phenotype virus-specific T-cells transduced with MP71 HA-1-TCR opt SS demonstrate more robust HA-1-specific IFNγ production against AML and ALL malignant cells compared to HA-1-TCR WT transduced T-cells.

significant HA-1-TCR cell-surface expression after transfer of the modified HA-1-TCR (Figure 4A).

To study whether this improved HA-1-TCR cell-surface expression was coincided with clinically relevant HA-1-specific functionality, weak and strong competitor phenotype T-cells transduced with either the unmodified or codon optimized and cysteine modified HA-1-TCR were analyzed for HA-1-specific cytotoxic activity (Figure 4B) and IFN-γ production (Figure 4C). Whereas weak competitor T-cells transduced with the unmodified HA-1-TCR exerted HA-1 specific cytotoxic reactivity and IFN-γ production against AML and ALL, introduction of the modified TCR enhanced HA-1-specific reactivity (Figure 4B and C, respectively). In addition, strong competitor T-cells transduced with the modified HA-1-TCR were able to demonstrate significant cytotoxic activity and IFN-γ production directed against HA-1+ malignant cells (Figure 4B and 4C, respectively).

In conclusion, these results confirm the generality of improved HA-1-TCR expression of introduced modified HA-1-TCRs into both weak as well as strong competitor phenotype T-cells, thus generating potent redirected HA-1-specific T-cells.

For use in clinical therapy the introduced TCR has to be encoded by a retroviral construct without potentially immunogenic marker genes. Therefore, we constructed a MP71 vector without marker gene encoding the modified HA-1-TCRα and ß chain, and analyzed whether weak and strong competitor T-cells (Figure 5) transduced with this clinically useful vector demonstrated similarly improved anti-leukemic reactivity. One week after transduction weak and strong competitor T-cells were analyzed for HA-1-specific reactivity against malignant target cells using IFN-γ ELISA (Figure 5). Transduction efficiency of the pLZRS and MP71 vector were based on NGF-R or HA-1-tetramer staining, and was demonstrated to be 15 and 2%, respectively. Whereas malignant cells were equally well recognized by weak competitor T-cells transduced with either the unmodified or the modified HA-1-TCR

(Figure 5), strong competitor T-cells transduced with the modified HA-1-TCR demonstrated markedly improved IFNγ production against AML en ALL target cells as compared to unmodified HA-1-TCR transduced T-cells.

In conclusion, TCR transfer with a codon optimized and cysteine modified HA-1-TCR resulted in efficient expression of introduced HA-1-TCRs and robust HA-1-specific functionality against clinically relevant target cells, both in weak as well as in strong competitor virus-specific T-cells.

DISCUSSION

To broaden the applicability of adoptive T-cell therapy in hematological malignancies, we aim to start a clinical study using MiHA-TCR transferred virus-specific T-cells. The MiHA HA-1-specific TCR is an attractive candidate for TCR gene transfer to treat hematological malignancies based on the hematopoiesis-restricted expression of HA-1, and the favorable population frequency. However, as previously described, HA-1-TCR cell surface expression after gene transfer is low[12]. In this study, we attempted to improve cell surface expression of the HA-1-TCR after gene transfer. HA-1-TCR expression appeared to be mostly hampered due to intrinsic properties of the HA-1-TCRβ chain, also reflected in low TCR cell surface expression on the parental HA-1-specific T-cell clones. By using a combined strategy of codon optimization and introduction of cysteine residues, we demonstrated a substantial improvement of the HA-1-TCR cell surface expression, resulting in TCR modified T-cells exhibiting clinically relevant HA-1-specific functional activity.

Since the transferred TCR has to compete for cell surface expression with the endogenous TCR and mixed TCR dimers, gene transferred TCRs need to exhibit high affinity for their specific peptide-HLA complex. Although the HA-1-TCR is known to exhibit high affinity for the HA-1-peptide-HLA-A2 complex, we anticipate that for optimal efficacy of the HA-1-TCR transferred T-cells the cell surface expression of the HA-1-TCR has to be high, allowing the HA-1-TCR modified virus-specific T-cells to also recognize clinically relevant target cells expressing endogenously processed HA-1 antigen. Differential TCR cell surface expressions after gene transfer have been described previously[35,36]. Which properties of the TCR chains influences cell surface expression has been subject to debate[30], and it has been postulated that interchain pairing and competition for CD3-complex formation may both play a role. In this study, we demonstrate that on the parental HA-1-specific T-cell clones the HA-1-TCR complex is relatively lowly expressed despite normal TCRα and ß chain mRNA levels, although here competition for CD3-complex formation does not play a role. From these results we conclude that low TCR expression is a characteristic of the HA-1-TCR that is also transferred into T-cells using retroviral transduction. We hypothesized that this characteristic is based upon mRNA or protein instability of the HA-1-TCRβ chain. Codon optimization is described to increase RNA stability as well as translational efficacy by knockout of cryptic splice sites and RNA destabilizing sequence elements, and optimization of codon usage and

GC content, resulting in substantially increased expression of the introduced TCRs[16]. Since codon optimization did not change the expression of the HA-1-TCRß chain at the cell surface, we anticipate that the underlying problem of low HA-1-TCRß cell surface expression is downstream of translation. When differences in sequence between highly expressed and inefficiently expressed TCR-BV6 chains after gene transfer were analyzed, most conspicuous differences were found in the CDR1 region. It has been postulated that the CDR1 region is important for HA-1-specificity, since all the HA-1-specific T-cell clones described thus far selectively use the TCR-BV6S4 chain[14,37]. Based on these results, we hypothesized that potentially the CDR1 region could be involved in inefficient expression of the HA-1-TCRß chain. Upon exchange of this CDR1 region with the HA-2-TCRβ CDR1 region, however, HA-1-specificity was abrogated, underlining the importance of the CDR1 region for HA-1-specific reactivity. Exchange of the HA-1-TCRß CDR1 region with the CDR1 region of the HA-2-TCR, in addition, did not improve HA-1-TCR cell surface expression.

For optimal TCR expression, selection of strong competitor phenotype TCRs for TCR gene transfer purposes is necessary. Alternatively, host T-cells with weak competitor phenotype TCRs can be selected. We have previously described that based on specificity permissive virus-specific T-cells exist that allow high expression of the transferred TCR at the cell surface[13]. Selection of these permissive virus-specific T-cells therefore would be a strategy to obtain sufficient expression of the introduced TCR. However, this would reduce the pool of host T-cells useful for HA-1-TCR gene transfer. Therefore, we aimed to find a strategy to obtain high HA-1-TCR expression both in virus-specific T-cells with weak and strong competitor phenotype. Codon optimization is described to increase RNA stability as well as translational efficacy by knockout of cryptic splice sites and RNA destabilizing sequence elements[16]. Inclusion of cysteine residues is described to facilitate matched TCR chain pairing by formation of an extra interchain disulfide bond[17-19]. Most notably forced preferential pairing of the HA-1-TCR chains by inclusion of cysteine residues resulted in high HA-1-TCR expression. In addition, we have previously reported that forced preferential pairing reduced mixed TCR dimer formation, as measured by reduced neoreactivity exerted by mixed TCR dimers[33]. Combining the two strategies increased the HA-1-TCR expression even more in both the weak and strong competitor phenotype T-cells, resulting in high numbers of high-avidity T-cells capable of recognizing primary AML and ALL cells.

Currently, we are starting up a phase I/II clinical trial to treat HLA-A*0201+ HA-1+ patients with refractory hematological malignancies for whom no other therapies are available with codon optimized and cysteine modified HA-1-TCR td virus-specific T-cells. Using this modified HA-1-TCR, selection for weak competitor phenotype T-cells for HA-1-TCR gene transfer might still result in the highest yield of high-avidity TCR modified T-cells, but also without prior selection, high numbers of high-avidity TCR modified T-cells can be obtained, allowing for a simple strategy to isolate and transduce the most dominantly present CMV- or EBV-specific T-cell populations, without the need for another selection based on high HA-1-TCR cell surface expression.

In conclusion, the combination of codon optimization and inclusion of cysteine residues in HA-1-TCR chains resulted in high HA-1-TCR expression after gene transfer. Even strong competitor phenotype virus-specific T-cells transduced with the clinical vector encoding the modified HA-1-TCR highly expressed the HA-1-TCR on their cell surface, resulting in robust HA-1-specificity against clinically relevant target cells. Therefore, for future clinical HA-1-TCR gene transfer studies, the MP71 vector encoding the codon optimized and cysteine modified HA-1-TCR will be used.

AUTHORSHIP AND DISCLOSURES

ML, JF and MH designed experiments, ML, RB, RH, and HE performed experiments, ML, RB, RH, HE and MH analyzed data, ML, RW, JF, and MH wrote the paper. All authors approved the final version of the manuscript for publication. The authors reported no potential conflicts of interest.

Summary and discussion

6

SUMMARY

TCR gene transfer is an attractive strategy to equip T-cells with defined antigen-specific TCRs using short-term in vitro procedures. Selection of host cells with a known specificity and introduction of a well characterized TCR may result in an off-the shelf therapy that combines high anti-leukemic reactivity with a minimal risk of GvHD. However, some potential drawbacks to TCR gene transfer exist. The introduced TCR is under regulation of a retroviral promotor to ensure high cell surface expression, and it is unknown whether this interferes with physiological downregulation and re-expression of the TCR after antigen-specific stimulation. TCR transfer leads to lower expression of the introduced TCR compared to parental T-cell clone due to competition for cell surface expression with the endogenous TCR. T-cells expressing different amounts of introduced and endogenous TCRs on their cell surface may skew after repetitive stimulations via one TCR to populations predominantly expressing the triggered TCR, resulting in loss of dual-specificity. TCR transfer may in addition lead to the formation of mixed TCR dimers, composed of introduced TCR chains pairing with the endogenous TCRα or β chain, harboring potentially harmful new reactivities. This thesis focused on benefits and threats of TCR gene transfer and possible solutions.

TCRs that are introduced in T-cells using gene transfer are mostly under regulation of a strong viral promotor to ensure high introduced TCR cell surface expression. For effective reactivity and in vivo survival of the TCR transduced T-cells, physiological stimulation via the introduced TCR is important. The internalization of CD3 complexes induced by TCR stimulation resulting in termination of all cellular interactions and T-cell non-responsiveness seems to be vital to provide a so called refractory period of activation. In chapter 2 we investigated whether specific stimulation of either the introduced or endogenous TCR of dual TCR engineered T-cells resulted in comodulation of the non-triggered TCR. In addition, we examined the impact of regulation via either the endogenous or retroviral promotor on re-expression of the introduced and endogenous TCR on TCR transduced T-cells and antigen-responsiveness via both TCRs. For this purpose, virus-specific T-cells were retrovirally transduced with several TCRs. We demonstrated that early after antigen-specific stimulation of TCR transduced T-cells both the endogenous and introduced TCR complexes were downregulated irrespective of which TCR was triggered and this resulted in marked reduction of functional activity both via the stimulated as well as the non-stimulated TCR. These results indicate that stimulation via

one TCR results in a protective refractory period comprising of non-responsiveness via both TCRs. TCR downregulation shortly after TCR triggering was not changed in TCR transduced T-cells, however, the introduced TCR under regulation of a retroviral promotor was rapidly re-expressed on the cell surface at 24h after TCR stimulation independent of which TCR was triggered. This rapid re-expression of the introduced TCR, however, did not lead to immediate restored functionality or activation induced cell death (AICD), illustrating that cell mechanisms other than TCR cell surface expression are also involved in providing a protective refractory period.

We suggest that in the case of rapid expression of the introduced TCR CD8 downregulation is involved in providing a protective refractory period. We conclude that TCR transduced virus-specific T-cells can be potentially useful for clinical purposes, since a protective refractory period is maintained in these T-cells even though the introduced TCR is rapidly re-expressed after TCR-triggering.

Persistence of TCR modified T-cells is important for a potent continuous anti-leukemic response. TCR transfer into T-cells specific for latent viruses may achieve persistence of TCR modified T-cells, since repetitive encounter with viral antigens latently present in the recipient may activate the TCR engineered T-cells via their endogenous TCR, and thereby increase in vivo survival. However, when frequent encounter of viral antigens would lead to selective survival of TCR modified virus-specific T-cells with predominant expression of the endogenous virus-specific TCR, this may result in loss of dual-specificity of the T-cells and eventually in relapse of the leukemia. In Chapter 3, we demonstrate that HA-2-TCR transduced virus-specific T-cells repetitively stimulated via one TCR remained dual reactive in response to triggering via both the endogenous and the introduced TCR. After repetitive stimulation of one TCR, HA-2-TCR transduced virus-specific T-cells preferentially expressed the triggered TCR, losing high avidity interaction via the previously non-triggered TCR. However, TCR expression reverted within one week after a single stimulation via the previously non-triggered TCR. When the dual-specific T-cells were sorted in opposing CMV-TCR^{hi} T-cells and HA-2-TCR^{hi} T-cells, both subsets still demonstrated cytotoxic activity against HA-2 peptide pulsed target cells and CMV peptide pulsed target cells, respectively. However, CMV-TCR^{hi} T-cells demonstrated only cytotoxic activity against target cells presenting endogenously processed pp65 and not HA-2, and vice versa, HA-2-TCR^{hi} T-cells recognized only target cells presenting endogenously processed HA-2, but not pp65. After additional stimulation, both subsets were able to re-express the HA-2 and CMV-TCR, respectively. When TCR expression was redistributed on the T-cells, high avidity functionality via both the endogenous and the introduced TCR was restored. In conclusion, after repetitive stimulation HA-2-TCR transduced CMV-specific T-cells appeared to skew to populations predominantly expressing the triggered TCR. However, populations predominantly expressing the triggered TCR were able to revert their TCR make up and redistribute the previously non-triggered TCR after a single stimulation. These results indicate that TCR transduced virus-specific T-cells maintain co-expression of both the endogenous

and introduced TCR after several stimulations and can therefore be implemented in future clinical studies.

Transfer of TCRs into T-cells will not only result in cell surface expression of both the endogenous TCR and the introduced TCR, but also in cell surface expression of mixed TCR dimers consisting of introduced TCR chains pairing with endogenous TCR chains. The specificity of these mixed TCR dimers is unknown, and therefore harmful reactivities can not be excluded. In chapter 4, we investigated whether TCR gene transfer can lead to the generation of new detrimental reactivities by creating T-cells that express mixed TCR dimers. We demonstrate that transfer of TCRαβ complexes as well as only TCRα or TCRβ chains into virus-specific T-cells resulted in new reactivities. The observation that transfer of only TCRα or β chains resulted in neoreactivities showed that mixed TCR dimers were responsible for these new specificities. The observed neoreactivities could be either HLA class I or class II restricted. Furthermore, we not only observed neoreactive mixed TCR dimers harboring alloreactivity, but also autoreactivity. Cysteine modification to induce preferential pairing of the introduced TCRα and β chain reduced the neoreactivity of TCR td virus-specific T-cells considerably. We conclude that the formation of neoreactive mixed TCR dimers is not a feature of a specific TCR, since we observed this in all virus-specific T-cells tested, with different introduced TCRα or TCRβ chains. We therefore underline the importance of facilitating matched pairing of introduced TCR chains, for example by cysteine modification of the introduced TCR, and in addition, diminishing the chance of formation of harmful neoreactive mixed TCR dimers by using T-cell populations with restricted TCR repertoire as host cells for TCR transfer.

TCRs directed against MiHA HA-1 are good candidates for TCR gene transfer to treat hematological malignancies because of the hematopoiesis-restricted expression of the HA-1 MiHA. For optimal efficacy of adoptive immunotherapy with TCR modified T-cells, high introduced TCR expression is necessary. However, it has been described previously that HA-1-TCR cell surface expression after gene transfer is low. In chapter 5, we analyzed what caused this low expression and studied different strategies to improve HA-1-TCR expression after gene transfer. We demonstrate that poor TCR cell surface expression was already present in parental HA-1-specific T-cell clones. The low HA-1-TCR expression after gene transfer was not due to specific pairing properties of the HA-1-TCRα and β chain, but due to intrinsic properties of the HA-1-TCRβ chain. Of different strategies explored to improve cell surface expression of the introduced TCR, a combination of codon optimization and cysteine modification resulted in most prominent HA-1-TCR cell surface expression after gene transfer. Even strong competitor phenotype virus-specific T-cells transduced with the modified HA-1-TCR highly expressed the HA-1-TCR on the cell surface, resulting in HA-1-specific reactivity against target cells expressing endogenously processed HA-1.

On bases of these results we created the clinical MP71 vector encoding for an optimized and cysteine modified HA-1-TCRβ and α chain linked with a self-cleaving T2A sequence. Transfer of this vector into strong and weak competitor

virus-specific T-cells resulted in T-cell populations exerting robust HA-1-specific reactivity against clinically relevant target cells. Based on these results, a clinical study will be initiated to treat patients with hematological malignancies that received allogeneic stem cell transplantation (allo-SCT) with virus-specific donor T-cells transduced with the MP71 vector encoding the codon optimized and cysteine modified HA-1-TCR.

GENERAL DISCUSSION

CHARACTERISTICS OF THE INTRODUCED TCR

Efficient functional avidity of the introduced TCR

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. Therefore, gene transferred TCRs need to exhibit high affinity for their specific peptide-HLA complex to overcome low TCR cell surface expression.

The HA-2 and HA-1-TCR described in this thesis are examples of high-affinity TCRs, and were isolated from individuals with GvL and minimal GvHD. HA-2 and HA-1 have been proven to be exclusively expressed on cells of the hematopoietic system[1-3] and on clonogenic leukemic precursor cells[1,2]. Adoptive transfer of HA-2- or HA-1-specific T-cells will potentially result in selective elimination of patient-derived malignant and non-malignant hematopoietic cells whereas donor-derived hematopoietic cells will not be recognized. HA-1 has a more favorable population frequency (35-69%)[4-6] than HA-2 (70%- 95%) [5-7]. Preferentially, for TCR gene transfer, we would like to use TCRs with a strong competitor phenotype[8-10]. As reported previously[11], all HA-1-TCRs isolated and characterized up till now which are interesting for clinical use on bases of specificity exhibit low TCR cell surface expression after gene transfer. As described in chapter 5, satisfying HA-1-TCR cell surface expression and functionality could be obtained by HA-1-TCR gene transfer into weak competitor phenotype virus-specific T-cells. We have previously described that to some extent it can be predicted on bases of specificity whether virus-specific T-cells exhibit a weak or strong competitor phenotype[12]. In addition, we have previously demonstrated that expression of the introduced TCR is not a random process but is determined by characteristics of both the introduced and the endogenously expressed TCR[9]. What exactly determines this TCR phenotype still remains unsolved. It is likely that a strong competitor phenotype TCR has a high interchain affinity, which might favor its TCR-CD3 complex assembly over a TCR with low interchain affinity. Since in a population of virus-specific T-cells recognizing the same viral epitope TCR repertoire diversity is limited, it seems plausible that the phenotype of the 'public' TCR dominating the viral immune response is determining the weak or strong competitor phenotype of the total virus-specific T-cell population. To broaden the pool of host cells suitable for HA-1-TCR transfer, we sought to improve HA-1-TCR cell surface expression. For this purpose, both codon optimization and cysteine modification

were studied. It is remarkable that HA-1-TCR cell surface expression in strong competitor virus-specific T-cells benefited the most from cysteine modification, whereas codon optimization on its own had little effect on HA-1-TCR cell surface expression. These results indicate that to be able to compete for cell surface expression in strong competitor phenotype virus-specific T-cells improving HA-1-TCR interchain affinity is more effective than codon optimization of the different HA-1-TCR chains. We hypothesize that the improved HA-1-TCR interchain affinity indeed favors HA-1-TCR-CD3 assembly, and this assembly with CD3 protects the HA-1-TCR chains from rapid degradation. Codon optimization has been described to improve TCR cell surface expression by improving mRNA stability and enhancing translation into protein[13]. Although low HA-1-TCR expression appeared mostly due to low HA-1-TCRβ chain expression, we demonstrate that the strongest effect of codon optimization was observed for cell surface expression of the HA-1-TCRα chain, indicating that not all TCR-chains might benefit equally of codon optimization. The combination of codon optimization and cysteine modification of the HA-1-TCR has resulted in high cell surface expression of this high-affinity TCR, irrespective of which T-cells were used as host cells (chapter 5). All TCRs that we have studied up till now benefit from this combination strategy of codon optimization and cysteine modification and demonstrate significantly improved TCR cell surface expression.

The improved HA-1-TCR expression described in chapter 5 reduces the complexity of isolation strategies. First, not only weak competitor phenotype virus-specific T-cells can be used for HA-1-TCR gene transfer, but also strong competitor phenotype T-cells, and this broadens the pool of suitable host cells. Second, no additional purification step of HA-1-TCR expressing virus-specific T-cells is required, which would reduce the yield of TCR modified cells and increase the complexity of the production process. In addition to improved HA-1-TCR cell surface expression, cysteine modification resulted in markedly reduced expression of mixed TCR dimers, as indicated by reduced neoreactivity (chapter 4).

HA-1-TCR transduced T-cells can be used if the patient is HLA-A*0201+ and HA-1+ and has been transplanted with a HLA-A*0201+ and HA-1-, or alternatively, HLA-A*0201 mismatched donor. To increase the number of patients that can be treated with TCR-modified T-cells, characterization of more MiHA-specific TCRs is needed. Although several MiHAs have been identified until now, few of them are described to be strictly expressed on hematopoietic cells. Alternatively, MiHAs presented in HLA class II might be safe target antigens, since HLA class II expression is mainly restricted to cells of the hematopoietic system and it can therefore be argued that all HLA class II expressed MiHAs have a hematopoiesis-restricted expression.

Another option to increase the number of different patients that can be treated with TCR-modified T-cells, is to target leukemia associated antigens (LAA) like WT1 and PR3. In different healthy donors, T-cells specific for WT1[14-19] and PR3[20-22] have been identified after multiple stimulations with peptide-pulsed APCs. However, most LAAs are monomorphic self antigens, which are not only highly expressed on leukemic cells, but

can also be expressed at low level in normal healthy tissue. Most likely, T-cells recognizing these antigens with high affinity have been deleted in the thymus to maintain self-tolerance[23,24], and therefore TCRs specific for LAAs exhibiting high affinity for these antigens are lacking.

Exploring the use of high-affinity TCRs / antigen of choice

By deleting T-cell precursors recognizing with high affinity self-antigens presented in the context of self-HLA, tolerance is induced. For this reason, no high affinity T-cells directed against non mutated overexpressed LAAs or tumor associated antigens (TAAs) which are mostly self-antigens are present in the T-cell repertoire. To bypass this limitation of the endogenous T-cell repertoire, high affinity T-cell responses directed against LAAs presented in allogeneic HLA (allo-HLA) molecules can be induced. High affinity T-cells directed against self-antigens presented in the context of allo-HLA can be present, since T-cells do not encounter foreign HLA molecules during thymic selection. For example, Stauss and collaborators engineered high affinity HLA-A2 restricted WT1-specific T-cells by stimulating HLA-A2 negative PBMCs using B cells coated with recombinant HLA-A2 monomers containing single peptide epitopes[25]. To separate the WT1-specific allo-HLA restricted T-cells from the non-WT1-specific allo-HLA restricted T-cells several rounds of isolation using WT1 tetramers were needed. In order to obtain high affinity TAA-specific T-cells, Schendel and collaborators used an in vitro stimulation approach based on the same concept by stimulating HLA-A2 negative PBMCs with DCs obtained from the same individual that were transfected with the HLA-A2 restriction molecule and pulsed with several different TAAs[26]. Furthermore, in our lab, we have recently characterized an allo-MHC restricted PRAME-specific T-cell derived from an immune response in a patient transplanted over an HLA-A2 mismatch (Amir et al, unpublished data). It is unclear whether engineering high-affinity T-cells directed against self-antigens in non-self HLA comprises T-cells that can crossreact with several peptides in the non-self HLA or can even crossreact with different non-self HLAs. Recently in our lab, we compared HLA-A2 restricted WT1-specific T-cells derived from HLA-A2 positive or negative individuals[27]. In this study, WT1-specific allo-HLA restricted T-cells were separated from the non-WT1-specific allo-HLA restricted T-cells by sorting the cells using WT1 tetramers. Although allo-HLA restricted WT1-specific T-cells were more tumor-reactive than their auto-HLA restricted counterparts, even after tetramer isolation the allo-HLA restricted T-cells were crossreactive against non-WT1 antigens. Another safety issue of engineering high-affinity T-cells directed against self-antigens in non-self HLA might result in T-cells able to recognize healthy tissue expressing low levels of the self-antigen. In the second clinical study using TCR modified T-cells of Rosenberg and colleagues in which they transduced patient T-cells with a high-affinity MART-1-specific and gp100-specific TCR on target autoimmune destruction of melanocytes in ear, skin and hair that required treatment was observed in several patients, illustrating that high-affinity TCRs directed against self-antigens can indeed result in on-target toxicity directed against healthy tissue[28].

An alternative approach for the production of high-affinity TAA-specific T-cells involves the use of genes that encode monoclonal antibody chains. Generally, first generation CARs consisted of a single-chain antibody-derived antigen-binding motif coupled to signalling modules that are normally present in the TCR complex, such as the CD3-chain, whereas second- and third-generation CARs also contain costimulatory or anti-apoptotic moieties. An advantage of tumour-specific T-cells that are generated in this manner is that they respond to antigen in a non-MHC-restricted manner, with the affinity of an antibody. The affinity of CAR-modified T-cells for their target antigen is generally higher than TCR-modified T-cells, which could have several consequences for the functionality. First, T-cells engineered with a CAR might unwantedly exert effector functions directed against healthy tissue expressing low levels of the target antigen. Second, this higher affinity of CAR-modified T-cells might result in unphysiologically strong activation signals. Lastly, the higher affinity for their target antigen might hamper detachement to the target cell, and limit the ability of the CAR-modified T-cells to serially kill target cells.

It has been described that administration of T-cells modified with a CAR 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[29]. Recently, a serious adverse event was described by Morgan and colleagues that occurred in a patient with widely metastatic colon cancer who received more than 10^{10} T-cells modified with a CAR targeting HER2 containing costimulatory moieties (CD28 and 4-1BB) after intensive lymphodepletion. Within 15 minutes after cell infusion the patient experienced respiratory distress, coinciding with a dramatic pulmonary infiltrate in association with very high cytokines followed by cardiac arrest and death 4 days later. They speculate that the large number of administered cells localized to the lung immediately following infusion and were triggered to release cytokines by the recognition of low levels of ERBB2 on lung epithelial cells[30]. The concern was raised that second- and third-generation CARs may be too easily triggered by low expression of antigen resulting in a potent activation signal that leads to a lethal cytokine storm[31]. However, the high amount of CAR-modified T-cells infused could also have made it difficult to shut down the immune response using steroids. Although it is still unclear whether the combination of lymphodepletion and the presence of costimulatory moieties in the second- and third-generation CARs used might have contributed to the cytokine storm, from their experiments it seems plausible that the lethal cytokine storm was a direct result from unwanted on-target reactivity directed against lung tissue expressing low levels of ERBB2 due to the high affinity of the CAR-modified T-cells.

Alternatively, TCRs with different affinities can be obtained by modification of TCRα or TCRβ CDR1, CDR2 or CDR3 sequences[32-37]. In chapter 5 we describe that small adjustments in amino acid sequence of the TCRβ chain, namely exchange of the 18 amino acid long CDR1 region, resulted in completely abolished HA-1-specific reactivity. This suggests that minimal changes in TCRα or β sequences can result in drastic changes of

specificity. In addition, we previously studied whether chimeric HA-2-TCRs consisting of HA-2-TCR chains derived from different HA-2-specific T-cell clones isolated from a CML patient with an ongoing antileukemic immune response would still remain their HA-2-specificity[38]. Several clones exhibiting different HA-2-TCRs were characterized and on bases of HA-2-TCR usage T-cell clones were classified in 10 different groups. The different HA-2-specific T-cell clones demonstrated restricted TCRα and β usage and in addition, the HA-2-TCRα and TCRβ chains of all isolated T-cell clones demonstrated many similarities in TCRα and TCRβ chain sequences [38], of which the most striking similarity was that all HA-2-TCRα chains used the Jα42 region. Therefore, we hypothesized that the TCRα chain was responsible for the HA-2-specificity of these T-cell clones, and that the TCRβ chains would probably be interchangeable. However, it could not be predicted whether a specific chimeric HA-2-TCR consisting of a HA-2-TCRα of one group combined with a HA-2-TCRβ of the other group would remain HA-2-specific on bases of similarities in TCR chains of the different groups[38]. Some chimeric HA-2-TCRs remained HA-2-specific, whereas others exhibited a new alloreactivity, as described in chapter 4. From these experiments we concluded that it is hard to predict the dominant TCR-regions involved in specific recognition on bases of restricted TCR chain repertoire used by the different T-cell clones and similarities in sequences of these TCR chains. Furthermore, it is difficult to test for or predict for crossreactivity when different random modifications in the CDR regions of both TCR-chains are induced.

Recently, Rufer and colleagues reported that above a defined TCR-peptide-HLA affinity threshold, T-cell function could not be enhanced, indicating that there might exist a plateau of maximal T-cell function[39]. They propose to limit affinity improvement of rationally designed TCRs to a given affinity threshold which should result in optimal T-cell function while maintaining a limited risk of crossreactivity.

In conclusion, to broaden the applicability of TCR-modified T-cells for clinical use, characterization of more different high affinity TCRs recognizing either potential useful MiHAs or LAAs/TAAs is required. Thorough analysis of antigen expression restricted to malignant cells is necessary since current strategies to engineer high-affinity TCRs have shown to result in reactivity directed against low expression levels on healthy tissue. In addition, selection of high-affinity TCRs involved in immune responses without adverse events like the HA-1- and HA-2-TCR might be warranted, since peptide specificity of in vitro engineered or selectedTCRs can not be guaranteed.

SPECIFICITY OF THE ENDOGENOUS TCR

Rationale behind usage of virus-specific T-cells as recipient cells for TCR gene transfer

Clinical studies have demonstrated that for optimal efficacy of adoptive immunotherapy persistence of the transferred T-cells is required[40-44]. In comparison to adoptive T-cell therapy for viral infections after allo-SCT[45-48] persistence of adoptively transferred tumor-specific T-cells in vivo was remarkably short[44,49]. To obtain

therapeutic cell numbers, several stimulations were required in upper mentioned studies and it is now recognized that long in vitro culture periods negatively influence the in vivo functional activity of the T-cells[50-52]. Two conclusions were drawn based on studies performed on in vivo persisting tumor-specific T-cells. First, it was observed that persisting tumor-specific T-cells possessed longer telomeres than non-persisting T-cells[53,54]. Second, persisting T-cells re-expressed CD27 and CD28 molecules on their cell surface and it was suggested that persisting T-cells demonstrated transition from late stage effector into an early effector phenotype[55]. From these results it was concluded that less differentiated T-cell populations should be used for adoptive immunotherapy of TCR transduced T-cells.

Simultaneously, profound differences in survival in vivo of different memory subsets were reported[56-58]. Roughly, two distinct memory subsets are described, namely effector memory (TEM) and central memory (TCM) T-cells[57]. T_{EM} T-cells are CD62L and CCR7 negative and have strong lytic capacity and can release high amounts of IFN-γ. T_{CM} are CD62L and CCR7 positive and are thought to exhibit stem-cell like self-renewal capacity, meaning that upon antigen re-exposure these T-cells are capable of undergoing massive proliferation and differentiate into effector T-cells. For improved persistence of TCR modified T-cells, T_{CM} T-cells were demonstrated to be superior over T_{EM} T-cells[59]. In this study, virus-specific T-cell clones derived from purified T_{CM} and T_{EM} T-cells were gene marked to be able to distinguish between the two subsets and the endogenous T-cell repertoire, and transferred into normal macaques. Although similar in function, and phenotype in vitro, T-cell clones derived from T_{CM} or T_{EM} T-cells exhibited different fates in vivo. Whereas T_{CM}-derived clones persisted long term in the blood, T_{EM}-derived clones consistently failed to persist in the blood longer than 5 days. In mouse models it was established that a T_{CM} phenotype could be induced in vitro by adding a WNT pathway inhibitor, glycogen synthase kinase 3β to the T-cell culture, thereby generating T-cells with improved persistence and anti-tumor efficacy[60].

By using EBV- and CMV-specific T-cells as host cells for TCR gene transfer both T_{EM} and T_{CM} T-cells will be transduced. Recent studies demonstrated that distinct memory subsets are raised in different viral infections [61,62]. Even within one virus-specific memory response distinct memory subsets of virus-specific CD8+ T-cells can be found[63]. Whereas CMV-specific T-cells mostly consist of T_{EM} T-cells, EBV-specific T-cells especially those recognizing latent antigens mostly consist of T_{CM} T-cells. We hypothesize that by using both EBV- and CMV-specific T-cells with distinct phenotypic characteristics, TCR modified virus-specific T-cells can be engineered with different functional characteristics and homing capacities and prolonged long-term persistence. It has been described that adoptively transferred unmodified EBV-specific T-cells to prevent or treat EBV positive lymphoproliferative can persist for up to 9 years[46]. We will transfer into patients both EBV- and CMV-specific T-cells modified with the HA-1-TCR. According to the current hypothesis that adoptively transferred T_{CM} will persist superior to T_{EM}, predominant persistence of HA-1-TCR modified EBV-specific T-cells compared to CMV-specific T-cells should be observed in treated patients.

Additional advantages of knowledge of the endogenous TCR specificity of recipient cells used for TCR gene transfer

Next to their potentially beneficial phenotype, using EBV- and CMV-specific T-cells as host cells for TCR gene transfer offers other attractive benefits. EBV and CMV are viruses which latently persist after initial infection and therefore T-cells will be repetitively stimulated with antigen in vivo. Based on the hypothesis that T-cells specific for latently present viruses would be activated and receive co-stimulation via their endogenous TCR, EBV-specific T-cells were proposed as host cells for CAR-modification as a solution to the low proliferative capacity of first generation CAR-modified T-cells[64]. 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[46]. Some virus-specific T-cells can exhibit alloreactivity, directed against non-self HLA molecules[65]. However, this will only have consequences for potential induction of GvHD in HLA-mismatched transplantations and can be easily tested for by using non-transduced virus-specific T-cells. Alloreactive virus-specific T-cells, already capable of producing IFN-γ after co-incubation with patient-derived DCs without transfer of the HA-1-TCR, can be excluded for further use.

We hypothesize that using virus-specific T-cells as host cells for TCR gene transfer will also enable us to monitor more sensitively the immunological responses in patients. HA-1-TCR modified virus-specific T-cells can be monitored directly ex vivo by staining with either virus-tetramers, HA-1-tetramers, or a combination of both tetramers. Furthermore, if HA-1-TCR modified virus-specific T-cells appear to be only present in low numbers in the patients' circulation, an enrichment step using virus-specific tetramers can be performed to calculate precise numbers in vivo. In a recent report, EBV-specific T-cells modified with a CAR directed to the GD2 antigen expressed on neuroblastoma cells were compared to unselected T-cells modified with the same CAR[66]. Although CAR-modified T-cells could be hardly visualized directly ex vivo, knowledge of the endogenous TCR facilitated analysis of persistence of CAR-modified EBV-specific T-cells, since stimulation of the endogenous EBV-specific TCR resulted in an enrichement and subsequent detection of CAR-modified T-cells[66].

Another potential advantage of using host cells with known specificities of the endogenous TCR has recently been described in a mouse model[67]. It was shown 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. In addition, T-cell stimulation is followed by increased activation of the retroviral promotor[68-70] resulting in improved expression of the introduced TCR as

also demonstrated in chapter 2 and 3. We hypothesize that activation of the endogenous TCR by latently present viral antigens can both result in increased numbers of TCR modified T-cells, as well as in increased introduced TCR expression.

Limiting cell surface expression of different mixed TCR dimers

Previous attempts to prevent formation of mixed TCR dimers comprising of introduced TCR chains pairing with endogenous TCR chains were mainly made to improve expression of the introduced TCR chain. Since space on the cell membrane may limit the number of TCR-complexes that can be expressed, formation and cell surface expression of mixed TCR dimers will further limit expression of the introduced TCR. Preferential pairing of the introduced TCR may be forced using different techniques. For example, murinization of the constant domains has been described to result in exclusive pairing of the introduced TCR chains[71]. Since murine domains may be potentially immunogenic in vivo[29,72], two recent reports describe the minimal amount of murinization needed to obtain preferential pairing of the introduced TCR chains [73,74]. In the TCRβ chain murinization can be limited to an exchange of five amino acids and in the TCRα it can be limited to an exchange of four amino acids. Also, inclusion of extra cysteine residues in the constant domains of the introduced TCR resulting in an additional disulfide bond induces preferential pairing[75,76]. As described in chapter 4, formation of mixed TCR dimers comprised of introduced TCR chains pairing with endogenous TCR chains might not only hamper introduced TCR expression but might also result in potentially harmful neoreactivities. While no evidence of mixed TCR dimer induced autoimmunity was observed in earlier murine experiments and first clinical trials using MART-1 and gp100-TCR modified T-cells, in a recent set of experiments an often lethal autoimmune pathology was observed under conditions that promote the expansion of adoptively transferred T-cells more strongly, and this pathology appeared dependent on the action of mixed TCR dimers[77]. Whereas chapter 5 focused on improving introduced TCR expression and functionality, in addition, chapter 4 demonstrates that the facilitated matched pairing induced by inclusion of cysteine residues in the introduced TCR chains results in decreased formation of mixed TCR dimers, since observed neoreactivities could be markedly reduced by transfer of cysteine modified TCRs.

Alternatively, formation of mixed TCR dimers can be prevented by transducing γδ-T-cells, since the γδ-TCR chains are not able to pair with αβ-TCR chains[78]. 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 functional activity and persistence of the cells in vivo was shown[79]. However, further analyses and comparative studies 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 and equal with respect to effector functions. Previously, the in vitro production of tumor-specific T-cells by TCR gene transfer into hematopoietic stem cells (HSCs) was proposed as an alternative to generate T-cells predominantly expressing the introduced TCR[80]. Transduction of

HSCs with TCRs results in inhibition of other TCRβ chain rearrangements, and therefore the TCR transferred HSCs expressed no endogenous TCRβ on their cell surface. However, it is known that allelic exclusion is far from complete for TCRα chains, and thus this approach limits but does not completely prevent the number of different mixed TCR dimers that can be formed. In addition, risk of insertional mutagenesis of transduced stem cells may be a reason not to favor this approach.

We propose virus-specific T-cell populations as host cells for TCR gene transfer as an elegant strategy to limit the diversity of the TCR repertoire of the recipient T-cells. Since virus-specific T-cell populations consist of a restricted TCR repertoire[81,82], the number of different mixed TCR dimers formed will be limited and from in vivo data this appears a viable strategy to prevent neoreactivity caused by mixed TCR dimers[83]. In contrast, total PBMCs consist of a broad TCR repertoire and vast number of different mixed TCR dimers can be formed. In addition, the known reactivity of virus-specific T-cells allows detection of harmful neoreactivities by introducing into these virus-specific T-cells as controls only the TCRα or TCRβ chain of interest and subsequent testing against different patient-derived cell types. By this procedure TCR td virus-specific T-cells can be selected that show no off-target toxicity.

In conclusion, we have demonstrated that off-target reactivity exerted by mixed TCR dimers possibly resulting in GvHD is markedly reduced due to inclusion of cysteine residues in the HA-1-TCR chains. Furthermore, as described in chapter 5, the neoreactivities observed in this study were mostly allo-HLA restricted, posing only risks in a HLA-mismatched setting. In addition, TCR td T-cells harboring autoreactive mixed TCR dimers will only be able to survive when the peptide recognized is not expressed on the T-cells themselves, since this may lead to fratricide of these T-cells. Finally, transduction of oligoclonal virus-specific T-cell populations limits the number of different mixed TCR dimers that can be formed, thereby limiting the formation of harmful off-target reactivities.

CLINICAL STUDY

Treatment of hematological malignancies with T-cell depleted alloSCT followed by DLI significantly reduces the risk and severity of GvHD, however, still this complication remains an important cause of morbidity and mortality. To selectively induce GvL, more defined T-cell populations with restricted anti-leukemic specificity should be used.

Based on a history of extensive research on MiHA-TCR transfer into virus-specific T-cells partly described in this thesis, we would like to proceed with a clinical trial in which HA-1-TCR transduced EBV- and/or CMV-specific donor T-cells will be adoptively transferred into patients with acute leukemia. We will use CMV- and EBV-specific T-cells derived from the stem cell donor as host cells for TCR transfer, since these T-cells do not recognize antigens expressed on normal recipient cells and will therefore likely not induce GvHD[45,47,48,84-89]. Furthermore, HA-1-specific T-cell clones have been screened against an EBV-LCL panel expressing most prevalent HLA-molecules and no crossreactivity was

observed. Therefore, HA-1-TCR gene transfer into virus-specific T-cells allows the rapid production of a T-cell product designed to result in GvL-effect without detrimental GvHD.

For our clinical study, two patient groups will be eligible. First, HLA-A*0201+ and HA-1+ patients with refractory hematological malignancies who have no other treatment option left can be included in the study. Normally, these patients would not receive allo-SCT because a rapid progression can be expected already early after allo-SCT when DLI can not be given due to high risk of GvHD. Since the HA-1-TCR transduced virus-specific T-cells are designed to result in GvL without induction of GvHD, we hypothesize that these T-cells can be transferred as early as 6 weeks after allo-SCT.

Second, HLA-A*0201+ and HA-1+ patients with relapsed hematological malignancies who fail to respond to DLI or patients with early relapses of aggressive malignancies which are unlikely to be controlled by DLI without development of severe GvHD are eligible for this study. Previously, these patients had to be transplanted with a HLA-A*0201+ HA-1- or HLA-A*0201- donor and sufficient donor material has to be available for isolation of virus-specific T-cells.

We have now designed an isolation and transduction protocol that can result in a HA-1-TCR modified virus-specific T-cell product in a timeframe of approximately 2 weeks. Potentially, the lymphodepleted state of patients early after allo-SCT can induce a proliferative burst of the adoptively transferred HA-1-TCR transduced virus-specific T-cells. Since the number of leukemic cells should still be reduced early after allo-SCT due to the conditioning regimen, this will potentially result in favorable in vivo effector-to-target ratio's, and offers the possibility to also treat rapidly growing hematological malignancies.

Nederlandse samenvatting

7

ACHTERGROND

Hematopoiese en het ontstaan van leukemie

Bloed bestaat uit verschillende celtypen. Rode bloedcellen of erythrocyten zijn verantwoordelijk voor het zuurstoftransport, bloedplaatjes of thrombocyten zijn betrokken bij de stolling en witte bloedcellen of leukocyten zijn belangrijk voor afweer tegen verscheidene pathogenen, zoals virussen en bacteriën. Omdat deze verschillende cellen een beperkte levensduur hebben, vindt in het lichaam een continue aanmaak plaats door hematopoietische stamcellen die zich in het beenmerg bevinden. Dit proces wordt hematopoiese genoemd. Soms treedt er een ontsporing op in de hematopoiese en ontstaan er kwaadaardige (maligne) cellen die ongecontroleerd doorgroeien zonder zich te ontwikkelen. Leukemie is een vorm van kanker die gekenmerkt wordt door verdringing van het normale hematopoietisch systeem uit het beenmerg door de maligne cellen, en het accumuleren van grote aantallen leukemiecellen in het bloed.

Stamceltransplantatie

Er bestaan verschillende typen leukemie, die verschillend behandeld worden. Een aantal typen leukemie wordt behandeld met stamceltransplantatie. Conventionele myeloablatieve stamceltransplantatie bestaat uit preconditionering met chemo- en radiotherapie met als doel om zoveel mogelijk leukemiecellen te vernietigen, gevolgd door transplantatie van donor hematopoietische stamcellen. Behandeling met chemo- en radiotherapie resulteert echter ook in vernietiging van gezonde hematopoietische stamcellen, waardoor alle verschillende hematopoietische cellen niet meer gevormd kunnen worden. Om te voorkomen dat de patiënt overlijdt, worden gezonde stamcellen (graft) getransplanteerd om de normale hematopoiese te herstellen. Deze kunnen afkomstig zijn van de patiënt zelf (autoloog) of van een gezonde donor (allogeen). Na toediening van het stamceltransplantaat aan de patiënt migreren de stamcellen naar het beenmerg van de patiënt en ontwikkelt zich een nieuw hematopoietisch systeem.

Immuunreacties na stamceltransplantatie

Transplantatie van donorstamcellen kan resulteren in verschillende immuunreacties tussen cellen van de patiënt en donor. Als nog aanwezige patiënt-T-cellen een sterke immuunreactie tegen donorstamcellen ontwikkelen en die vernietigen, is er sprake van afstoting, ook wel host versus graft (HvG) reactie genoemd. Dit is een ernstige complicatie, omdat er dan geen

normale hematopoiese meer plaatsvindt. Daarnaast kunnen er immuunreacties plaatsvinden als donor-T-cellen aanwezig in het donorstamceltransplantaat patiëntweefsels herkennen, ook wel transplantaat versus ontvanger ziekte of Graft versus Host Disease (GvHD) genoemd. Deze immuunreacties zijn meestal gericht tegen de huid, de longen, lever en darmen, en kunnen levensbedreigende vormen aannemen. Om deze ernstige complicatie van allogene stamceltransplantatie (allo-SCT) te voorkomen, kunnen donor-T-cellen selectief uit het transplantaat verwijderd worden. Verwijdering van donor-T-cellen uit het transplantaat resulteert echter in een verhoogde kans op terugkeer van de leukemie. Donor-T-cellen zijn blijkbaar in staat om leukemiecellen te vernietigen die bestraling en chemotherapie hebben overleefd. Wanneer donor-T-cellen selectief reageren tegen resterende hematopoietische cellen van de patiënt, waaronder nog aanwezige leukemiecellen, wordt er gesproken van Graft versus Leukemia (GvL) effect. Omdat het stamceltransplantaat van de donor het hematopoietisch systeem van de patiënt vervangt, leidt deze reactiviteit niet tot problemen, maar is juist curatief. GvL is belangrijk gebleken om terugkeer van de leukemie tegen te gaan.

Immunotherapie na stamceltransplantatie

De erkenning dat donor-T-cellen in staat zijn om specifiek de maligne cellen van de patiënt op te ruimen, leidde tot de ontwikkeling van nieuwe stamceltransplantatiestrategieën. Patiënten worden getransplanteerd met donorstamceltransplantaat waar selectief de donor-T-cellen uit verwijderd zijn. Na 6 maanden worden ongeselecteerde donorlymfocyten als immunotherapie gegeven. Deze donorlymfocyteninfusies (DLI) induceren bij veel patiënten remissie van de ziekte. Helaas resulteert deze therapie niet alleen in een GvL effect, maar ook in gevaarlijke GvHD reacties. Uit patiënten die wel de curatieve GvL maar geen schadelijke GvHD reacties ontwikkelden, zijn donor-T-cellen geïsoleerd en gekarakteriseerd. Een aantal van deze donor-T-cellen herkenden selectief patienthematopoietische cellen, waaronder ook de leukemiecellen, zonder dat ze het gezonde niet-hematopoietische weefsel van de patiënt herkennen. Toediening van T-cellen met deze reactiviteit zou kunnen resulteren in selectieve herkenning van de leukemie zonder de levensbedreigende GvHD.

T-cellen en de T-celreceptor

T-cellen zijn afweercellen die belangrijk zijn in bestrijding tegen pathogenen, zoals bacteriën en virussen, en tegen lichaamsvreemde of ontspoorde cellen, zoals kankercellen. T-cellen moeten in staat zijn te detecteren of een cel gezond is, geïnfecteerd is met een pathogeen of getransformeerd is tot kankercel. T-cellen detecteren dit door middel van hun T-celreceptor (TCR). De TCR bestaat uit een TCRα- en TCRβ-keten. Door middel van zijn TCR kan een T-cel kleine fragmenten van eiwitten, peptides genaamd, herkennen wanneer zij gepresenteerd worden in HLA-moleculen. Op alle cellen in het lichaam komen HLA-moleculen tot expressie. HLA-klasse-I-moleculen presenteren peptides afkomstig van eiwit dat een cel zelf maakt en HLA-klasse-II-moleculen presenteren voornamelijk peptides afkomstig van eiwit dat cellen op kunnen nemen uit hun omgeving.

Binnen het T-celcompartiment wordt onderscheid gemaakt tussen T-cellen die de CD4 co-receptor tot expressie brengen en T-cellen die de CD8 co-receptor tot expressie brengen. CD4+ helper T-cellen kunnen peptides herkennen in HLA-klasse-II-moleculen. Na herkenning van geïnfecteerde of getransformeerde cellen is hun voornaamste rol het produceren van cytokines, die effectorfuncties van CD8+ cytotoxische T-cellen en de aanmaak van antilichamen door B-cellen bevorderen. CD8+ cytotoxische T-cellen kunnen peptides herkennen in HLA-klasse-I-moleculen. Herkenning resulteert in directe lysis van geïnfecteerde of getransformeerde cellen door productie van perforine en granzyme B.

Specificiteit van T-cellen; minor-antigenen

Er is een correlatie tussen het optreden van GvHD en de mate van HLA-verschillen tussen donor en patiënt. De voorkeur gaat daarom uit naar een donor die dezelfde HLA-moleculen heeft als de patiënt. In sommige gevallen van transplantaties met een HLA-identieke donor treedt er nog steeds GvHD bij de patiënt op. In dit geval komt de reactiviteit door donor-T-cellen die minor-antigenen herkennen. In het DNA bestaan er tussen individuen kleine verschillen, ook wel single nucleotide polymorphisms (SNPs) genoemd. Deze kleine verschillen in DNA zorgen ervoor dat op het niveau van aminozuren veranderingen optreden, die soms kunnen resulteren in een verschillend eiwit. Als door deze kleine verschillen patiëntcellen andere peptides presenteren als donorcellen, kan er een immuunreactie ontstaan. Op het moment dat deze verschillende peptides in staat zijn om een immuunreactie bij donor-T-cellen op te wekken, worden ze minor-antigenen genoemd. Als minor-antigenen op verscheidene patientcellen door het hele lichaam tot expressie komen, kan dit leiden tot GvHD. Er zijn echter ook minor-antigenen beschreven die alleen tot expressie komen op cellen van het hematopoietisch systeem, zoals HA-1 en HA-2. Donor-T-cellen in staat om HA-1 en HA-2 gepresenteerd in HLA-A*0201 te herkennen, vernietigden wel de patientbloedcellen en leukemiecellen, maar niet de andere gezonde patiëntcellen of donorbloedcellen. In verschillende patiënten resulteerde deze HA-1 en HA-2 donor-T-celrespons in GvL zonder GvHD, en sommige van deze patiënten zijn nog steeds ziektevrij.

DIT PROEFSCHRIFT

Donor-T-celresponsen spelen een belangrijke rol in zowel het gewenste GvL effect, als de levensbedreigende GvHD. Om op een snelle manier donor-T-cellen te genereren waarvan bekend is dat ze alleen de patiëntbloedcellen en leukemiecellen herkennen, maar geen ander gezond weefsel, kan gebruik worden gemaakt van TCR-gentherapie. Omdat de TCR de specificiteit van een T-cel bepaalt, resulteert introductie van de HA-1-TCR of HA-2-TCR in generatie van HA-1- of HA-2-specifieke T-cellen. Omdat bekend is dat immunotherapie met ongeselecteerde donorlymfocyten GvHD kan induceren, zou het introduceren van de HA-1- of HA-2-TCR in een populatie van diverse verschillende donor-T-cellen nog steeds GvHD kunnen

veroorzaken. Daarom heeft het de voorkeur om deze minor-antigenspecifieke TCR-en over te zetten in een andere goed gekarakteriseerde T-celpopulatie. Een aantrekkelijk alternatief om donor T-cellen te genereren met verwachte selectieve anti-leukemie-reactiviteit, is HA-1-TCR of HA-2-TCR gentransfer in virusspecifieke T-cellen, zoals cytomegalovirus (CMV)- of Epstein-Barr-virus (EBV)-specifieke T-cellen.

Er zijn nog wel een aantal verbeterpunten. Introductie van een TCR in verschillende CMV- of EBV-specifieke T-cellen leidt niet alleen tot expressie van de endogene en geïntroduceerde TCR op het celoppervlak, maar ook tot expressie van TCR-dimeren. TCR-dimeren zijn combinaties van de geïntroduceerde TCR-ketens gepaard met de endogene TCRα-, of β-ketens. Introductie van één TCR in één T-cel kan dus leiden tot expressie van vier verschillende TCRαβ-complexen op het celoppervlak, en leidt dus niet altijd tot eenzelfde TCR-opmaak op het celoppervlak. Er is bewezen dat er competitie plaatsvindt voor expressie op het celoppervlak, niet alleen tussen de geïntroduceerde TCR en de endogene virusspecifieke TCR, maar ook tussen TCR-dimeren.

Het onderzoek, beschreven in dit proefschrift, was erop gericht uit te zoeken wat de consequenties van verschil in TCR-opmaak waren, en of er oplossingen gevonden konden worden.

Effect van regulatie op de expressie van de geïntroduceerde TCR

TCR-en die door middel van retrovirale gentransfer in donor-T-cellen geïntroduceerd worden, worden gereguleerd door virale promotoren die de expressie van de TCR-en bepalen. Het voordeel hiervan is dat deze regulatie tot een hoge expressie van de geïntroduceerde TCR leidt. Het nadeel hiervan is dat virale promotoren anders werken dan de promotoren die de expressie van de eigen TCR reguleren. Normaliter resulteert herkenning van een specifiek peptide-HLA-complex door een TCR tot internalizatie van de TCR-complexen. Dit resulteert in beëindiging van alle TCR-HLA-interacties tussen T-cel en de herkende cel. T-cellen zijn dan tijdelijk niet in staat om op nieuwe stimuli te reageren. Zij ondergaan een soort van "time-out", ook wel refractaire periode genoemd, die ze in staat stelt om verschillend DNA af te schrijven, zodat ze kunnen delen en allerlei verschillende cytokines produceren. Deze refractaire periode zou T-cellen beschermen tegen activatie geïnduceerde celdood (AICD).

In hoofdstuk 2 wordt onderzocht of specifieke stimulatie van zowel de geïntroduceerde als de eigen endogene TCR in TCR-gemodificeerde T-cellen leidt tot zo'n refractaire periode. TCR-gemodificeerde T-cellen lieten na specifieke stimulatie van zowel de endogene virusspecifieke TCR als de geïntroduceerde minor-antigenspecifieke TCR vergelijkbare internalizatie van TCR-complexen zien. Stimulatie via één van beide TCR-en resulteerde in internalizatie van beide TCR-en, maar de geïntroduceerde TCR werd telkens veel sneller weer tot expressie gebracht op het celoppervlak. Ondanks deze snelle herexpressie ondergingen TCR-gemodificeerde T-cellen toch een normale refractaire periode, en bleven ze ongevoelig voor stimulaties via één van beide TCR-en. Een mogelijke verklaring voor het feit dat de snelle herexpressie van de geïntroduceerde TCR niet leidt tot de mogelijkheid om de TCR-gemodificeerde T-cel weer

te kunnen activeren via de geïntroduceerde TCR, is de gecoördineerde internalizatie van de co-receptor CD8. We concluderen dat, ondanks de niet fysiologische regulatie via de virale promotor die tot een snelle herexpressie van de geïntroduceerde TCR leidt, T-cellen zich toch fysiologisch gedragen en nog niet in staat zijn om geactiveerd te worden via hun TCR. Het lijkt erop dat T-cellen verschillende manieren gebruiken om hun refractaire periode na stimulatie veilig te stellen, en daardoor hoeven TCR-gemodificeerde T-cellen niet te worden geacht gevoeliger te zijn voor AICD door verlies van hun refractaire periode.

Persisterende expressie van de geïntroduceerde TCR

Introductie van TCR-en in CMV- of EBV-specifieke T-cellen is niet alleen aantrekkelijk omdat deze T-cellen geen GvHD veroorzaken, maar ook omdat deze T-cellen in staat zijn om lange tijd aanwezig te blijven. Op die manier zou ook de anti-leukemiereactiviteit via de geïntroduceerde TCR voor lange tijd aanwezig blijven. CMV- en EBV-specifieke T-cellen blijven waarschijnlijk zo lang aanwezig in het bloed, omdat ze af en toe weer hun functie moeten uitoefenen om het virus onder controle te houden. Op die momenten worden ze via hun TCR gestimuleerd met specifieke virale peptides. Als deze herhaaldelijke stimulaties via de endogene virusspecifieke TCR zou resulteren in uitgroei van TCR-gemodificeerde T-cellen die voornamelijk de endogene TCR tot expressie brengen, maar niet meer de geïntroduceerde TCR, kan dit tot verlies van de anti-leukemiereactiviteit leiden. Om te testen of dit optreedt, hebben we van HA-2-TCR-gemodificeerde CMV-specifieke T-cellen óf de endogene CMV-specifieke TCR óf de geïntroduceerde HA-2-TCR herhaaldelijk gestimuleerd.

In hoofdstuk 3 beschrijven we hoe in eerste instantie deze herhaaldelijke stimulaties van óf de endogene CMV-TCR óf de geïntroduceerde HA-2-TCR leken te resulteren in specifieke uitgroei van TCR-gemodificeerde T-cellen die voornamelijk de CMV-TCR of de HA-2-TCR, respectievelijk, tot expressie brachten. Deze specifieke TCR-opmaak waarbij óf de endogene óf de geïntroduceerde TCR domineerde op de T-cellen, kon echter ongedaan gemaakt worden door éénmaal de andere TCR te stimuleren. In aanvulling op deze experimenten werden de TCR-gemodificeerde T-cellen gesorteerd op dominante expressie van óf de endogene CMV-TCR óf de geïntroduceerde TCR. Direct na het sorteren waren deze T-celpopulaties wel in staat om via beide TCR-en peptidebeladen targetcellen te herkennen. Echter, de TCR-gemodificeerde T-cellen konden alleen via de TCR op basis van welke expressie ze gesorteerd waren ook sterke interacties aangaan met niet-peptidebeladen targetcellen die wel óf CMV-geïnfecteerd waren, óf HA-2 positief. Ook deze T-celpopulaties waren in staat om na een additionele stimulatie hun TCR-opmaak weer te veranderen, en beide TCR-en weer goed tot expressie te brengen op het celoppervlak. Wanneer de TCR-opmaak was veranderd, waren ook de sterke interacties via beide TCR-en hersteld. Uit deze experimenten concluderen we dat TCR-gemodificeerde virus-specfieke T-cellen goed te gebruiken zijn voor gentherapie, omdat de kans op selectieve uitgroei van virus-specifieke T-cellen, die niet meer de

geïntroduceerde TCR tot expressie brengen of kunnen brengen, heel miniem is.

Vóórkomen en voorkómen van TCR-dimeren met mogelijk gevaarlijke reactiviteit

Introductie van TCR-en in T-cellen resulteert in de expressie van vier verschillende TCR-complexen op het oppervlak. Niet alleen komen de geïntroduceerde TCR en de endogene TCR tot expressie, maar ook 2 TCR-dimeercomplexen bestaande uit de geïntroduceerde TCRα-, en β-keten die gepaard zijn met de endogene TCR-ketens. De specificiteit van de geïntroduceerde TCR en de endogene TCR is bekend, maar van de TCR-dimeren is onbekend of en wat voor specificiteit ze hebben. Daarom kan niet uitgesloten worden dat ze potentieel een gevaarlijke specificiteit hebben die mogelijk in GvHD kan resulteren.

In hoofdstuk 4 hebben we onderzocht of en hoe vaak TCR gentransfer resulteert in vorming van TCR-dimeren met een gevaarlijke nieuwe specificiteit. Om dit te onderzoeken zijn zes verschillende virusspecifieke T-cellen uit diverse gezonde individuen getransduceerd met zeven goed gekarakteriseerde TCR-en. De resultaten laten zien dat zowel introductie van complete TCR-en, als transductie van enkel TCRα-, of β-ketens leidde tot nieuwe specificiteiten, die niet toegewezen konden worden aan de endogene TCR of de geïntroduceerde TCR. Voornamelijk de observatie dat enkel transductie van een TCRα-, of β-keten al resulteerde in nieuwe specificiteiten, wijst erop dat deze veroorzaakt werden door TCR-dimeren. De nieuwe specificiteiten konden gericht zijn tegen HLA-klasse-I-moleculen, maar ook HLA-klasse-II-moleculen, en resulteerde in zowel alloreactieve als autoreactieve T-cellen. Door cysteïnes aan te brengen in de geïntroduceerde TCR, werd een extra zwavelbrug gecreëerd. Dit resulteerde in selectieve paring van beide geïntroduceerde TCR ketens, zodat de neoreactiviteit significant verminderd werd. We concluderen dat TCR gentransfer regelmatig resulteert in vorming van TCR-dimeren met potentieel gevaarlijke neoreactiviteit, omdat dit fenomeen in alle virusspecfieke T-cellijnen die we hebben getest optrad na introductie van verschillende TCRα-, of β-ketens. We pleiten ervoor om het risico op ontstaan van potentieel gevaarlijke TCR-dimeren te verkleinen door selectieve paring te bevorderen van de geïntroduceerde TCR-ketens, en T-celpopulaties te transduceren met een gerestricteerd TCR-gebruik.

Verbeterde expressie en functionaliteit van codon geoptimaliseerde en cysteïne gemodificeerde HA-1-TCR-en

TCR-en gericht tegen minor-antigen HA-1 zijn aantrekkelijk voor TCR gentransfer om patiënten met hematologische maligniteiten te behandelen vanwege de exclusieve expressie van HA-1 op bloedcellen. Voor sterke reactiviteit tegen de leukemiecellen is een goede expressie van de HA-1-TCR op het celoppervlak van de donor T-cel van het grootste belang. Helaas hebben we in voorgaande studies al gezien dat de HA-1-TCR laag tot expressie komt op het celoppervlak na TCR gentransfer.

In hoofdstuk 5 werd getracht de oorzaak van deze lage expressie te achterhalen, en werden verschillende strategieën bestudeerd om de HA-1-TCR-expressie te verbeteren na TCR

gentransfer. In dit hoofdstuk laten we zien dat de HA-1-TCR-expressie al laag is op de oorspronkelijke HA-1-specifieke T-celklonen uit de patiënt, en dus niet alleen laag is na TCR gentransfer. Verder tonen we aan dat de lage HA-1-TCR-expressie na TCR gentransfer niet veroorzaakt wordt door inefficiënte paring van de HA-1-TCRα- en β-ketens onderling, maar dat vooral lage expressie van de HA-1-TCRβ-keten een limiterende factor is. Van de verschillende strategieën die bestudeerd zijn, werd de meest verbeterde HA-1-TCR-expressie verkregen door het aanbrengen van een extra zwavelbrug in de HA-1-TCR-ketens te combineren met codon optimalisatie van de HA-1-TCR-ketens. Deze aanpassing van de HA-1-TCR leidde zelfs tot een hoge expressie van de HA-1-TCR in virusspecifieke T-cellen die daarvoor predominant hun endogene TCR tot expressie brachten op het celoppervlak na TCR gentransfer. Door de hoge HA-1-TCR-expressie konden TCR-gemodificeerde virusspecifieke T-cellen ook targetcellen herkennen die niet met HA-1-peptide beladen waren, maar van zichzelf HA-1-positief waren, en dit duidt op een sterke interactie tussen de T-cel en de targetcel.

Op basis van deze resultaten is een virus geconstrueerd dat voor klinische toepassing bruikbaar is. In de al in klinische studies toegepaste virale MP71 vector zijn de codon geoptimaliseerde en cysteïne gemodificeerde HA-1-TCRβ- en α-keten verbonden met een T2A sequentie ingebracht. Transfer via deze vector van de HA-1-TCR leidde zowel in virusspecifieke T-cellen met een zwak competiterend en sterk competiterend fenotype tot sterke reactiviteit tegen verschillende HA-1-positieve leukemiecellen.

Op basis van uitgebreid onderzoek, mede beschreven in dit proefschrift, zal een klinische studie worden gestart. Patiënten met hematologische maligniteiten na allo-SCT zullen behandeld worden met virusspecifieke donor-T-cellen getransduceerd met de MP71 vector coderend voor de geoptimaliseerde HA-1-TCR.

KLINISCHE STUDIE

Immunotherapie voor patiënten na allo-SCT met donor T-cellen met anti-leukemiespecificiteit zou kunnen resulteren in exclusief GvL zonder GvHD. Echter, het is niet altijd mogelijk om uit donormateriaal T-cellen met een duidelijke en exclusieve anti-leukemiereactiviteit te induceren.

Door TCR gentransfer van minor-antigenspecifieke TCR-en kan in een korte kweekperiode een T-celproduct gegenereerd worden met een selectieve anti-leukemiereactiviteit. Om te voorkomen dat de T-cellen die getransduceerd worden alsnog zelf in staat zijn om GvHD te veroorzaken, gaat de voorkeur uit naar virusspecifieke T-cellen als ontvanger cellen. Door CMV- en EBV-specifieke T-cellen te transduceren, wordt gebruik gemaakt van de capaciteit van deze cellen om lange tijd aanwezig te blijven in het lichaam, en op die manier een langdurige anti-leukemiereactiviteit te garanderen.

Op basis van uitgebreid onderzoek, mede beschreven in dit proefschrift, zal binnenkort gestart worden met een klinische studie waarin patiënten die HLA-A*0201 en HA-1 positief

zijn, behandeld kunnen worden met HA-1-TCR-gemodificeerde virusspecifieke donor-T-cellen. Een voorwaarde is wel dat de stamceldonor HA-1 negatief is, omdat de T-cellen anders in staat zijn om zichzelf en het donortransplantaat te herkennen.

Er zullen twee groepen patiënten in aanmerking komen voor de behandeling met allo-SCT gevolgd door immunotherapie met HA-1-TCR getransduceerde virusspecifieke T-cellen. De eerste patiëntengroep die in aanmerking komt voor behandeling, zijn HLA-A*0201+ en HA-1+ patiënten met refractaire leukemieën die normaliter niet in aanmerking komen voor allo-SCT. Omdat hun leukemie na bestraling en chemotherapie niet voldoende vernietigd is, is de verwachting dat, indien deze patiënten wel getransplanteerd worden, de leukemie binnen 6 maanden weer in grote hoeveelheden in het bloed aanwezig is. Door de hoge kans op GvHD kan dan nog geen DLI gegeven worden. Omdat het HA-1-TCR-gemodificeerde virusspecifieke T-celproduct ontworpen is om geen GvHD te induceren, maar wel hoge anti-leukemische reactiviteit te bezitten, kan in deze patiënten veel vroeger na allo-SCT deze immunotherapie toegediend worden.

De tweede te includeren patiëntengroep bestaat uit HLA-A*0201+ en HA-1+ patiënten met een recidief hematologische maligniteit, die niet in aanmerking komen voor DLI door een verhoogde kans op GvHD, of omdat ze niet gereageerd hebben op DLI. Voorwaarde voor deze groep patiënten is dat ze getransplanteerd zijn met een HA-1 negatieve donor, en dat er genoeg donormateriaal ingevroren is om virus-specifieke T-cellen te kunnen isoleren.

Indien deze nieuwe behandeling bij beide groepen leidt tot selectieve anti-leukemiereacties zonder GvHD in patiënten die geen andere behandelopties meer hebben, kan de behandeling ook een goed alternatief voor DLI zijn in patiënten na allo-SCT met een recidief. Verder kan bij bewezen effectiviteit de therapie uitgebreid worden door andere TCR-en te includeren. Voor deze eerste studie is gekozen voor de HA-1-TCR, omdat over dit minor-antigen en deze TCR veel bekend is. Op dit moment is de voorwaarde dat patiënten zowel HLA-A*0201 en HA-1 positief moeten zijn, en de donor HA-1 negatief, beperkend voor toepassing in alle patiënt-donor combinaties. Deze combinatie zal namelijk in ongeveer 15% van de patiënten voorkomen. Verscheidene andere TCR-en zijn nu in ons laboratorium uitgebreid gekarakteriseerd, en kunnen bij slagen van deze eerste klinische studie mogelijk ook geïmplementeerd worden.

Nawoord

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Promotie-onderzoek. Je kan het er maar druk mee hebben. Vrienden die naast hun artsenspecialisatie, waarbij ze onregelmatige diensten moesten draaien, ook nog een sociaal leven weten te onderhouden, zijn mij regelmatig tot inspiratie geweest. Ook familie, die naast het hebben van 2 eigen bedrijven, het opvoeden van 2 kinderen en het om de week naar Spanje moeten om daar systemen op de rails te zetten, nog tijd overhielden om een nieuwe zorginstelling in Zoetermeer op te richten, lieten me inzien dat 'het druk hebben' ook kan betekenen dat je je vooral 'druk maakt'. Waarmee niet gezegd is dat het niet fijn is om er een dikke streep onder, en een grote vink vóór te zetten; mijn proefschrift is af!

Natuurlijk wil ik ook graag wat mensen bij naam noemen. Het is niet meer dan logisch om te beginnen met het bedanken van Renate de Boer en Renate Hagedoorn. Samen met RenateH heb ik in een paar jaar tijd verscheidene vectoren geknutseld, met constructen in combinatie met allerlei markergenen, omdat dit zo extreem handig is. Nog steeds is zij een altijd beschikbare vraagbaak bij moleculaire proeven. Voor RenatedB lijkt geen enkel experiment te groot. Verder is het ontzettend prettig samenwerken met haar, en moppert ze nooit als ik een experiment weer eens op het laatste moment omgooi. Ik ben ontzettend blij dat zij naast mij willen staan als paranimf!

Verder heb ik het altijd prettig gevonden om binnen het TCR gen transfer groepje te vallen, dat ondertussen eigenlijk die naam ontgroeid is. Lars, Manja, Esther en Menno hebben het transduceren, sorteren en vervolgens kweken van enorme aantallen clonen tot een kunst verheven. Ik ben blij dat ik daar wat van mocht oppikken. Dankzij Avital, Dirk, Hetty, Monique en Pleun is de expertise uitgebreid naar karakterisatie van diverse T-cel responsen, waarvan die tegen schimmels misschien nog wel het nuttigst voor T-cel kweken is…De studenten Gerdien Volbeda en Vishant Jankipersadsing wil ik bedanken voor hun enthousiasme en inzet tijdens hun stage op de afdeling.

Met mijn werkplekje op de 5e verdieping heb ik als AIO veel mazzel gehad. Inge en Marieke waren, en zijn, nooit te beroerd om even hun licht te laten schijnen op ingewikkelde experimenten, waardoor ze de eretitel 'schaduwbegeleiders' van Jorrit toebedeeld kregen. Ook de rest van mijn kamergenoten op achtereenvolgens de 5e, 7e en 6e verdieping wil ik bedanken voor zowel de wetenschappelijke als niet-wetenschappelijke discussies.

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Holst en Reinier van der Linden had dit proefschrift nog véél langer op zich laten wachten.

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En natuurlijk zijn er de mensen die ik niet bij name hóéf te noemen. Jullie onophoudelijke steun en geloof in mij wordt nooit als vanzelfsprekend aangenomen; bedankt!

Curriculum vitae

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Marleen van Loenen werd geboren op 10 januari 1980 te Oss. In 1998 behaalde zij haar gymnasiumdiploma aan het Titus Brandsmalyceum te Oss en begon in datzelfde jaar met de studie Biomedische Wetenschappen aan de Universiteit Leiden. Tijdens deze studie liep zij twee wetenschappelijke stages. Onder begeleiding van dr. Jan Wondergem en ir. Marjan Boerma heeft zij bij de vakgroep Klinische oncologie in het Leids Universitair Medisch Centrum (LUMC) onderzoek gedaan naar het effect van ioniserende straling op het hart in een rattenmodel. Haar tweede stage liep ze op de afdeling Hematologie binnen het LUMC onder begeleiding van dr. Mirjam Heemskerk. Met behulp van de IFN-γ capture assay werd getracht minor-antigen- of leukemie-specifieke T-cellen direct ex vivo te isoleren. Zij behaalde haar doctoraalexamen in 2003. In datzelfde jaarbegon zij aan het in dit proefschrift beschreven promotieonderzoek onder leiding van dr. Mirjam Heemskerk en prof. dr. Fred Falkenburg op het laboratorium voor Experimentele Hematologie van de afdeling Hematologie (hoofd prof.dr. Roel Willemze). Sinds maart 2008 is ze werkzaam als wetenschappelijk onderzoeker op dezelfde afdeling en betrokken bij het opzetten van een fase I/II klinische studie. In deze klinische studie zal de effectiviteit van behandeling met HA-1-TCR gemodificeerde T-cellen na allogene stamceltransplantatie als therapie voor acute leukemie onderzocht worden.

LIST OF PUBLICATIONS

Accepted publications

1. **M.M. van Loenen, R.S. Hagedoorn, R. de Boer, HM. van Egmond, J.H.F. Falkenburg, M.H.M. Heemskerk**
   Rapid re-expression of retrovirally introduced versus endogenous TCRs in engineered T cells after antigen-specific stimulation.
   J Immunother. 2011 Mar;34(2):165-74.
2. **M.M. van Loenen, R. de Boer, R.S. Hagedoorn, H.M. van Egmond, J.H.F. Falkenburg, M.H.M. Heemskerk**
   Optimization of the HA-1-specific T-cell receptor for gene therapy of hematological malignancies.
   Haematologica. 2010 Nov 25. [Epub ahead of print]
3. **M.M. van Loenen, R. de Boer, A.L. Amir, R.S. Hagedoorn, G.L. Volbeda, R. Willemze, J.J. van Rood, J.H. Falkenburg, M.H. Heemskerk**
   Mixed T cell receptor dimers harbor potentially harmful neoreactivity.
   Proc Natl Acad Sci USA. 2010 Jun 15;107(24)10972-7.
4. **A.L. Amir, L.J. D'Orsogna, D.L. Roelen, M.M. van Loenen, R.S. Hagedoorn, R. de Boer, M.A. van der Hoorn, M.G. Kester, I.I. Doxiadis, J.H. Falkenburg, F.H. Claas, M.H. Heemskerk**
   Allo-HLA reactivity of virus-specific memory T cells is common.
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5. **M.M. van Loenen, R.S. Hagedoorn, M.G. Kester, M. Hoogeboom, R. Willemze, J.H. Falkenburg, M.H. Heemskerk**
   Kinetic preservation of dual specificity of coprogrammed minor histocompatibility anigen-reactive virus-specific T-cells.
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6. **A.U. van Lent, M. Nagasawa, M.M. van Loenen, R. Schotte, T.N. Schumacher, M.H. Heemskerk, H. Spits, N. Legrand**
   Functional human antigen-specific T cells produced in vitro using retroviral T cell receptor transfer into hematopoietic progenitors.
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7. **L.T. van der Veken, R.S Hagedoorn, M.M. van Loenen, R. Willemze, J.H. Falkenburg, M.H. Heemskerk**
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8. **M. Boerma, J.J. Kruse, M. van Loenen, H.R. Klein, C.I. Bart, C. Zurcher, J. Wondergem**
   Increased deposition of von Willebrand factor in the rat heart after local ionizing irradiation.
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Submitted publications

1. **A.L. Amir, D.M. van der Steen, M.M. van Loenen, R.S. Hagedoorn, R. de Boer, M.D.G. Kester, A.H. de Ru, G.J. Lugthart, C. van Kooten, P.S. Hiemstra, I. Jedema, M. Griffioen, P. van Veelen, J.H. F. Falkenburg, M.H.M. Heemskerk**
   PRAME specific allo-HLA restricted T-cells with potent antitumor reactivity as source for therapeutic TCR gene transfer.

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CHAPTER 1 - INTRODUCTION

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CHAPTER 2 - RE-EXPRESSION

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CHAPTER 3 - MINOR HISTOCOMPATIBILITY

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CHAPTER 4 - MIXED DIMERS

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