T-control: T-cell therapy in the context of allogeneic stem cell transplantation
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Citation
Roex, M. C. J. (2021, May 27). *T-control: T-cell therapy in the context of allogeneic stem cell transplantation*. Retrieved from https://hdl.handle.net/1887/3166759

Version:	Publisher's Version
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Cover Page

The handle http://hdl.handle.net/1887/3166759 holds various files of this Leiden University dissertation.

Author: Roex, M.C.J.
Title: T-control: T-cell therapy in the context of allogeneic stem cell transplantation
Issue date: 2021-05-27

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T-CELL THERAPY IN THE CONTEXT OF ALLOGENEIC STEM CELL TRANSPLANTATION

MARTHE ROEX

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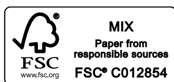
ISBN:	978-94-6419-193-6
Cover painting:	Evelien Jagtman | www.evelienjagtman.com
Lay-out:	Ilse Modder | www.ilsemodder.nl
Printed by:	Gildeprint Enschede | www.gildeprint.nl



Printing of this thesis was financially supported by Miltenyi Biotec.

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T-CONTROL:
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Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op donderdag 27 mei 2021
klokke 15.00 uur

door

Marthe Catharina Johanna Roex
Geboren te Enschede in 1987

Promotor:	Prof. dr. J.H.F. Falkenburg
Co-promotor:	Dr. I. Jedema
Leden promotiecommissie:	Prof. dr. H.J. Guchelaar Prof. dr. M.J. Kersten, Amsterdam UMC Prof. dr. T. Mutis, Amsterdam UMC

The work presented in this thesis was performed at the Department of Hematology of the Leiden University Medical Center and was financially supported by the European Union's seventh Framework Program (FP/2007-2013).

TABLE OF CONTENTS

Chapter 1	General introduction and aim of the thesis	9
Chapter 2	Effect of alemtuzumab-based T-cell depletion on graft compositional change *in vitro* and immune reconstitution early after allogeneic stem cell transplantation *Cytotherapy 2021; 23(1): 45-56.*	37
Chapter 3	The simultaneous isolation of multiple high and low frequent T-cell populations from donor peripheral blood mononuclear cells using the major histocompatibility complex I-*Strep*tamer isolation technology *Cytotherapy 2018; 20(4): 543-555.*	67
Chapter 4	Generation and infusion of multi-antigen-specific T cells to prevent complications early after T-cell depleted allogeneic stem cell transplantation – a phase I/II study *Leukemia 2020; 34(3): 831-844.*	93
Chapter 5	A minority of T cells recognizing tumor-associated antigens presented in self-HLA can provoke antitumor reactivity *Blood 2020; 136(4): 455-467.*	125
Chapter 6	Summary and general discussion	161
Appendices	Nederlandse samenvatting	185
	Author affiliations	194
	Abbreviations	198
	List of publications and conference presentations	202
	Curriculum vitae	208
	Dankwoord	210

CHAPTER 1

GENERAL INTRODUCTION AND AIM OF THE THESIS

1

GENERAL INTRODUCTION

HEMATOPOIETIC STEM CELL TRANSPLANTATION

The ideal therapy to treat patients with hematologic malignancies is to eliminate malignant cells in the body of the patient, while healthy cells remain unaffected. Unfortunately, strategies that only specifically and efficiently attack hematopoietic malignant cells are not available thus far. The best available treatment regimens consist of remission induction therapy followed by consolidation therapy using chemotherapy, immunotherapy and irradiation, with severe side-damage to healthy hematopoietic cells. In patients with high-risk malignancies, hematopoietic stem cell transplantation can be performed as part of the consolidation therapy to rebuild a healthy hematopoietic system. After transplantation, stem cells migrate to the bone marrow and have the ability to proliferate and differentiate into mature healthy blood cells. The infusion of stem cells harvested from the patient before the consolidation therapy is called autologous stem cell transplantation (autoSCT). As an alternative, stem cells from a healthy donor can be used for an allogeneic stem cell transplantation (alloSCT).[1-3] Hematopoietic stem cells can be harvested directly from the bone marrow (BM) but are nowadays usually acquired via leukapheresis from the peripheral blood (PB) after mobilization from the bone marrow by administration of granulocyte colony stimulating factor (G-CSF).[4,5] In alloSCT, both stem cells from a related or an unrelated donor can be used.

Traditionally, myeloablative (MA) conditioning regimens have been used in SCT to maximally eradicate malignant cells and allow engraftment of the stem cells into the bone marrow of the patient using high doses of chemotherapy, immunotherapy, total body irradiation (TBI) and immune suppression. Due to considerable toxicity, feasibility of this therapy was limited to fit and young patients. However, the conditioning regimen alone has shown to be not sufficient to prevent relapse of the hematologic malignancy. This is illustrated by the high relapse rates after autoSCT or genetically identical (using an identical twin) stem cell transplantations in patients with high-risk acute leukemia, in contrast to alloSCT.[6] The long-term curative effect of alloSCT is mediated by donor-derived T cells that are able to recognize and persistently eradicate residual malignant cells of the patient. This beneficial phenomenon is known as the graft-versus-leukemia (GVL) effect.[7-9] Since this GVL effect has shown to be responsible for the curative potential of alloSCT, less toxic reduced intensity or non-myeloablative (NMA) conditioning regimens have been developed to broaden the curative potential of alloSCT to patients of higher age and with co-morbidities.[10,11] These conditioning regimens aim to allow engraftment of donor hematopoietic stem cells without fully eliminating the hematopoietic system of the patient, but have the disadvantage of higher relapse rates compared to MA conditioning.

Although donor-derived T-cell responses are able to initiate GVL after alloSCT, donor-derived T-cell

responses can also be directed against healthy cells in the tissues and organs of the patient which can result in detrimental graft-versus-host disease (GVHD). The major challenge in the field of alloSCT is to find a balance between the prevention of GVHD while maintaining GVL and immunity for the protection against foreign pathogens like viruses. As these harmful and beneficial immune responses after alloSCT are mediated by T cells, knowledge about the biology of T cells is necessary to understand and further develop strategies to improve the curative effect of alloSCT.

BIOLOGY OF T CELLS

Within the hematopoietic system, two types of T cells can be distinguished: T-cell receptor (TCR) gamma-delta (γ/δ) T cells that are part of the innate immune system and TCR alpha/beta (α/β) T cells that play a major role in adaptive immune responses to control viral infections. In this thesis, we focus on α/β T-cell responses.

TCR-HLA interaction

To provoke an adaptive immune response, TCR α/β T cells need to be stimulated via their TCR that recognizes peptides (antigens) in the context of human leukocyte antigen (HLA) molecules, encoded by the major histocompatibility complex (MHC) on antigen-presenting cells. The TCR of an individual T cell is specific for a particular peptide-HLA combination. The strength by which a TCR interacts with a peptide-HLA complex is termed TCR-peptide-HLA affinity. This TCR-peptide-HLA affinity in combination with additional interactions between the T cell and antigen-presenting cells via adhesion and costimulatory molecules determines the strength by which a T cell binds to a target cell, called T-cell avidity. All nucleated cells express HLA class I molecules (HLA-A, -B, -C), which present peptides derived from intracellular proteins on their cell surface to $CD8^{pos}$ T cells. HLA class II molecules (HLA-DP, -DQ, -DR) are under physiological conditions mainly present on cells of the hematopoietic system, which can process and present peptides derived from both intra- and extracellular proteins to $CD4^{pos}$ T cells.[12,13]

Thymic selection of T cells

During T-cell development, TCR are generated by a complex process including recombination of variable, joining and constant gene segments followed by random insertion and deletion of nucleotides, as well as the pairing of different α- and β-TCR chains.[14] Therefore, the diversity of randomly generated TCR is enormous. Thymocytes (T-cell precursors) derived from the bone marrow are educated in the thymus to ensure a peripheral T-cell repertoire consisting of mature T cells containing TCR that are able recognize pathogens from outside the body, like viruses, but do not elicit harmful immune responses against cells of the own body.[15,16] In the thymic cortex, thymocytes expressing an α/β TCR that are able to recognize peptides presented in the context of self-HLA with at least low affinity are positively selected, while thymocytes that do not interact with peptides in self-HLA at all are eliminated. After $CD4^{pos}$ or $CD8^{pos}$ lineage commitment, these

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T-cell precursors undergo negative selection in the thymic medulla. Hereby, thymocytes that recognize self-peptides in the context of HLA with high affinity are eliminated in order to prevent self-directed immune responses which may cause auto-immune diseases.[17-19] Eventually, the T-cell repertoire in the peripheral blood of an individual is expected to consist of a huge variety of T cells that are capable of recognizing all kinds of peptide-HLA complexes different from the combination of self-peptides presented in self-HLA.[20,21]

T-cell subsets

Several subtypes of mature CD4^{pos} and CD8^{pos} T cells can be distinguished based on their phenotype and their functional characteristics.[22] T cells that have not yet encountered their particular antigen are called naïve T cells. To provoke an immune response, naïve T cells need stimulation via professional antigen-presenting cells, that besides high levels of peptide-HLA complexes also express co-stimulatory molecules and adhesion molecules. After this initial stimulation, naïve T cells rapidly expand and differentiate into effector cells that are able to migrate to infected tissue and carry out specialized T-cell functions like cytokine production and cytotoxic activity. After elimination of infected cells, most proliferated T cells die, while a minority of T cells differentiate into resting memory T cells. Whereas the naïve T-cell repertoire directed against a certain antigen contains a broad range of avidities, the memory T-cell repertoire specific for the same antigen primarily exists of high-avidity T cells. Upon a second encounter with the same antigen, these memory T cells can become easily activated, resulting in rapid and effective expansion, differentiation and elimination of infected cells.[23] Besides the mentioned T-cell subsets that stimulate immune responses, a special type of CD4^{pos} T cells suppresses and downregulates induction and proliferation of effector T cells. These regulatory T cells are thought to modulate immune responses, maintain tolerance to self-antigens and prevent auto-immunity.[24]

ALLOREACTIVE T-CELL RESPONSES: GVL AND GVHD

Allogeneic stem cell grafts contain 1-5% donor-derived hematopoietic stem cells, implying that other immune cells like lymphocytes of donor-origin are part of the stem cell graft and are transferred into the patient. In general, PB-derived grafts contain 5-10 times more stem cells compared to BM-derived grafts, which favors stem cell engraftment in the patient but also results in higher T-cell counts in the graft.[4,25] Because donor T cells in the graft are educated in the thymus of the donor, these T cells are only tolerant to self-antigens of donor origin. After alloSCT, alloreactive T-cell responses occur when donor-derived T cells that are educated in the donor, recognizing patient cells as foreign. Dependent on the tissue distribution of the recognized antigen, the immune response will result in GVL or GVHD. GVL is initiated if only hematopoietic cells (containing the malignant cells) of patient origin are recognized by donor-derived T cells, while GVHD is induced if healthy tissue cells of the patient are attacked by donor-derived T cells. The risk of GVHD is increased after HLA-mismatched transplantation, because donor-derived T

cells transferred with the graft might recognize allogeneic HLA molecules on healthy tissue cells of the patient as foreign. Therefore, HLA-matched alloSCT is preferred over HLA-mismatched alloSCT. To allow the separation of GVL from GVHD after HLA-matched alloSCT, knowledge about antigens that have the potential to induce beneficial alloreactive T-cell responses is essential.

Minor histocompatibility antigens

Also after HLA-matched alloSCT, strong alloreactive immune responses can occur. Genetic differences between patient and donor that give rise to polymorphic peptides presented in matched HLA-molecules on patient cells that are recognized by donor T cells are called minor histocompatibility antigens (MiHA).[26,27] These polymorphisms occur at the level of single or multiple base pairs, due to singe nucleotide polymorphisms, base pair insertions or deletions, or copy number variations. Generally, the immune system of an individual has not been exposed to MiHA of another individual. Therefore, MiHA-specific T-cell responses after alloSCT have exclusively been described in MiHA^{pos} patients receiving grafts from MiHA^{neg} donors, because high-avidity MiHA-specific T cells are supposed to pass thymic selection only in MiHA^{neg} donors.[28-31] As a consequence, MiHA-specific T cells in MIHA^{neg} donors are expected to be present in the naïve T-cell repertoire. High-avidity donor T cells that are capable of recognizing immunogenic peptides on patient cells may lead to destruction of the cells expressing this MiHA, without impairment of donor-derived cells. Both the tissue distribution of the gene encoding the MiHA as well as the expression of HLA molecules determines the clinical effect of the alloreactive T-cell response. Since HLA class I molecules are normally expressed by all nucleated cells, donor T cells that recognize HLA class I-restricted MiHA derived from proteins exclusively expressed by (malignant) hematopoietic cells of the patient are likely to cause GVL, while donor T cells that recognize HLA class I-restricted MiHA that are broadly expressed on both hematopoietic and non-hematopoietic tissues of the patient can mediate both GVL and GVHD at the same time.[26,31-33] In contrast, HLA class II-restricted MiHA can be considered as relatively hematopoiesis-specific, because HLA class II expression is under non-inflammatory conditions limited to mainly hematopoietic cells. After the initial stimulation, differentiation and expansion of MiHA-specific T-cells of donor origin, the immune response will decline when MiHA^{pos} patient cells are eradicated and a memory T-cell response may develop. This memory response is relevant for sustained and prolonged suppression of MiHA^{pos} cells. Several clinical observational studies have shown a direct association between emergence of T cells specific for hematopoiesis-restricted MiHA (eg, HA-1 and HA-2), and elimination of malignant cells post-alloSCT in the absence of extended GVHD.[29] Therefore, many effort has been made to identify hematopoiesis-restricted MiHA via several strategies.[26,34-37] However, the tissue distribution of MiHa is not the only determinant that separates GVL from GVHD. Inflammatory environmental circumstances can render non-hematopoietic cells susceptible to T-cell recognition of broadly expressed MiHa. Because the magnitude and diversity of alloreactive T-cells responses in patients with selective GVL reactivity have shown to be lower than in patients with GVL

combined with GVHD, it is suggested that limited GVHD also benefits GVL.[28]

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Tumor-related antigens

Another group of antigens that have the potential to give rise to beneficial T-cell responses after alloSCT are tumor-related antigens. Tumor cells can express proteins that are essential or associated with their malignant phenotype. Within this group of tumor-related antigens, several categories can be distinguished, like tumor-associated virus antigens, tumor-specific antigens and tumor-associated antigens (TAA).[38-42] For example the Epstein-Barr virus (EBV) and human T-cell lymphotropic virus type 1 (HTLV-1) can be involved in the formation of hematologic malignancies, especially lymphoid neoplasms.[43] Since these antigens are non-self and are (over)expressed by tumor cells, donor-derived T cells can recognize these antigens as foreign. However, only a limited number of hematologic cancers is initiated by viruses and express these antigens. Tumor-specific antigens or neoantigens are antigens derived from mutated oncogenes or tumor suppressor genes, or chromosomal translocations. Since these mutations or translocations only occur in the tumor cells and not in healthy cells, these newly formed antigens are tumor-specific. Examples of mutations that give rise to mutant antigens in AML patients are internal tandem duplications of the FMS-like tyrosine kinase 3 gene (Flt3) and mutations in the nucleophosmin 1 (NPM1) gene.[44,45] Nonetheless, the majority of tumor-related antigens belong to the group of TAA comprising non-mutated monomorphic self-antigens like differentiation antigens, aberrantly expressed antigens (eg, WT1, RHAMM, proteinase-3)[46-49] and cancer-germline antigens (also known as cancer-testis antigens; eg, PRAME, NY-eso-1).[50-52] These antigens are expected to be overexpressed in malignant cells, while expression is absent or low in healthy cells. Several studies have suggested that T cells recognizing these self-antigens may contribute to antitumor reactivity after HLA-matched alloSCT.[38,39,49,53-65] A relation has been proposed between expansion of TAA-specific T cells in the peripheral blood of patients and better relapse-free survival.[66] In addition, disease relapses have been observed in patients in the absence of TAA-specific T cells.[67-72] Furthermore, multiple phase I/II vaccination studies have targeted TAA in patients with hematologic malignancies.[56-65] Although in a minority of patients clinical responses coincided with increased frequencies of TAA-specific T cells in peripheral blood, a causative relation between the induction of high-avidity TAA-specific T cells and clinical effect has not been proven so far.[56-65] Due to these inconsistent results, the value of TAA as targets to boost GVL-responses needs to be exploited in more detail.

PREVENTION OF GVHD

Although donor-derived T-cells transferred with the graft can elicit alloreactive T-cell responses resulting in GVL, the administration of T cells together with stem cells of donor origin will simultaneously induce GVHD in the majority of patients. GVHD can present in an acute or chronic state, in several grades of severity, commonly affecting patient’s skin, gut, liver and/or lungs.[73-76,15,16] Since this dominant complication is associated with high morbidity and mortality,

several strategies have been studied to reduce the risk of GVHD.[73,74,77,78]

HLA-matching

Since most tissue cells express HLA class I, mismatches for HLA class I-molecules between patient and donor often result in GVHD when donor T cells recognize healthy patient tissues, or graft rejection when residual patient T cells recognize donor stem cells. Therefore, it is preferred to match HLA-alleles between donor and patient. Only 25% of a patient's siblings are statistically HLA-identical, limiting the chance to find an HLA-matched related donor for a specific patient. Since the variation in HLA-polymorphisms is enormous, the chance of finding a matched unrelated donor in an international data bank is highly dependent on the patient's genetic background. For Caucasian patients, the chance of finding a 10/10 HLA-matched donor (only mismatched on HLA-DP allele(s)) is about 50-70%, but this chance will drop to 10-15% for patients with a non-Caucasian background.[79-81] Although the risk of (extensive) GVHD can be diminished by HLA-matching, HLA-matching alone is not sufficient to prevent GVHD.

Immune suppressive medication

Many transplant centers do not manipulate the composition of the grafts before administration to the patient, which is called a T-cell replete alloSCT. Using this approach, long-term prophylactic immunosuppressive medication like cyclosporine A is indicated for several months to years to prevent GVHD. Since pre-clinical models have demonstrated that especially donor-derived TCR α/β T cells with a naïve phenotype are the major players in the development of GVHD, strategies have focused on either the reduction of potentially alloreactive T cells in the patient (*in vivo)* or in the graft before administration to the patient (*in vitro).*[82]

In vivo T-cell depletion

In vivo T-cell depletion (TCD) using antibodies can be applied as part of the conditioning regimen before transplantation. A lot of experience has been obtained with alemtuzumab (ALT) and anti-thymocyte globulin (ATG). ALT is a humanized monoclonal antibody of the IgG1 type which targets the glycophosphatidylinositol (GPI)-anchored protein CD52.[83] This antigen is expressed on mature lymphocytes at different levels and not (or only marginally) on hematopoietic stem and progenitor cells.[84] ATG is a polyclonal antibody that targets several antigens that are mainly expressed on T cells in blood and peripheral lymphoid tissue.[85] When ALT and/or ATG are administered to the patient before infusion of the graft, the effect is both directed against residual patient-derived T cells that survived the chemotherapy and/or TBI included in the conditioning regimen, as well as against donor-derived T cells that are administered together with the stem cells during transplantation. Elimination of patient-derived T cells may favor the risk of graft rejection and may thereby support engraftment after transplantation.[86] Especially in HLA-mismatched transplantation, reducing the risk of graft rejection is of major importance. Elimination of donor-derived T cells is of importance

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to reduce the risk of acute and chronic GVHD.

In vitro T-cell depletion

In vitro TCD is achieved by manipulation of the graft before infusion into the patient. One strategy is the elimination or selection of specific cell populations by using antibody-coated magnetic beads and magnetic separation.[87-93] Selection of CD34^{pos} cells can be achieved by the physical positive isolation of CD34^{pos} cells to create a purified graft of stem and progenitor cells, while only a very limited number of T cells is preserved.[88,89] Although clinical applications showed stable engraftment and low incidence of acute and chronic GVHD in the absence of immunosuppressive therapy, concerns regarding the risk for disease relapse and opportunistic viral infections remained. Therefore, the application of limited T-cell addback to CD34^{pos} selected T-cell grafts has been explored to support early T-cell reconstitution and preserve protective immunity after alloSCT.[92] These observations suggest that a limited amount of T cells in the graft is necessary for early immune reconstitution and protection against viral reactivations. Another strategy is the *in vitro* selective depletion of TCR α/β T cells from stem cell grafts.[90,91] This approach is thought to results in the efficient depletion of TCR α/β T cells while TCR γ/δ T cells are expected to be preserved in the grafts, leading to a significant reduction in GVHD incidence while a sustained reactivity against pathogens is maintained.

ALT can also be added directly to the stem cell graft before infusion, known as *in vitro* TCD or 'ALT to the bag'. Using this strategy, donor-derived CD52^{pos} cells can be depleted already before graft infusion into the patient. Although the exact effect of ALT addition to grafts on graft composition is not studied extensively, it has shown to be a very effective and fast method for prevention of GVHD as part of both MA and NMA conditioning.[94-98]

Although these mentioned approaches to prevent GVHD result in a significant decrease in incidence and severity of GVHD, beneficial T-cell responses are also impaired by the use of immunosuppressive medication and/or TCD. The GVL effect will be diminished, leading to a high risk of disease relapse. Furthermore, ALT- and/or ATG-based TCD may lead to an increased incidence of viral reactivations compared to non-TCD alloSCT protocols, resulting in substantial morbidity.[99,100] However, the incidence of viral disease has shown to be comparable between these two protocols, suggesting that close monitoring and pre-emptive antiviral therapy might prevent the progression from viral reactivation to viral disease.[96,101-103]

VIRAL COMPLICATIONS AFTER TCD alloSCT

The major viral pathogens causing serious morbidity and mortality after alloSCT are the cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human adenovirus (AdV). In immune competent individuals, infections with these viruses normally occur during childhood or

adolescence and are accompanied by mild or even absent symptoms and have a self-limiting character. Professional antigen-presenting cells are required for the induction of a primary T-cell responses upon first infection, leading to a rapid increase of effector T cells and the formation of memory T cells. Because these viruses will not be entirely cleared from their host but remain latently present in immune and tissue cells, this immunological memory is very important to remain protected against viral reactivations. However, during a state of immunodeficiency as in the period after alloSCT, this protective immunity is destroyed by the conditioning regimen and reactivations are not any longer controlled by memory T cells. In the absence of protective immunity, viral infected cells are not eliminated and the virus can replicate uncontrolled.

Multiple factors influence the timing, rate and diversity of cellular immune reconstitution after alloSCT, like conditioning regimen (including TCD), patient age, stem cell source, occurrence of GVHD and the use of immunosuppressive therapy.[104-107] T-cell reconstitution can be considerably delayed due to TCD of the graft, and is often incomplete. Sources for T-cell reconstitution can be both patient-derived T cells that survived the conditioning regimen and donor-derived T cells that are transferred with the transplant. These mature T cells from patient or donor origin may also comprise virus-specific T cells with a memory phenotype that can readily provide protection against viral reactivations. Besides that, *de novo* generated naïve T cells derived from the transplanted donor stem cells and educated in the patient thymus are another source of T-cell reconstitution. However, these latter T cells need time to develop, to receive adequate stimulation by professional antigen-presenting cells and to expand until appropriate cell numbers are reached to fight a primary viral infection or viral reactivation. Although these *de novo* developing T cells are of importance for rebuilding the immune system in the long term, these cells only have a limited role in the control of viral complications in the critical first months after TCD alloSCT.

<u>Cytomegalovirus</u>

CMV is responsible for the majority of viral reactivations following alloSCT. The risk of reactivation of CMV is dictated by the serostatus of both patient and donor.[108,109] Between 45-60% of patients that receive an alloSCT, have encountered CMV before (seropositive patients) and are at risk for an endogenous CMV reactivation after transplantation.[110,111] CMV infection of a CMV-seronegative patient via a stem cell graft from a CMV-seropositive donor can occur, but is less common. Ultimately, around 80% of CMV-seropositive patients will encounter a CMV reactivation after alloSCT.[112] The CMV reactivation may progress to CMV disease characterized by potentially fatal organ involvement, such as CMV pneumonia, colitis or encephalitis.[113] The availability of antiviral agents like (val)ganciclovir and foscarnet have contributed to a significant reduction in CMV-related morbidity and mortality following TCD alloSCT. However, administration of these drugs is limited by adverse effects and possible development of resistance, and antiviral therapy has only a temporary effect.[114,115] Subsequently, the incidence of CMV disease is still 10% in the first year

after alloSCT in CMV-seropositive patients.[113]

Epstein-Barr virus

Almost 90% of the population is EBV seropositive at adult age.[116] After an active infection, the virus latently resides in B cells. In patients after alloSCT, both residual patient-derived B cells that survived the conditioning regimen as well as transferred donor-derived B cells with the graft, are a source for EBV reactivations. An impaired immune system may not be able to prevent the massive expansion of EBV-infected B cells leading to potentially fatal post-transplant lymphoproliferative disease (PTLD) in about 0.5-17% of patients.[117] In case TCD strategies only focus on the elimination of T cells, donor-derived B cells in the graft remain an important risk factor. However, using *in vitro* ALT-based TCD strategies, the risk of EBV-PTLD is not significantly increased compared to T-cell replete alloSCT, because B cells also highly express CD52 and are eliminated by ALT.[118,119] Both prevention and therapy of EBV-PTLD rely on rituximab targeting the CD20-antigen which is expressed by both EBV-infected B cells as well as healthy B cells.

Adenovirus

AdV reactivations after alloSCT show a high incidence particular in pediatric patients, while the incidence in adult patients is around 3-20%.[100,120] AdV infections can progress to severe localized or disseminated disease, which is associated with high mortality rates. Reconstitution of AdV-specific T cells has been demonstrated to be essential to control AdV infections after alloSCT.[121,122] The efficacy of antiviral treatment for AdV infections like cidofovir is still under investigation and is associated with severe toxic effects.[123]

DONOR LYMPHOCYTE INFUSION

TCD can efficiently reduce the risk of acute and chronic GVHD. However, by reducing or eliminating T cells from the graft, the curative GVL effect and viral immunity are abrogated as well as. To (re)introduce GVL effect and viral protection after TCD alloSCT, the concept of donor lymphocyte infusions (DLI) has been developed.[124] In this approach, unselected lymphocytes from the stem cell donor are administered to patients after TCD alloSCT, with the aim to induce durable remission of persistent or relapsed disease. Timing, clinical setting and dosing of DLI determines whether GVL effect and viral immunity can be promoted while the risk and intensity of GVHD remains acceptable.[125] A longer time period between alloSCT and DLI is associated with lower intensity of GVHD and allows infusion of higher doses of DLI. By postponing the administration of donor lymphocytes, the inflammatory environment and the tissue damage caused by the conditioning regimen is gradually resolved. Moreover, the majority of patient antigen-presenting cells have been replaced by donor antigen-presenting cells reducing the presentation of patient-derived antigens and alloreactive T-cell activation directed against (healthy) patient tissue. Because immunosuppressive drugs are not indicated after TCD, transferred T cells of the DLI can proliferate

and function without exogenous inhibition, and the post-transplant lymphopenic condition of patients allows the homeostatic proliferation of T cells.[126] DLI can be applied in a prophylactic, pre-emptive or therapeutic settings. Prophylactic administration is usually administered at a pre-defined moment to prevent disease relapse, regularly 3-6 months post-alloSCT. Later application of prophylactic DLI lowers the risk of GVHD, but comes at the cost of increased risk of disease relapse in the meantime. Pre-emptive and therapeutic administration of DLI can be applied in case patients suffer from mixed-chimerism or early disease relapses.[127,128] Besides the introduction of a GVL effect, the therapeutic administration of unmanipulated DLI from virus-seropositive donors has also been administered in the setting of refractory viral reactivations to boost the virus-specific immune system.[129-131] The optimal dosing of DLI depends on the conditioning regimen, donor source, time after transplantation and clinical setting. For example, DLI after NMA conditioning, obtained from an unrelated donor, infusion early after alloSCT or applied in a prophylactic setting only allows low doses of DLI to reduce the risk of GVHD.[125,132,133]

ADOPTIVE T-CELL THERAPY

Although the two step approach of TCD alloSCT followed by unmanipulated DLI may be an efficient and relatively safe way to treat patients with hematologic malignancies, patients are vulnerable to disease relapses and viral reactivations in the period between TCD alloSCT and unmanipulated DLI.[127,128] Therefore, the adoptive transfer of selected T-cell populations with exclusively beneficial effects is highly desirable early after TCD alloSCT. Prerequisites for the broad application of selective T-cell therapy is the knowledge of targetable antigens that are shared between patients and the feasibility of isolation methods for clinical application.

Virus specific T-cell therapy

Much experience has been gained with the generation of virus-specific T-cell products to prevent or treat viral reactivations or viral disease.[134,135] Initially, virus-specific T-cell lines were created by *in vitro* repetitive antigenic stimulation of T cells with (pools of) overlapping peptides followed by long-lasting expansion in the presence of interleukin-2 to generate virus-specific T-cell products.[136-139] However, the *in vivo* efficacy and long-term survival of virus-specific T-cell lines after administration was disappointing, attributed to the abrupt withdrawal from IL-2 in combination with functional and phenotypical changes of T cells initiated during the culture period.[140,141] Furthermore, long-term culture periods make this strategy not applicable for the treatment of patients with rapid progressive viral disease. Further efforts have been made to develop methods to directly isolate $CD4^{pos}$ and/or $CD8^{pos}$ virus-specific T cells from the blood of seropositive donors followed by short time culturing or direct infusion without *in vitro* expansion. These T cells are supposed to proliferate more efficiently under physiological conditions *in vivo* compared to extensively *in vitro* cultured T cells. In this respect, peripheral blood of virus seropositive donors were stimulated with viral peptides, after which activated $CD4^{pos}$ and $CD8^{pos}$ T cells were isolated

based on an activation-induced effect, like the secretion of cytokines (eg, interferon gamma) or the expression of activation markers (eg, CD137) at the cell surface.[142-150] These studies show that the generation of CMV, EBV and AdV-specific T-cell lines is feasible, administration is safe and enduring efficacy could be demonstrated by simultaneous appearance of virus-specific T cells in the peripheral blood in concordance with viral control. The isolation of CD8^{pos} peptide-specific T cells can also be achieved using MHC I-multimers (tetramers) or MHC I-*Strep*tamers (Figure 1). Both are techniques to isolate T cells based on the specificity of their T-cell receptor and are independent of T-cell kinetics of cytokine production or activation marker expression. However, these approaches require knowledge of defined viral peptides restricted to prevalent MHC I molecules and are not available for the isolation of CD4^{pos} T cells due to the lack of functional MHC class II-multimers or -*Strep*tamers. In contrast to MHC I-tetramers, the MHC I-*Strep*tamer technology is designed for clinical grade isolation of T-cell populations, since MHC I-*Strep*tamer-complexes can be dissociated from the T cells resulting in noncoated, unlabelled antigen-specific T-cell products with a preserved T-cell function, suitable for direct clinical administration.[151,152] The feasibility of this Good Manufacturing Practice (GMP) compliant technology was demonstrated in various (pre-)clinical studies by the isolation of very pure CD8^{pos} T-cell products containing acceptable numbers of contaminating T cells.[153-157] Although CD4^{pos} helper T cells are thought to contribute to *in vivo* survival, persistence and function of CD8^{pos} T cells, the infusion of CD8^{pos} CMV- or EBV-specific T-cell populations reported promising results regarding T-cell expansion and clinical outcomes as applied in the therapeutic setting. Furthermore, previous observations suggested that the adoptive transfer of a minimum of 250–5,000 virus-specific T cells/kg body weight of the patient is sufficient for virus control in the therapeutic setting, encouraging the infusion of direct *ex vivo* isolated T cells without *in vitro* expansion.[146,156,158,159] Recently, the generation of T-cell products specific for multiple antigens derived from different viruses has been proposed to provide viral disease prophylaxis or treatment after (TCD) alloSCT.[156,160,161] However, the optimal strategy for fast generation, safe administration and clinical efficacy of multi-virus specific T-cell products needs improvement to incorporate their application in standard clinical practice.

MiHA-specific T-cell therapy

MiHA can be therapeutically relevant for treatment strategies aimed to promote GVL without initiating GVHD. Theoretically, the adoptive transfer of high-avidity T cells directed against hematopoiesis-restricted MiHA isolated from MiHA^{neg} donors seems a suitable strategy. However, T-cells directed against MiHA are expected to derive from the donors naïve T-cell repertoire, implying a very low frequency in peripheral blood. This makes the enrichment of MiHA-specific T cells even more complex than the obtainment of virus-specific T-cells from seropositive healthy donors, and theoretically similar to the isolation of virus-specific T cells from seronegative donors. This was illustrated before by the generation of leukemia-specific cytotoxic T-cell lines by the stimulation of T cells of HLA-matched donors with leukemic cells of the patients, followed by long-term *in vitro*

culturing under stringent GMP conditions before infusion into the patient.[162,163] Although some patients did benefit from this therapy, the strategy was logistically complex, time consuming and only successful in a limited number of cases. Afterwards, studies focussed on the application of T-cell lines after alloSCT directed against specific hematopoiesis-restricted MiHA with a balanced allele frequency in the population, like HA-1H. However, the isolation and adoptive transfer of HA-1H-specific T-cell lines has so far not resulted in durable *in vivo* persistence of HA-1H-specific T cells.[164,165] As already mentioned for virus-specific T cells, long-term *in vitro* culture may limit the *in vivo* expansion capacity of T cells. Therefore, other strategies for adoptive transfer of hematopoiesis-restricted MiHA-specific T cells need to be developed. Besides the isolation and administration of unmanipulated T-cells, T cells can be genetically engineered by TCR gene transfer for hematopoiesis-restricted MiHA to obtain high numbers of MiHA-specific T cells.[153,166,167] Furthermore, vaccination strategies with donor and patient antigen-presenting cells loaded with MiHA peptides have been explored to boost donor-derived MiHA-specific T-cell responses after alloSCT.[168,169]

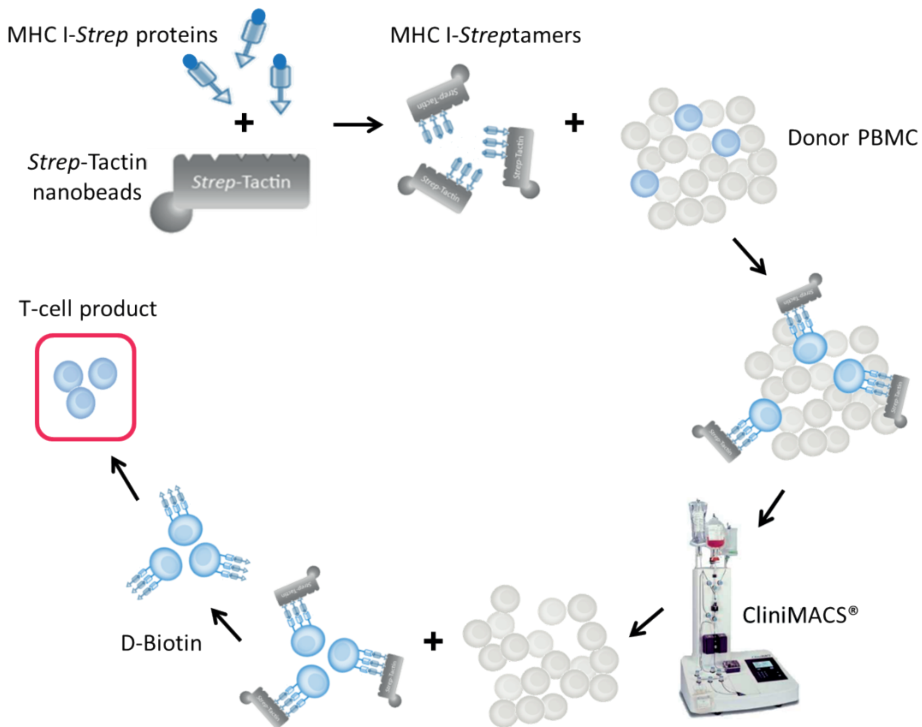

Figure 1. MHC I-*Strep*tamer technology for isolation of antigen-specific T cells from peripheral blood mononuclear cells (PBMC). MHC I-*Strep*tamers are generated by the incubation of peptide-loaded MHC I-*Strep*-proteins with magnetically labelled *Strep*-Tactin nanobeads, and are incubated with PBMC to allow binding of MHC I-*Strep*tamers to antigen-specific T cells. Isolation of MHC I-*Strep*tamer bound cells is performed on a CliniMACS or MidiMACS separator device. After dissociation of MHC I-*Strep*tamers from antigen-specific T cells using D-Biotin, the result is a T-cell product containing unmanipulated antigen-specific T cells.

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Tumor-related T-cell therapy

Tumor-specific and tumor-associated antigens are both explored as targets for adoptive T-cell therapy in the setting of hematologic malignancies.[170] As WT1 is classified as the 'priority antigen' by the American National Cancer Institute, many clinical trials have focussed on WT1 as tumor-target.[171] Besides many active immunotherapy approaches like vaccination studies, only a limited number of clinical trials made use of passive immunotherapeutic approaches like the adoptive transfer of genetically unmanipulated donor-derived TAA-specific T cells with the aim to induce antitumor responses. In two study, T-cell lines directed against BCR-ABL, WT1 and proteinase-3 versus only WT1 were generated by stimulation of donor-derived T cells with donor-derived mature dendritic cells loaded with peptides of the mentioned antigens.[170,172] After several rounds of stimulation, T-cell products were prophylactically or therapeutically administered to leukemia patients after alloSCT. Although generation and administration of T-cell products was feasible and safe, it is hard to prove efficacy. Again, using this approach, several *in vitro* stimulation and expansion rounds are needed, making it a time-consuming and labour-intensive approach. Wang et al demonstrated the direct *ex vivo* isolation of WT1-specific T cells from alloSCT donor-derived leukapheresis products using MHC I-*Strep*tamers.[173] Although WT1-specific T cells could be enriched from donor peripheral blood mononuclear cells (PBMC), T-cell products contained only a few million cells due to the very low precursor frequency of WT1-specific T cells in the peripheral blood of healthy individuals. Therefore, the direct isolation of TAA-specific T cells for adoptive T-cell therapy approaches is as complex as for MiHA-specific T cells and needs further investigation. Currently, several trials are enrolling patients to test genetically unmanipulated T-cell immunotherapeutic approaches targeting TAA.[50,174] However, at the same time, it is still under debate whether TAA are effective targets for passive or active immunotherapy strategies. The most important question that needs to be addressed is whether TAA-specific T cells that are able to recognize TAA-expressing malignant cells are actually present in the T-cell repertoire of healthy individuals, as negative thymic selection is supposed to delete high-avidity TAA-specific T cells to prevent auto-immunity. An argument supporting the value of TAA in immunotherapy is the dysregulated overexpression of TAA in malignant cells which may allow the immune system to discriminate TAA-expressing malignant cells from their healthy counterparts.[48,51,175,176]

AIM OF THE THESIS

Donor-derived T cells play a key role in alloSCT. In the period around the transplantation, donor-derived T cells are depleted or suppressed to reduce the risk of harmful GVHD. However, in the complete absence of donor-derived T cells, the curative GVL effect of alloSCT and the virus-specific immunity is abrogated. Therefore, the major challenge in the field of alloSCT is to find a balance between the GVL effect and viral protection versus GVHD. The research described in this thesis focusses on the manipulation of donor-derived T cells during the process of alloSCT.

To reduce the risk of GVHD after alloSCT, several strategies for TCD have been proposed. In **chapter 2** we study the effect of *in vitro* ALT addition on the composition of allogeneic stem cell grafts before infusion into the patients. The depletion efficiencies of different lymphocytes and T-cell subsets are investigated by comparing the composition of grafts before and after the incubation with ALT. Subsequently, we analyze whether the composition of the grafts at the moment of infusion into the patients are predictive for T-cell reconstitution and the development of GVHD early after ALT-based TCD alloSCT. These observations result in better understanding of the effect of ALT on different lymphocyte subsets and suggestions for the *in vitro* application of ALT for the depletion of T cells in allogeneic stem cell grafts.

As long-term immunosuppression is not indicated after TCD alloSCT, this transplantation strategy is an ideal platform for the application of adoptive T-cell therapy to reduce complications early after TCD alloSCT. In **chapter 3** the development of a widely applicable method for the simultaneous isolation of multiple antigen-specific T-cells populations from donor PBMC is studied. The MHC I-*Strep*tamer technology was previously developed for the detection and isolation of antigen-specific T-cell populations under GMP conditions. So far, MHC I-*Strep*tamers were used for the isolation of virus-specific T-cell populations from virus-seropositive donors with a relatively high precursor frequency in the donor's peripheral blood. Furthermore, the clinically applied T-cell products isolated with this approach targeted a limited number of different virus-specific T-cell antigens. Although T cells directed against a single antigen can control viral reactivations, the inclusion of T cells with different target antigen specificities in one product may be preferred for viral control. Therefore, we assess how many T-cell populations can be simultaneously targeted in one isolation procedure while the purity of the product is maintained and the isolation of potentially harmful alloreactive T cells remains limited. In addition, we investigate whether T-cell populations with high frequencies in the peripheral blood can be isolated in the same procedure as T-cell populations with very low frequencies in the peripheral blood of healthy individuals, like virus-specific T cells from seronegative individuals, MiHA- and TAA-specific T cells. Based on our findings, we define optimal technical conditions to isolate multi-antigen specific T-cell products from donor PBMC using the MHC I-*Strep*tamer technology for direct clinical application.

In **chapter 4** we investigate the clinical application of MHC I-*Strep*tamer isolated multi-antigen specific T-cell products for the prevention of viral reactivations and disease relapses early after TCD alloSCT in a phase I/II trial. The feasibility of patient/donor inclusion and donor-derived T-cell products generation is assessed. We aim to isolate personalized T-cell products targeting CMV-, EBV-, AdV-, TAA- and MIHA-specific antigens based on the technical knowledge obtained in **chapter 3**, to boost both virus-specific and tumor-specific T-cell immunity. Furthermore, the safety of prophylactic infusion early after TCD alloSCT is analyzed with respect to infusion-related complications and the initiation of GVHD. Patient follow-up provides information on the relation

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between T-cell product infusions, the occurrence of clinical events like viral reactivations and disease relapses, and the expansion of target-antigen-specific T cells in the peripheral blood of the patients. The results of this study give insight in the safety and feasibility of personalized adoptive T-cell therapy using MHC I-*Strep*tamers and might hint for patient groups that benefit of prophylactic interventions to prevent complications early after TCD alloSCT.

In literature, TAA are proposed as powerful target antigens for immunotherapeutic approaches to stimulate antitumor reactivity. Since the isolation and administration of TAA-specific T cells is also aimed in the clinical study of **chapter 4**, we investigate in **chapter 5** whether TAA-specific T cells in the repertoire of healthy individuals actually have the potential to recognize endogenously processed and presented TAA. TAA-specific T cells are isolated using MHC I-*Strep*tamers from healthy donors and are functionally analyzed to demonstrate whether clinically relevant antitumor responses directed against these self-antigens in the context of self-HLA are expected to develop from the autologous or HLA-matched repertoire after immunotherapeutic interventions like TAA-specific vaccination, stem cell transplantation or adoptive immunotherapy.

In **chapter 6** the results of this thesis are summarized and discussed and conclusions based on the results of this thesis and recent literature are drawn.

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CHAPTER 2

EFFECT OF ALEMTUZUMAB-BASED T-CELL DEPLETION ON GRAFT COMPOSITIONAL CHANGE IN VITRO AND IMMUNE RECONSTITUTION EARLY AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Cytotherapy 2021; 23(1): 45-56.

ABSTRACT

BACKGROUND

To reduce the risk on graft-versus-host disease (GVHD) after allogeneic stem cell transplantation (alloSCT), T-cell depletion (TCD) of grafts can be performed by the addition of alemtuzumab (ALT) 'to the bag' (*in vitro*) before transplantation. In this prospective study, we analyzed the effect of *in vitro* incubation with 20 mg ALT on the composition of grafts prior to graft infusion. Furthermore, we assessed whether graft composition at the moment of infusion was predictive for T-cell reconstitution and development of GVHD early after TCD-alloSCT.

METHODS

Sixty G-CSF-mobilized stem cell grafts were obtained from ≥9/10 HLA-matched related and unrelated donors. The composition of the grafts was analyzed by flow cytometry before and after the *in vitro* incubation with ALT. T-cell reconstitution and the incidence of severe GVHD were monitored until 12 weeks after transplantation.

RESULTS

In vitro incubation of grafts with 20 mg ALT resulted in an initial median depletion efficiency of T-cell receptor (TCR) α/β T cells of 96.7% (range 63.5-99.8%) followed by subsequent depletion *in vivo*. Graft volumes or the absolute leukocyte counts of grafts before the addition of ALT were not predictive for the efficiency of TCR α/β T-cell depletion. $CD4^{pos}$ T cells were depleted more efficient than $CD8^{pos}$ T cells, and naïve and regulatory T cells were depleted more efficient than memory and effector T cells. This differential depletion of T-cell subsets was in line with their reported differential CD52-expression. *In vitro* depletion efficiencies or absolute numbers of (naïve) TCR α/β T cells in the grafts after ALT incubation were not predictive for T-cell reconstitution or the development of GVHD post-alloSCT.

CONCLUSIONS

We conclude that the addition of ALT 'to the bag' is an easy, fast and general applicable strategy to prevent GVHD in patients receiving alloSCT after myeloablative or non-myeloablative conditioning, due to the efficient differential depletion of donor-derived lymphocytes and T cells.

INTRODUCTION

Allogeneic stem cell transplantation (alloSCT) is a potentially curative treatment for patients with a variety of malignant and non-malignant hematologic diseases.[1,2] The therapeutic effect of alloSCT is mediated by alloreactive donor T-cell responses directed against (malignant) hematopoietic cells of the patient.[3,4] However, donor-derived T cells can also elicit immune responses directed against other healthy cells in tissues and organs of the patient, causing detrimental acute or chronic graft-versus-host disease (GVHD).[5] Although long-term immunosuppressive treatment post-transplantation can strongly reduce the risk of GVHD, this strategy also suppresses disease- and pathogen-specific immune responses. To control the T-cell compartment of the graft, *in vitro* T-cell depletion (TCD) can be achieved. By controlling only the number of T cells in the grafts, such as through physical purification of CD34^{pos} stem cells (positive selection), or selective depletion of total T-cell receptor (TCR) alpha/beta (α/β) T cells (negative selection) using antibody-coated magnetic beads and magnetic separation, no clear discrimination between these opposed T-cell effects can be achieved.[6-11] Therefore, several strategies have been explored to manipulate stem cell grafts in a way that potentially harmful alloreactive T cells are depleted, while beneficial T cells are preserved. Pre-clinical models have demonstrated that donor-derived naïve TCR α/β T cells are the major inducers of GVHD.[12,13] For direct protection against viral complications after alloSCT, peripheral expansion of memory TCR α/β T cells derived from seropositive donors is important,[14] whereas α/β-expressing regulatory T cells are similarly instrumental for the maintenance of tolerance and suppression of alloreactive T-cell responses.[15] In this regard, selective depletion of naïve TCR α/β T cells from grafts have been explored.[16,17] Furthermore, the use of alemtuzumab (ALT, Campath-1H) is so far widely known for both *in vitro* and/or *in vivo* aspecific TCD,[18-20] but might differentially target TCR α/β-expressing T-cell subsets more than other T-cell subsets.

ALT is a humanized monoclonal IgG1 antibody targeting the glycophosphatidylinositol (GPI)-anchored protein CD52, which is expressed on the surface of mature lymphocytes but not (or only marginally) on hematopoietic stem cells.[20-23] Since CD52 is not homogenously expressed on lymphocyte subsets, its susceptibility to ALT-induced TCD may vary.[21] *In vivo* application of ALT as part of the pre-transplant conditioning regimen aims to prevent graft rejection by elimination of patient T cells before graft infusion and to prevent the development of GVHD by depletion of donor T cells after graft infusion.[24-26] ALT can also be added directly to the stem cell graft - known as *in vitro* TCD or 'ALT to the bag'. This fast and easy applicable strategy has been shown to be effective in the prevention of GVHD when combined with both myeloablative (MA) and non-myeloablative (NMA) conditioning regimens.[27-31] Although TCD results in delayed immune reconstitution post-transplant, the incidence of cytomegalovirus (CMV) disease is not increased after *in vitro* ALT-based TCD alloSCT compared with non-TCD alloSCT strategies, suggesting that protective immunity is at least partly conserved.[29,32-34] Soon after ALT-based TCD alloSCT, expansion

of CD52^{neg} T cells that are insensitive to ALT has been observed.[14,35-40]

In this study, we investigated the effect of *in vitro* ALT addition to grafts on depletion efficiencies of lymphocyte subsets and changes in graft composition before infusion into patients. Furthermore, we analyzed whether graft composition was predictive for T-cell reconstitution and development of GVHD soon after ALT-based TCD alloSCT.

MATERIALS AND METHODS

PATIENTS AND TRANSPLANTATION PROTOCOLS

In this prospective study, 60 patients treated with an ALT-based TCD alloSCT for a hematologic disease at Leiden University Medical Center (LUMC, Leiden, The Netherlands) were included. Patient characteristics are provided in Table 1. Granulocyte colony-stimulation factor (G-CSF)-mobilized peripheral blood stem cell (PBSC) grafts were obtained from ≥9/10 HLA-matched related and unrelated donors. Informed consent was obtained in accordance with the Declaration of Helsinki. Patients received myeloablative or non-myeloablative conditioning according to the protocols indicated in Supplementary Table 1. The applied form of *in vivo* T-cell depletion differed slightly depending on the conditioning regimen and donor source. In the case of NMA conditioning and/or an unrelated donor, patients received 15 mg ALT (MabCampath, Sanofi Genzyme, Naarden, The Netherlands) intravenously (iv) twice the week before transplantation. Because patients receiving only ALT-based NMA conditioning and a graft from an unrelated donor still had an relatively high risk of GVHD, in addition to ALT, this group also received 1 mg/kg rabbit-derived anti-thymocyte globulin (ATG; Sanofi Genzyme, Naarden, The Netherlands) for additional T-cell depletion. Prophylactic immune suppression using cyclosporine A was given only temporarily to patients receiving MA conditioning and a graft from an unrelated donor. Irrespective of the conditioning regimen, 20 mg ALT was added to each graft for *in vitro* T-cell depletion. After 30 minutes of incubation on a roller bank at room temperature, the graft was immediately infused into the patient.

PROCESSING AND ANALYSIS OF GRAFT SAMPLES

Stem cell graft volumes were determined by weighing (1 mg = 1 mL). Samples were taken of each graft prior to and 30 minutes after the addition of 20 mg ALT. The concentration of leukocytes in these samples was determined using a Sysmex KX-21N (Sysmex, Etten-Leur, The Netherlands). Samples were centrifuged, and cells were resuspended in red blood cell lysis buffer (8.4g/L NH_4Cl and 1 g/L $KHCO_3$, pH 7.4; LUMC Pharmacy) and incubated for 10 minutes at 4°C. After centrifugation, cells were resuspended in Iscove's Modified Dulbecco's Medium (Lonza, Basel, Switzerland) containing 10% heat-inactivated human serum, 3 mM L-glutamine (Lonza) and 100 U/ml penicillin/

streptomycin (Lonza) at a concentration of 1-2*10^6 cells/ml and stored overnight at 4°C. The next day, the concentration and percentage of viable cells was determined using a hemocytometer and eosin. The total numbers of viable cells in the grafts were calculated by multiplying the leukocyte concentrations of the grafts by the graft volumes, followed by multiplication by the percentages of viable cells after overnight storage. The percentages of TCR α/β T cells ($CD3^+α/β^+$), TCR gamma/delta (γ/δ) T cells ($CD3^+γ/δ^+$), B cells ($CD19^+$), NK cells ($CD56^+$), $CD4^{pos}$ and $CD8^{pos}$ naïve T cells ($CD3^+CD4^+CD27^+CD45RA^+$/$CD3^+CD8^+CD27^+CD45RA^+$), $CD4^{pos}$ and $CD8^{pos}$ memory T cells ($CD3^+CD4^+CD45RO^+CD45RA^-$/$CD3^+CD8^+CD45RO^+CD45RA^-$), $CD4^{pos}$ and $CD8^{pos}$ effector T cells ($CD3^+CD4^+CD27^-CD45RA^+$/$CD3^+CD8^+CD27^-CD45RA^+$), and regulatory T cells ($CD3^+CD4^+CD25^+CD127^-FoxP3^+$) in the graft samples before and after ALT incubation were analyzed by flow cytometry. Cells were stained with Alexa Fluor 647-labelled TCR α/β antibodies (ITK Diagnostics, Uithoorn, The Netherlands), Alexa Fluor 700-labelled CD45RO antibodies (ITK Diagnostics), allophycocyanin (APC)-H7-labelled CD3 antibodies (Beckton Dickinson (BD) Biosciences, San Diego/San Jose, USA), fluorescein isothiocyanate (FITC)-labelled CD27 and TCR γ/δ antibodies (BD Biosciences), phycoerythrin (PE)-labelled CD25 antibodies (BD Biosciences), phycoerythrin-cyanine7 (PeCy7)-labelled CD56 (BD Biosciences) and CD127 antibodies (Invitrogen, Waltham, MA, USA), PE-Texas-Red-labelled CD19 and CD45RA antibodies (Invitrogen), peridinin-chlorophyll-protein complex (PerCP)-labelled CD4 antibodies (BD Biosciences), V450-labelled CD8 antibodies (BD Biosciences) or V500-labelled CD4 antibodies (BD Biosciences). Intracellular FoxP3 staining was performed using the FoxP3 staining kit (FoxP3-APC monoclonal antibody, Invitrogen) according to the manufacturer's instructions. Cells were measured on an LSR II (BD Biosciences) and analyzed using Diva Software (BD Biosciences). Peripheral blood mononuclear cells (PBMC) of a healthy control were regularly measured as normal control. The absolute numbers of lymphocyte and T-cell subsets in the grafts were calculated by multiplying the absolute numbers of leukocytes in the grafts with the percentages of the cell subsets analyzed by flow cytometry. Depletion efficiencies (%) of specific cell populations were calculated as (1-(number of viable cells in the graft after ALT incubation / number of viable cells in the grafts before ALT incubation)) x 100%.

GVHD AND IMMUNE RECONSTITUTION AFTER alloSCT

Severe acute GVHD was defined as acute GHVD requiring (additional) systemic immunosuppressive therapy within a follow-up period of 12 weeks after TCD alloSCT. To investigate early immune reconstitution, peripheral blood (PB) samples were collected at 3 and 6 weeks after transplantation. PB samples for assessment of immune reconstitution collected between day 11 and day 31 were categorized as 3 weeks post-alloSCT and those collected between day 32 and day 52 as 6 weeks post-alloSCT. Absolute numbers of circulating T cells ($CD45^+CD3^+$) were determined as part of routine clinical evaluation on fresh blood samples using TruCount Tubes (BD Biosciences), following the manufacturer's instructions. Samples were stained with APC-labelled CD3 (BD Biosciences) antibodies and V500-conjugated CD45 (BD Biosciences) antibodies.

The percentages of CD52^{pos} (CD45^{+}CD3^{+}CD52^{+}FLAER^{+}) and CD52^{neg} (CD45^{+}CD3^{+}CD52^{-}FLAER^{-}) T cells were determined by staining follow-up samples with Alexa Fluor 488-labelled GPI-anchor-specific inactivated toxin pro-aerolysin (FLAER-AF488, SanBio, Uden, The Netherlands), APC-labelled CD52 antibodies (ITK Diagnostics), APC-H7-labelled CD3 antibodies (BD Biosciences) and V500-labelled CD45 antibodies (BD Biosciences). Cells were measured on an LSR II (BD Biosciences) and analyzed using Diva Software (BD Biosciences). PBMC of a healthy control were regularly measured as normal control. Absolute numbers of CD52^{pos} and CD52^{neg} T cells were calculated by multiplying the percentages of these cells within the CD3^{pos} cell populations by the absolute T-cell counts obtained via measurements on fresh blood samples using TruCount Tubes (BD Biosciences).

STATISTICAL ANALYSIS

Absolute lymphocyte and T-cell counts (Table 2) and percentages of lymphocytes (Supplementary Table 2) in grafts before and after *in vivo* ALT incubation were compared using Wilcoxon matched-pairs signed-rank test. The Friedman test was used to detect a significant difference between depletion efficiencies of the different lymphocyte subsets (Figure 1A) and T-cell subsets (Figure 3A) in the total study cohort. For this analysis, the alpha level was corrected using the Bonferroni method (alpha = 0.05 / 6 = 0.008). As post hoc analysis, the Wilcoxon matched-pairs signed-rank test was used to calculate the difference between depletion efficiencies of two specific cell subsets in Figures 1A and 3A. The Mann-Whitney test was used to analyze the differences in absolute numbers and depletion efficiencies of naïve and regulatory T cells and the ratio between the numbers of naïve and regulatory T cells between patients who did or did not experienced GVHD early after transplantation (Figures 5A-E). Level of significance was <0.05 (2-sided). Statistical analysis were performed using Prism 8.0 (GraphPad, La Jolla, CA, USA).

RESULTS

IN VITRO ALT-BASED T-CELL DEPLETION OF STEM CELL GRAFTS LEADS TO DIFFERENTIAL DEPLETION OF LYMPHOCYTE SUBSETS

In vitro TCD of allogeneic stem cell grafts for the prevention of GVHD was applied by the addition of 20 mg ALT to the bag, followed by an incubation of 30 minutes and direct subsequent infusion into patients. Lysis of part of the lymphocytes in the grafts was expected to take place directly *in vitro*, followed by anticipated ongoing lysis of both donor and patient lymphocytes *in vivo* after infusion of the graft into the patient. To measure the magnitude of the direct *in vitro* effect of ALT on the number and distribution of lymphocyte subsets in the grafts, we analyzed the composition of 60 G-CSF mobilized PBSC grafts before and after *in vitro* incubation with 20 mg ALT. Stem cell grafts were obtained from 20 related and 40 unrelated donors for patients undergoing TCD alloSCT for a variety of hematologic diseases (Table 1).

Table 1. Patient and transplantation characteristics of study cohort. MA, myeloablative; neg, negative; NMA, non-myeloablative.

Median age, years (range)	60 (20-73)
Gender, *n* (%)	
Male	36 (60)
Female	24 (40)
Diagnosis, *n* (%)	
Acute myeloid leukemia	28 (47)
Acute lymphoblastic leukemia	7 (12)
Multiple myeloma	10 (17)
Myelodysplastic syndrome	4 (7)
Hodgkin lymphoma	1 (2)
B-cell lymphoma	3 (5)
T-cell lymphoma	2 (3)
Myeloproliferative syndrome	4 (7)
Severe aplastic anemia	1 (2)
Conditioning regimen and donor type (HLA-matching), *n* (%)	
MA related (12/12)	6 (10)
MA unrelated (≥9/10)	14 (23)
NMA related (12/12)	14 (23)
NMA unrelated (≥9/10)	26 (43)

Graft composition was analyzed 0 days (*n* = 5), 1 day (*n* = 48), or 2 days (*n* = 7) after the donor leukapheresis procedure, depending on logistics. Before the addition of ALT, the grafts had a median volume of 379 mL (range, 75-789 mL) and contained a median of $70*10^9$ leukocytes (range, $15.1\text{-}147.7*10^9$). Subset analysis of lymphocytes showed that before ALT addition, grafts were dominated by TCR α/β T cells, followed by B cells, NK cells and TCR γ/δ T cells (Supplementary Figure 1A). After incubation with ALT, the absolute numbers of all these lymphocyte subsets in the grafts significantly decreased (Table 2 and Supplementary Figure 1B). TCR α/β T cells were depleted significantly more efficient than TCR γ/δ T cells, B cells and NK cells (TCR α/β T cells versus TCR γ/δ T cells, B cells or NK cells: all $p<0.001$), as illustrated by median depletion efficiencies of 96.7% (range, 63.5-99.8%), 86.9% (range, 8.7-98.7%), 88.6% (range, 0-99%) and 72.5% (range, 14.3-93.6%), respectively (Figure 1A). In 7 grafts, the depletion efficiencies of TCR α/β T cells were below 90% (grafts 5, 19, 20, 35, 38, 44, and 46, indicated by green symbols in all figures). These grafts were among the grafts with the highest absolute numbers of TCR α/β T cells at the moment of infusion into the patient (Supplementary Figure 1B). The differential depletion of lymphocyte subsets resulted in significant compositional changes of grafts. Before incubation with ALT, a median of 71.9% (range, 55.8-85.5%) of lymphocytes consisted of TCR α/β T cells (Figure 1B), whereas after incubation with ALT this decreased to a median of 33.7% (range, 4.8-81.0%; $p<0.001$) (Figure 1C and Supplementary Table 2). Since TCR γ/δ T cells, B and NK cells were depleted less efficiently compared to TCR α/β T cells, these cell types comprised a significantly larger proportion of cells in the graft after ALT incubation than before ALT incubation (Figure 1C

and Supplementary Table 2). No differences in depletion efficiencies or graft compositions were observed between grafts obtained from related or unrelated donors (Supplementary Figure 2A) or between grafts that were analyzed on the same day or 1 or 2 days after the donor leukapheresis procedure (Supplementary Figure 2B), suggesting no selective loss of lymphocytes during transport or *in vitro* depletion.

Next, we investigated whether the magnitude of direct *in vitro* depletion of TCR α/β T cells in the grafts could be predicted based on graft characteristics before the addition of ALT. A trend was observed between the levels of direct *in vitro* depletion efficiency of TCR α/β T cells and leukocyte concentrations in the grafts before ALT addition ($R^2 = 0.059$, $p = 0.060$) (Figure 2A). However, no correlation was found between the levels of direct *in vitro* depletion of TCR α/β T cells and absolute leukocyte counts in the grafts before ALT addition ($R^2 = 0.005$, $p = 0.579$, data not shown) or the volumes of the grafts ($R^2 = 0.013$, $p = 0.385$) (Figure 2B). Interestingly, if only samples with TCR α/β T-cell depletion efficiencies higher than 90% (excluding the green outliers) are considered in the analysis, significant correlations with levels of *in vitro* depletion of TCR α/β T cells are seen for both leukocyte concentrations in the grafts (R^2=0.1405; p=0.0057) and graft volumes (R^2=0.1084; p=0.0161) (data not shown). However, we do not have an indication as to the underlying cause of the outliers.

These observations illustrate that lymphocyte subsets were unequally depleted from the grafts during the 30 minutes *in vitro* incubation with ALT, resulting in a major compositional change of the grafts already before infusion into the patient. Of lymphocyte subsets, TCR α/β T cells were the most efficiently depleted *in vitro*. The extent of direct *in vitro* TCR α/β TCD was not predictable based on graft volume or absolute leukocyte count in the graft before ALT addition.

EFFICIENT *IN VITRO* DEPLETION OF NAÏVE AND REGULATORY T CELLS BY THE ADDITION OF ALT TO STEM CELL GRAFTS

Since donor-derived memory and effector T cells from the graft are mainly expected to contribute to (early) protective immunity against pathogens post-transplantation, whereas the presence of naïve T cells and absence of regulatory T cells derived from the donor have been associated with the development of GVHD post-transplantation, we investigated the effect of ALT on the *in vitro* depletion of different T-cell subsets. T cells with a naïve, memory and effector phenotype within the $CD8^{pos}$ and $CD4^{pos}$ TCR α/β T-cell compartments and $CD4^{pos}$ regulatory T cells were quantified before and 30 minutes after addition of ALT to the grafts.

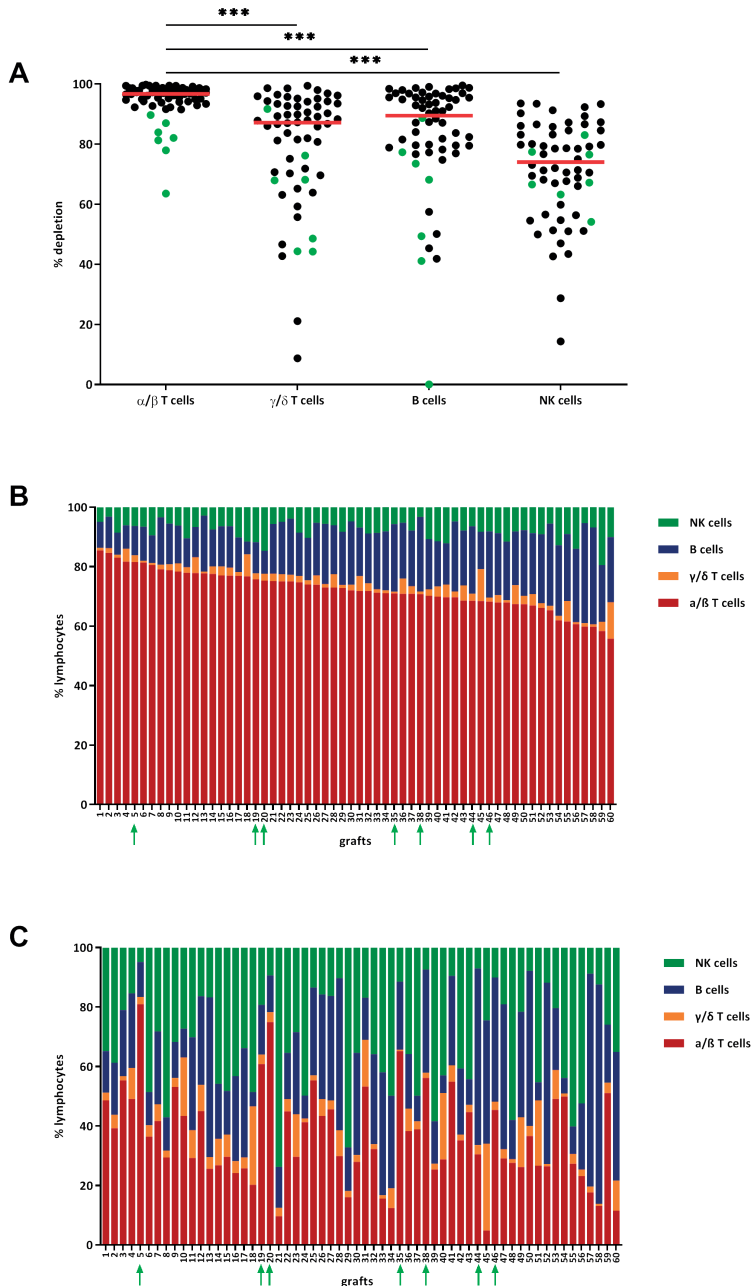

Figure 1. Lymphocytes in alloSCT grafts before and after *in vitro* ALT incubation (n=60). The seven grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols (dots in A, arrows in B and C). **A**, Depletion efficiencies (%) of lymphocytes after in vitro ALT incubation. The red solid lines indicate medians. The Wilcoxon matched-pairs signed-rank test was used for statistical analysis. *** means p<0.001. **B**, Compositions of the lymphocyte compartment in the grafts before *in vitro* ALT incubation. **C**, Compositions of the lymphocyte compartment in the grafts after *in vitro* ALT incubation.

Table 2. Absolute numbers of lymphocytes in the grafts before and after *in vitro* incubation with ALT.

		Before ALT addition		**After ALT addition**		**Significance of differences**
		Median	*Range*	*Median*	*Range*	*P value**
TCR α/β T cells (*10^9)		12.4	3.67 – 41.0	0.45	0.03 – 5.13	<0.001
CD8^{pos} (*10^7)	Naïve T cells	118	12.3 – 758	6.62	0.74 – 411	<0.001
	Memory T cells	56.5	1.00 – 260	6.62	0.16 – 37.3	<0.001
	Effector T cells	24.8	1.04 – 182	29.4	1.40 – 245	0.098
CD4^{pos} (*10^7)	Naïve T cells	540	62.5 – 2350	4.73	0.07 – 426	<0.001
	Memory T cells	484	100 – 1230	2.59	0.08 – 34.0	<0.001
	Effector T cells	6.69	0.28 – 60.9	0.61	0.00 – 6.05	<0.001
	Regulatory T cells	20	0.00 – 122	0.25	0.00 – 5.77	<0.001
TCR γ/δ T cells (*10^9)		0.34	0.05 – 2.18	0.04	0.003 – 0.61	<0.001
B cells (*10^9)		2.84	7.21 – 9.06	0.23	0.02 – 10.0	<0.001
NK cells (*10^9)		1.16	0.42 – 3.06	0.30	0.04 – 1.18	<0.001

** Wilcoxon matched-pairs, signed-rank test*

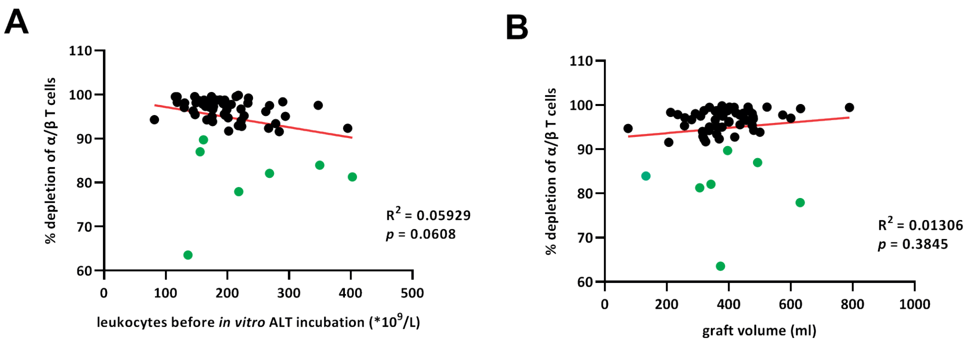

Figure 2. Correlations between the efficiencies of α/β T-cell depletion and graft characteristics (*n*=60). The 7 grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The solid lines indicate the linear regression analysis. **A**, Relation between the TCR α/β T-cell depletion efficiencies (%) and the leukocyte counts in the grafts before ALT addition. **B**, Relation between the TCR α/β T-cell depletion efficiencies (%) and the graft volumes.

The absolute numbers of all TCR α/β T-cell subsets, except for CD8^{pos} effector T cells, decreased significantly after ALT incubation *in vitro* (Table 2 and Supplementary Figure 3). Depletion efficiencies revealed that CD4^{pos} T cells were more efficiently depleted than CD8^{pos} T cells (Figure 3A). Within CD4^{pos} T cells, the median depletion efficiencies of naïve, memory, effector and regulatory T cells were 99.4% (range, 72.8-100%), 99.5% (range, 94.6-100%), 92.8% (range, 0-100%), and 98.6% (range, 0-100%), respectively, showing that CD4^{pos} effector T cells had a significantly lower depletion efficiency compared with the other CD4^{pos} T-cell subsets (p<0.0001).

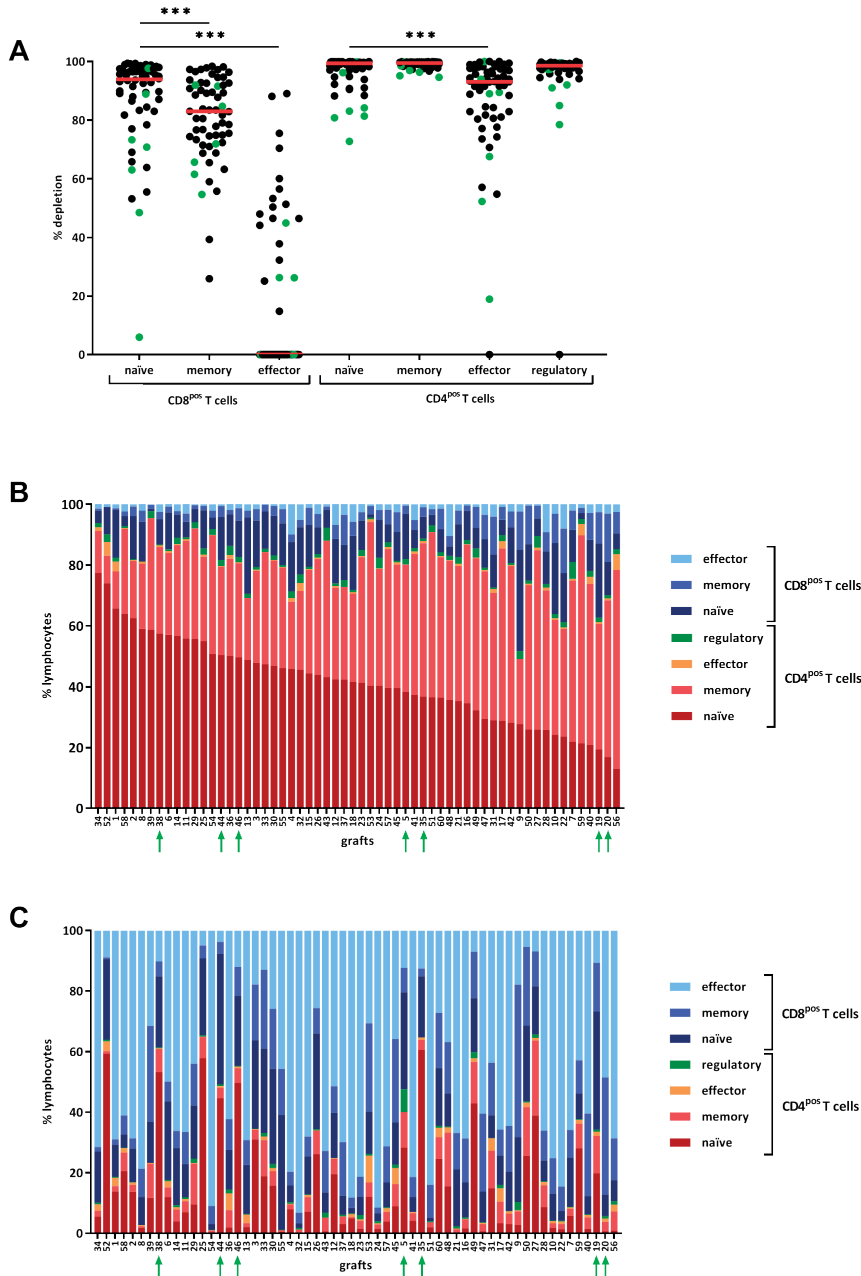

Figure 3. T cells in alloSCT grafts before and after *in vitro* ALT incubation (*n*=60). The 7 grafts with α/β T-cell depletion efficiencies below 90% are indicated by green symbols (dots in A, arrows in B and C). **A**, Depletion efficiencies (%) of T cells after *in vitro* ALT incubation. The red solid lines indicate medians. The Wilcoxon matched-pairs signed-rank test was used for statistical analysis. *** means *p*<0.001. **B**, Compositions of the T-cell compartment in the grafts before *in vitro* ALT incubation. **C**, Compositions of the T-cell compartment in the grafts after *in vitro* ALT incubation.

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In the CD8^{pos} T-cell compartment, the depletion effect of ALT on naïve T cells in the grafts was significantly stronger than the effect on memory (naïve versus memory CD8^{pos} T cells, *p*=0.0003) and effector T cells (naïve versus effector CD8^{pos} T cells, *p*<0.0001), as illustrated by median depletion efficiencies of 93.6% (range, 6.0-99.4%), 82.3% (range, 25.9-98.4%), and 0% (range, 0-89.1%), respectively. Directly after *in vitro* ALT incubation, the compositions of the T-cell compartments in the grafts changed substantially. Hence, although before ALT addition the T-cell compartments were dominated by naïve and memory CD4^{pos} T cells in the majority of grafts, after ALT incubation the majority of grafts contained mainly effector and memory CD8^{pos} T cells (Figures 3B and C, Supplementary Table 2).

These results illustrate that during the *in vitro* incubation of stem cell grafts with ALT, the differential depletion of T-cell subsets resulted in an alteration of the T-cell compartment of the grafts. The most efficient depletion was observed for CD4^{pos} naïve, memory, and regulatory T cells, whereas no or only a marginal depletion of CD8^{pos} effector T cells was achieved. Although the ALT-based TCD of the grafts was already substantial after *in vitro* incubation, we cannot rule out that the process continues *in vivo* after graft infusion into the patients, thereby further influencing the graft composition. However, this effect can only be assessed indirectly by measuring immune cell reconstitution in patients.

THE ABSOLUTE NUMBER OF T CELLS IN THE GRAFTS AT THE MOMENT OF TRANSPLANTATION IS NOT PREDICTIVE FOR T-CELL RECONSTITUTION AT 3 OR 6 WEEKS POST-alloSCT

To investigate whether the graft compositions after *in vitro* ALT incubation were predictive for T-cell reconstitution after graft infusion, we determined the absolute numbers of circulating T cells in PB at 3 and 6 weeks post-transplantation. Because T-cell reconstitution can also be influenced by the pre-transplant conditioning, patients were analyzed per conditioning regimen as indicated in Supplementary Table 1. The six patients who received MA conditioning and a related donor graft did not receive *in vivo* ALT iv in the week before transplantation as part of the conditioning regimen, whereas the remaining 54 patients did receive *in vivo* ALT iv. Patients who underwent NMA conditioning and had an unrelated donor graft also received ATG iv. Patients with MA conditioning and a graft from an unrelated donor received cyclosporine A in the first weeks post-transplantation.

Follow-up samples were available for 54 and 57 patients at 3 and 6 weeks, respectively, after alloSCT. Correlation analysis per conditioning regimen revealed that the absolute numbers of T cells in the grafts at the moment of infusion in the patients were not predictive for the numbers of circulating T cells in peripheral blood at 3 weeks (data not shown) or 6 weeks after transplantation (Figure 4A). The three patients with relatively high numbers of T cells 6 weeks after transplantation suffered from an active viral reactivation (Epstein Barr virus reactivation, CMV reactivation, or

combined CMV and varicella zoster virus reactivation in patients receiving grafts 18, 39 and 41, respectively, indicated by red symbols). The patients who received grafts that showed *in vitro* TCR α/β T-cell depletion efficiencies below 90% (green symbols) had no advantage in T-cell reconstitution post-transplantation.

As ALT targets only $CD52^{pos}$ T cells, T cells that have lost membrane expression of CD52 are insensitive to ALT. We and others have previously shown that after ALT-based TCD alloSCT, reconstitution of T cells is partly due to the expansion of $CD52^{neg}$ T cells.[35,36] Figure 4B illustrates that both $CD52^{pos}$ and $CD52^{neg}$ T cells contributed to T-cell reconstitution soon after TCD alloSCT, although the reconstitution was variable among conditioning regimens. In 23 of 24 evaluable patients who received NMA conditioning and a related donor graft, MA conditioning and a related donor graft or MA conditioning and an unrelated donor graft, $CD52^{neg}$ T cells at a concentration of $>10^6$/L were detectable in the PB at 6 weeks after TCD alloSCT. This level of $CD52^{neg}$ T-cell reconstitution was observed in only nine of 22 evaluable patients that received NMA conditioning and an unrelated donor graft and also received ATG as part of the conditioning regimen.

Since the T-cell compartments of the grafts directly after *in vitro* ALT incubation were dominated by effector T cells, whereas memory T cells are especially expected to contribute to T-cell reconstitution in the weeks after transplantation due to their expansion capacity, we evaluated whether the absolute numbers of memory T cells in the grafts at the moment of infusion correlated with reconstitution of $CD52^{pos}$ T cells 3 and 6 weeks after transplantation. For all conditioning regimens, the absolute numbers of memory T cells in the grafts were not predictive for the reconstitution of $CD52^{pos}$ T cells at 3 weeks (data not shown) or 6 weeks after transplantation (Figure 4C).

These results indicate that T-cell reconstitution early after TCD alloSCT cannot be predicted based on the composition of the graft at the moment of infusion into the patient.

OCCURRENCE OF EARLY ACUTE GVHD CANNOT BE PREDICTED BASED ON GRAFT COMPOSITION AFTER *IN VITRO* ALT INCUBATION

The ultimate aim of *in vitro* ALT-based TCD is to reduce the risk of GVHD post-alloSCT. Hence, we determined the incidence of early acute GVHD requiring immunosuppressive therapy in the study cohort. Since donor-derived naïve T cells are thought to be the main initiators of GVHD and regulatory T cells the main suppressors of alloreactive immune responses, an imbalance between these cell populations in the infused grafts might affect the chance of developing GVHD after transplantation. Therefore, we compared the absolute numbers, depletion efficiencies and ratio between absolute numbers of naïve and regulatory T cells in the grafts at the moment of infusion between patients who did or did not develop GVHD.[41]

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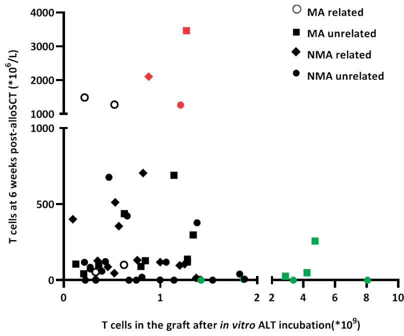

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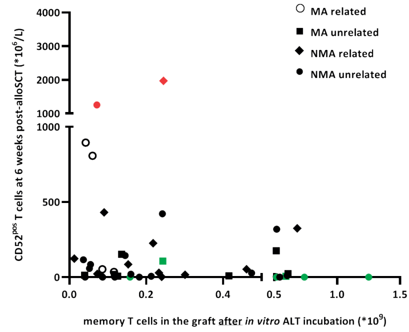

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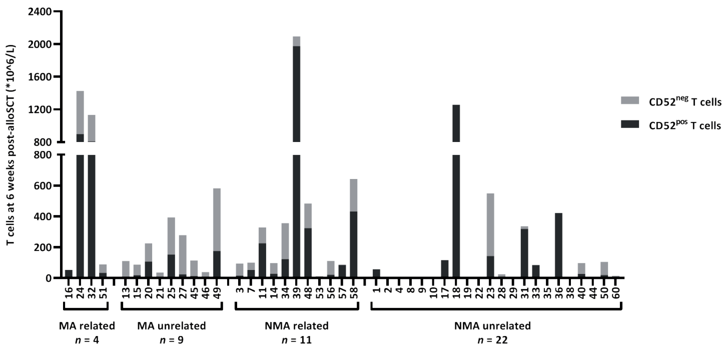

Figure 4. Correlations between the absolute numbers of T cells in the grafts after *in vitro* ALT incubation and T-cell reconstitution at 6 weeks after TCD alloSCT. Squares represent patients that received MA conditioning and a graft from an unrelated donor. Dots represent patients that received NMA conditioning and a graft from an unrelated donor. Diamonds represent patients that received NMA conditioning and a graft from a related donor. **A**, Relation between the absolute numbers of total T cells in the grafts after *in vitro* ALT incubation and the concentrations of total T cells in the peripheral blood at 6 weeks post-transplantation (*n* = 57). The 7 grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The 3 red symbols indicate patients with viral reactivations. **B**, Concentrations of CD52^{neg} and CD52^{pos} T cells in the peripheral blood at 6 weeks after alloSCT (*n* = 46). **C**, Relation between the absolute numbers of memory T cells in the grafts after *in vitro* ALT incubation and the concentrations of CD52^{pos} T cells in the peripheral blood at 6 weeks post-transplantation (*n* = 42). The 6 grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The 2 red signs indicate patients with viral reactivations.

Within our cohort of 60 patients, eight patients required start of (additional) systemic immunosuppressive therapy due to the development of acute GVHD at a median of 29 days (range, 22-70 days) after transplantation. Of these eight patients, five patients received MA conditioning and an unrelated donor graft (grafts 5, 20, 21, 25, and 49), two patients received NMA conditioning and an unrelated donor graft (grafts 40 and 43), and one patient received

MA conditioning and a related donor graft (graft 51). All eight patients suffered from skin GVHD; patient 43 also developed liver GVHD. Figures 5A and 5B illustrate that no significant differences were observed between patients who did or did not develop GVHD regarding the absolute counts of naïve and regulatory T cells in the grafts at the moment of infusion into the patients (p=0.362 and p=0.217, respectively). As the ALT-based lytic effect is expected to continue *in vivo* after graft infusion, we hypothesized that the depletion efficiency of naïve and regulatory T cells *in vitro* might be used as an indication whether naïve and regulatory T cells were sensitive to ALT-based depletion. However, no differences in *in vitro* depletion efficiencies of these two T-cell subsets were observed between the two groups of patients (p=0.871 and p=0.613, respectively) (Figures 5C and 5D). Interestingly, the ratio between the numbers of naïve and regulatory T cells in the grafts before infusion was significantly higher in patients without GVHD after TCD alloSCT compared with patients who developed GHVD soon after TCD alloSCT (p=0.016) (Figure 5E). Furthermore, no significant differences were found in concentrations of circulating total or CD52^{pos} T cells in the PB of patients with versus without development of GVHD 3 weeks post-transplantation (data not shown).

The reported low incidence of severe acute GVHD shows that the risk of developing acute (skin) GVHD requiring (additional) systemic immunosuppressive therapy was very limited after the described conditioning regimens and ALT-based *in vitro* TCD strategy of grafts. Because of this low incidence, the few patients who did develop early acute GVHD post-alloSCT could not be identified based on the *in vitro* depletion efficiency or absolute cell count of naïve or regulatory T cells in their graft at the moment of transplantation.

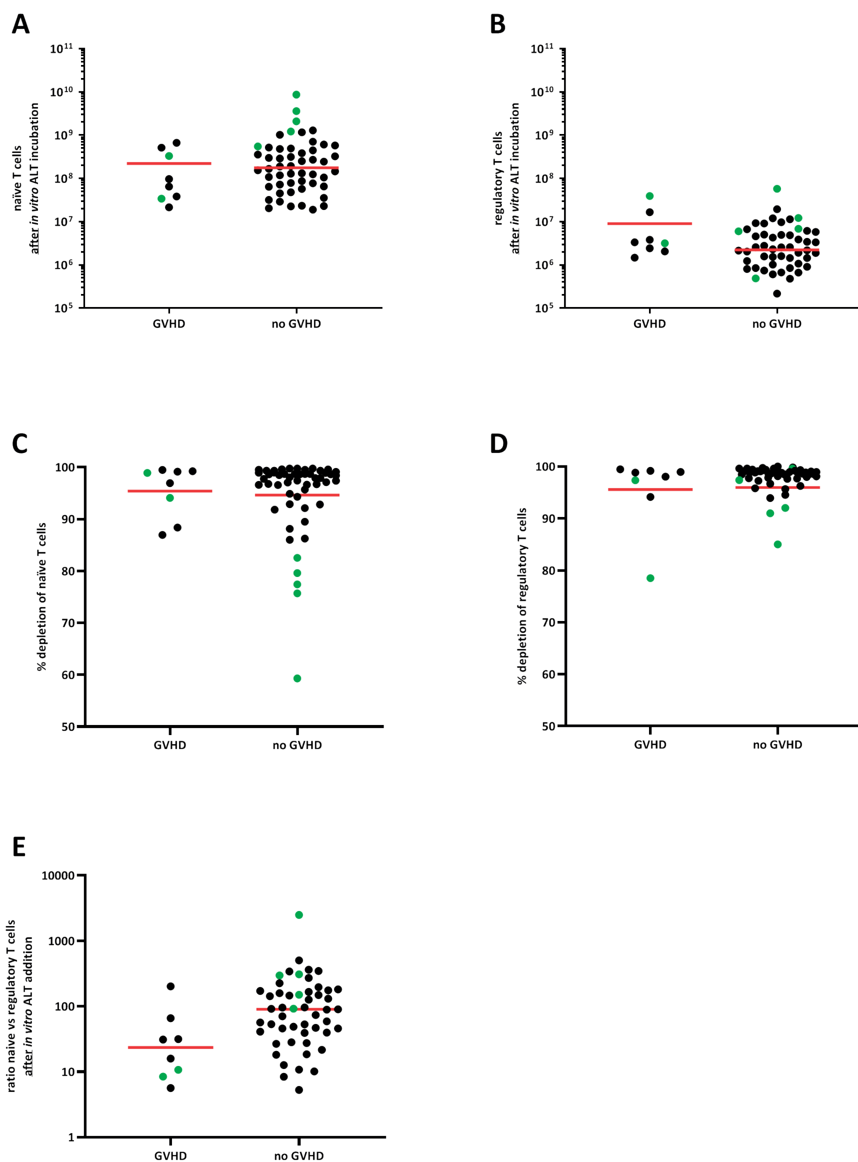

Figure 5. The absolute amounts nor the depletion efficiencies of naïve or regulatory T cells in the grafts can identify patients at risk of developing acute GVHD early after TCD alloSCT. After alloSCT, 8 of 60 patients developed acute GVHD requiring start of or change in systemic immunosuppressive therapy within 12 weeks post-alloSCT. The 7 grafts with α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The Mann-Whitney test was used for statistical analysis. **A**, The absolute numbers of naïve T cells and **B**, regulatory T cells in the grafts after the incubation with ALT in patients with or without GVHD. The median is indicated by the red line. **C**, The depletion efficiencies of naïve T cells and **D**, regulatory T cells in the grafts due to the *in vitro* incubation with ALT in patients with or without GVHD. The median is indicated by the red line. **E**, Ratio between the numbers of naïve and regulatory T cells in the grafts after the incubation with ALT.

DISCUSSION

In this study, we show that the *in vitro* incubation of allogeneic stem cell grafts with ALT resulted in a differential depletion of lymphocytes, leading to a significant compositional change in the grafts before infusion into the patient. However, notwithstanding graft variability, no predictive parameters for reconstitution of T cells at 3 or 6 weeks or GVHD development after transplantation could be defined. This can be explained by continuation of TCD *in vivo* after graft infusion, whereas the low reported incidence of acute GVHD within 12 weeks after transplantation implies that the described conditioning regimens containing *in vitro* ALT-based TCD of grafts resulted in efficient GVHD prophylaxis.

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In our ALT 'to the bag' protocol, 20 mg of ALT was added *in vitro* to every graft irrespective of graft characteristics. This resulted in a significant reduction in lymphocytes after 30 minutes of *in vitro* incubation. Among the lymphocyte subsets, TCR α/β T cells were depleted the most efficiently compared with TCR γ/δ T cells, B cells and NK cells. Among the TCR α/β T-cell subsets, $CD4^{pos}$ T cells in the grafts were depleted more efficiently than $CD8^{pos}$ T cells, and naïve and regulatory T cells were depleted more efficiently than effector T cells. The median absolute number of effector T cells seemed to increase slightly after *in vitro* incubation with ALT, which is most likely within the measurement's margin of error. The differences in observed depletion efficiencies of lymphocyte and T-cell subsets were in line with their reported CD52-expression levels and resulted in major compositional changes in the grafts.[21] The T-cell compartment in the grafts consisted of mainly $CD4^{pos}$ T cells with a naïve or memory phenotype before ALT addition, whereas the remaining T cells in the grafts at the moment of infusion were in the majority of patients dominated by $CD8^{pos}$ T cells with an effector or memory phenotype. Although the absolute numbers of T cells in the grafts were significantly reduced by the direct effect of ALT *in vitro*, the grafts were not completely depleted of T cells at the moment of graft infusion. Furthermore, the absolute numbers of infused TCR α/β T cells or memory T cells were not predictive for immune reconstitution 3 or 6 weeks after transplantation.

We have previously demonstrated that in the majority of patients no circulating T cells are found immediately after or in the days following infusion of stem cell grafts pre-incubated with ALT, suggesting that the process of ALT-based TCD most likely continues *in vivo* after graft infusion.[42] Accordingly, reliable measurements of the absolute numbers of lymphocytes and T cells that are actually depleted by our ALT-based TCD cannot be assessed, but the reported depletion efficiencies do give insight in the proportions of depletion among cell subsets. The efficiency of TCD of the ALT-based TCD strategy is therefore difficult to compare with other *ex vivo* TCD strategies, such as physical isolation of $CD34^{pos}$ cells or selective depletion of TCR α/β T cells using antibody-coated magnetic beads and magnetic separation, since the complete effect of these TCD strategies can be evaluated before graft infusion.[6,8]

The composition of the grafts varied widely in quantity and quality of T cells already before ALT addition. This might be due to differences in lymphocyte composition among donors as well as technique and duration of leukapheresis.[43,44] Graft characteristics, such as absolute leukocyte counts and leukocyte concentrations of the grafts before ALT addition, and graft volumes did not predict depletion efficiency of TCR α/β T cells. Furthermore, graft source (related versus unrelated) or the time interval between the donor leukapheresis procedure and actual graft infusion into the patient did not influence *in vitro* depletion efficiency or graft compositional change of lymphocytes or T-cell subsets. These observations illustrate that our *in vitro* TCD strategy works equally well for grafts obtained from national and international donor centers with variable volumes and leukocyte counts.

Analysis of grafts before and after the addition of ALT *in vitro* showed that naïve T cells and regulatory T cells were depleted in similar proportions. Previous studies using selective naïve T-cell depletion have shown that an efficient depletion of naïve T cells is associated with a decreased risk of acute GVHD post-transplantation.[11] By contrast, efficient depletion of regulatory T cells from the graft might reverse this effect, whereas high frequencies of regulatory T cells in stem cell grafts are associated with a decreased chance of developing GVHD.[45-47] However, in our cohort, only eight of 60 patients developed acute GVHD requiring (additional) systemic immunosuppressive therapy within 12 weeks after transplantation. This low incidence of mainly limited acute GVHD suggests that, in the majority of patients, alloreactive T cells were efficiently depleted from donor stem cell grafts and/or that remaining alloreactive T cells were adequately suppressed after transplantation. The observed balanced depletion of both naïve and regulatory T cells from grafts might therefore contribute to GVHD prevention. It has been suggested that the ratio of regulatory T cells to total CD4^{pos} T cells at 2 weeks after alloSCT is an indicator for the development of GVHD in patients after HLA-mismatched, non-TCD alloSCT.[48] With regard to naïve or regulatory T cells, we were not able to find a difference in *in vitro* depletion efficiency or absolute cell counts after *in vitro* ALT incubation between patients who did or did not develop GVHD post-transplantation. This might be explained by the low incidence of GVHD in our cohort. However, the ratio between the numbers of naïve and regulatory T cells was significantly higher in patients without GVHD compared to patients who developed GHVD soon after TCD alloSCT. This unexpected result is difficult to interpret because of the low number of patients who experienced GVHD. In addition, the absolute numbers of naïve and regulatory T cells were very low after *in vitro* ALT incubation, and small differences in depletion efficiency between naïve and regulatory T cell subsets may result in large differences in the ratio. Based on the results, it was not possible to predict beforehand which patients were at risk of developing GVHD based on graft composition after *in vitro* ALT incubation.

We have shown that 6 weeks after TCD alloSCT, T-cell reconstitution was mediated by both CD52^{pos} and CD52^{neg} T cells. In almost all patients, only ALT was used as T-cell depleting agent, and reconstitution of CD52^{neg} T cells was observed. As these CD52^{neg} T cells can give adequate protection

against viral reactivations, delayed reconstitution of CD52^{pos} T cells in this group of patients seems not to be problematic.[35] Importantly, patients who receive NMA conditioning and an unrelated donor graft have a relatively high risk of GVHD development, and therefore received both ALT and ATG as T-cell depleting agents. As CD52^{neg} T cells are sensitive to ATG, CD52^{neg} T-cell reconstitution was not predominant over CD52^{pos} T-cell reconstitution in these patients. Theoretically, an excess of unbound ALT infused with the graft, in combination with ongoing lytic levels of ATG in PB could result in a further delay in T-cell reconstitution. In the patients who received both ALT and ATG, T-cell reconstitution was indeed delayed compared with T-cell reconstitution in patients receiving only ALT. Based on these observations, we conclude that the addition of 20 mg ALT to stem cell grafts *in vitro* as TCD strategy for patients receiving MA or NMA conditioning is sufficient to prevent GVHD without extreme delays in protective T-cell repopulation. Furthermore, since long-term immunosuppression is not indicated after *in vitro* ALT-based TCD, this alloSCT protocol is a suitable platform for application of post-transplant cellular therapies such as donor lymphocyte infusion or adoptive transfer of *in vitro* selected T-cell populations to specifically boost the graft-versus-leukemia effect or pathogen-specific immunity.[49-54]

In conclusion, we have shown that the *in vitro* addition of 20 mg ALT to allogeneic stem cell grafts is an easy, fast and generally applicable method for the efficient depletion of donor-derived T cells from allogeneic stem cell grafts. The heterogenous expression of CD52 results in the differential depletion of lymphocyte and T-cell subsets, leading to a major compositional change in the graft before infusion into the patient. The continuation of TCD *in vivo* results in a limited incidence of GVHD, which can be explained by the balanced depletion of naïve and regulatory T cells by ALT.

ACKNOWLEDGEMENTS

The authors thank patients, donors and medical staff involved in this study; Employees of the Laboratory for Stem Cell Therapy, Laboratory for Specialized Hematology and the Flow Cytometry Core Facility for logistical and technical assistance; Liesbeth de Wreede for statistical advice. This study was financially supported by the Minerva fellowship of the LUMC Cancer Fund.

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SUPPLEMENTARY MATERIAL

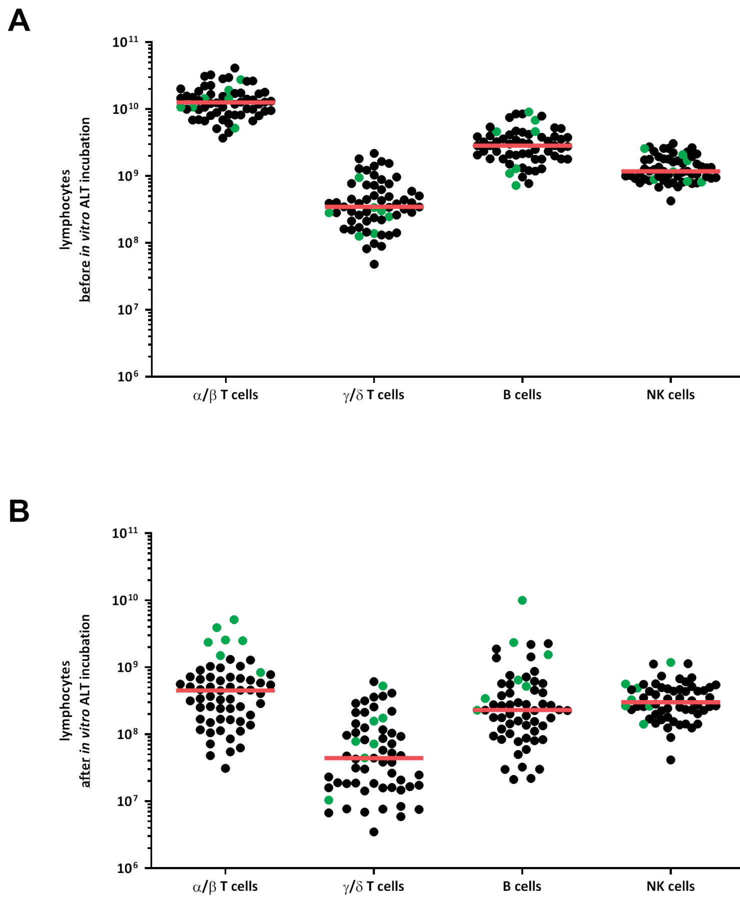
Supplementary Table 1. ALT-based TCD conditioning regimens for G-CSF mobilized peripheral blood allogeneic stem cell transplantations. T-cell depleting agents are underlined. iv, intravenously; MA, myeloablative; NMA, non-myeloablative; TBI, total body irradiation.

Donor	Conditioning intensity	Agents	Dose	Administration days related to transplantation (day 0)
Related	MA *n* = 6	Cyclophosphamide	60 mg/kg iv	-6,-5
		Mesna	60 mg/kg iv	-6,-5,-4
		TBI	9 Gy	-1
		Alemtuzumab added to the graft	20 mg ‘in the bag’	0
		or		
		Busulfan	3.2 mg/kg iv	-9 until-6
		Cyclophosphamide	60 mg/kg iv	-4,-3
		Mesna	60 mg/kg iv	-4,-3,-2
		Alemtuzumab added to the graft	20 mg ‘in the bag’	0
	NMA *n* = 14	Fludarabine	50 mg/m2 oral	-10 until-5
		Busulfan	3.2 mg/kg iv	-7,-6
		Alemtuzumab	15 mg iv	-4,-3
		Alemtuzumab added to the graft	20 mg ‘in the bag’	0
		or		
		Fludarabine	30 mg/m2 iv	-14 until-10
		Cytarabine	2000 mg/m2 iv	-13 until-10
		Amsacrine	100 mg/m2 iv	-13 until-10
		Busulfan	3.2 mg/kg iv	-6,-5
		Alemtuzumab	15 mg iv	-4,-3
		Alemtuzumab added to the graft	20 mg ‘in the bag’	0
Unrelated	MA *n* = 14	TBI	9 Gy	-8 or-7
		Cyclophosphamide	60 mg/kg iv	-6,-5
		Mesna	60 mg/kg iv	-6,-5,-4
		Alemtuzumab	15 mg iv	-6,-5
		Cyclosporine A	3 mg/kg iv	-1 until +30, then tapered off
		Alemtuzumab added to the graft	20 mg ‘in the bag’	0
		or		
		Busulfan	3.2 mg/kg iv	-9 until-6
		Cyclophosphamide	60 mg/kg iv	-4,-3
		Mesna	60 mg/kg iv	-4,-3,-2
		Alemtuzumab	15 mg iv	-6,-5
		Cyclosporine A	3 mg/kg iv	-1 until +30, then tapered off
		Alemtuzumab added to the graft	20 mg ‘in the bag’	0
	NMA *n* = 26	Fludarabine	50 mg/ m2 oral	-10 until-5
		Busulfan	3.2 mg/kg iv	-7,-6
		Alemtuzumab	15 mg iv	-4,-3
		Anti-thymocyte globulin	1 mg/kg iv	-2
		Alemtuzumab added to the graft	20 mg ‘in the bag’	0
		Prednisolone	1 mg/kg iv or oral	1 until 10, then tapered off
		or		
		Fludarabine	30 mg/m2 iv	-14 until-10
		Cytarabine	2000 mg/m2 iv	-13 until-10
		Amsacrine	100 mg/m2 iv	-13 until-10
		Busulfan	3.2 mg/kg iv	-6,-5
		Alemtuzumab	15 mg iv	-4,-3
		Anti-thymocyte globulin	1 mg/kg iv	-2
		Alemtuzumab added to the graft	20 mg ‘in the bag’	0
		Prednisolone	1 mg/kg iv or oral	1 until 10, then tapered off

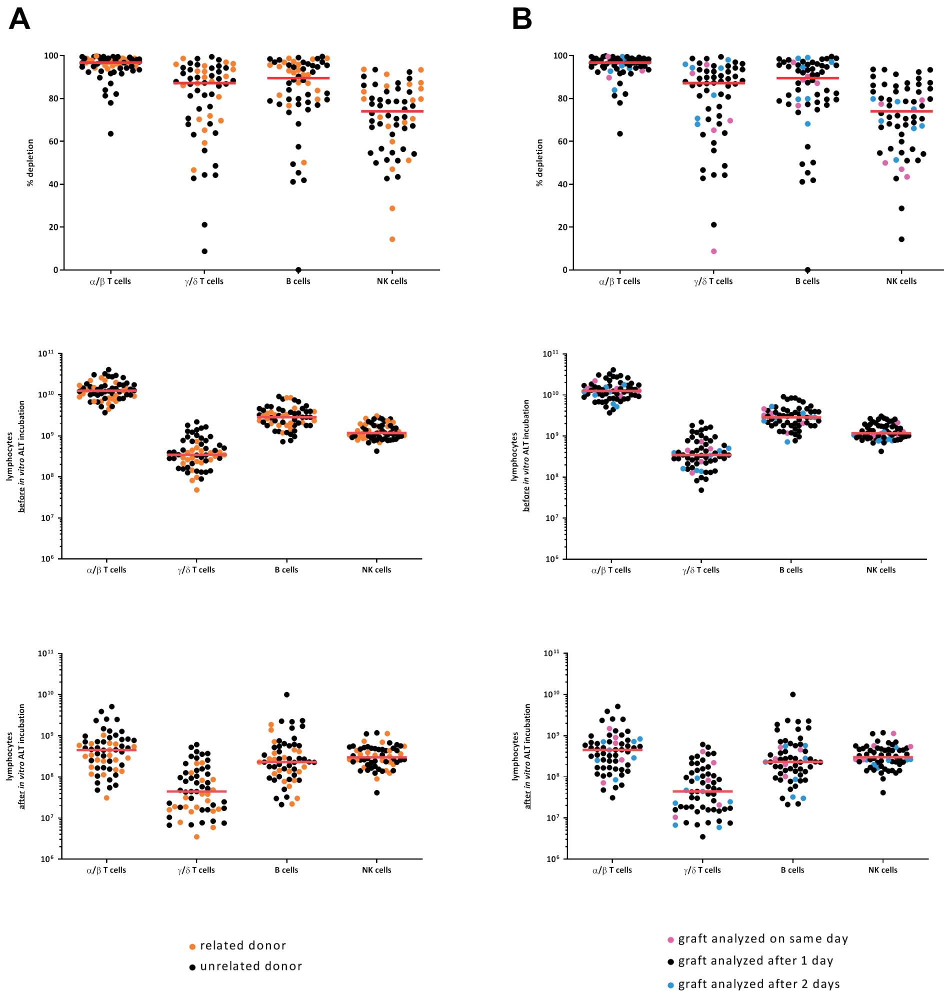
Supplementary Table 2. Percentages of lymphocyte subsets in the grafts before and after the *in vitro* incubation with ALT. The significance of differences in percentages of lymphocytes in grafts before and after *in vitro* ALT incubation was calculated.

		Before ALT addition		After ALT addition		Significance of differences
		Median	*Range*	*Median*	*Range*	*P value**
TCR α/β T cells (%)		71.9	55.8 – 85.5	33.7	4.8 – 81.0	<0.001
$CD8^{pos}$ (%)	Naïve T cells	10.5	0.8 – 33.3	12.8	1.1 – 43.1	0.003
	Memory T cells	3.3	0.1 – 15.9	10.0	0.6 – 50.4	<0.001
	Effector T cells	2.0	0.1 – 10.5	60.8	3.8 – 93.2	<0.001
$CD4^{pos}$ (%)	Naïve T cells	40.9	13.1 – 77.5	7.6	0.2 – 60.5	<0.001
	Memory T cells	37.9	9.0 – 68.4	3.7	0.5 – 24.7	<0.001
	Effector T cells	0.5	0.0 – 5.1	0.8	0.0 – 8.9	0.019
	Regulatory T cells	1.5	0.0 – 5.6	0.4	0.0 – 7.6	<0.001
TCR γ/δ T cells (%)		2.1	0.6 – 12.3	3.2	0.5 – 29.2	<0.001
B cells (%)		17.3	4.3 – 33.7	22.3	4.8 – 73.8	<0.001
NK cells (%)		7.6	2.8 – 19.5	29.3	4.9 – 73.8	<0.001

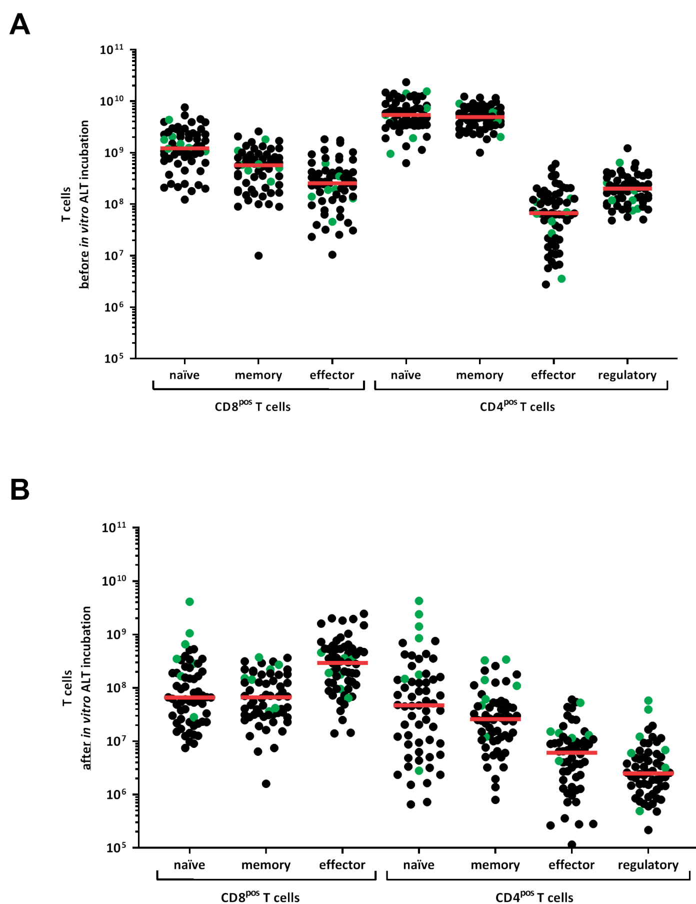
** Wilcoxon matched-pairs, signed-rank test*



Supplementary Figure 1. Absolute numbers of lymphocytes in grafts (n = 60). The 7 grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The red solid lines indicate medians. **A**, Before *in vitro* ALT incubation. **B**, After *in vitro* ALT incubation.



Supplementary Figure 2. Depletion efficiencies (%) and absolute numbers of lymphocytes in grafts before and after *in vitro* ALT incubation (n = 60). **A**, grafts of related donors are indicated by orange symbols, grafts of unrelated donors are indicated by black symbols. **B**, grafts analyzed 0, 1 or 2 days after the donor leukapheresis procedure are indicated by pink, black and blue symbols, respectively.



Supplementary Figure 3. Absolute numbers of T cells in grafts (n = 60). The 7 grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The red solid lines indicate medians. **A**, Before *in vitro* ALT incubation. **B**, After *in vitro* ALT incubation.

2

CHAPTER 3

THE SIMULTANEOUS ISOLATION OF MULTIPLE HIGH AND LOW FREQUENT T-CELL POPULATIONS FROM DONOR PERIPHERAL BLOOD MONONUCLEAR CELLS USING THE MAJOR HISTOCOMPATIBILITY COMPLEX I-STREPTAMER ISOLATION TECHNOLOGY

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Cytotherapy 2018; 20(4): 543-555.

ABSTRACT

BACKGROUND

Adoptive transfer of donor-derived T cells can be applied to improve immune reconstitution in immune-compromised patients after allogeneic stem cell transplantation. The separation of beneficial T cells from potentially harmful T cells can be achieved by using the major histocompatibility complex class I (MHC I)-*Strep*tamer isolation technology, which has proven its feasibility for the fast and pure isolation of T-cell populations with a single specificity. We have analyzed the feasibility of the simultaneous isolation of multiple antigen-specific T-cell populations in one procedure by combining different MHC I-*Strep*tamers.

METHODS

First, the effect of combining different amounts of MHC I-*Strep*tamers used in the isolation procedure on the isolation efficacy of target-antigen-specific T cells and on the number of off-target co-isolated contaminating cells was assessed. The feasibility of this approach was demonstrated in large-scale validation procedures targeting both high and low frequent T-cell populations using the Good Manufacturing Practice (GMP)-compliant CliniMACS Plus device.

RESULTS

T-cell products targeting up to 24 different T-cell populations could be isolated in one, simultaneous MHC I-*Strep*tamer procedure, by adjusting the amount of MHC I-*Strep*tamers per target antigen-specific T-cell population. Concurrently, the co-isolation of potential harmful contaminating T cells remained below our safety limit. This technology allows the reproducible isolation of high and low frequent T-cell populations. However, the expected therapeutic relevance of direct clinical application without *in vitro* expansion of these low frequent T-cell populations is questionable.

DISCUSSION

This study provides a feasible, fast and safe method for the generation of highly personalized MHC I-*Strep*tamer isolated T-cell products for adoptive immunotherapy.

INTRODUCTION

Immune-compromised patients after allogeneic hematopoietic stem cell transplantation (alloSCT) are vulnerable to viral infections and disease relapses. Although donor T cells can mediate graft-versus-leukemia (GVL) responses and restore pathogen specific immunity, the administration of T cells with the graft or unmodified donor lymphocyte infusions (DLI) early after a T-cell-depleted graft is also associated with a significant risk of graft-versus-host disease (GVHD).[1-3] Therefore, the adoptive transfer of selected T-cell populations with solely beneficial effects is highly desirable, especially in the period between T cell-depleted alloSCT and DLI. This requires a widely applicable, fast and Good Manufacturing Practice (GMP)-compliant technique to isolate well defined T-cell populations from donor peripheral blood mononuclear cells (PBMC) and avoid the co-isolation of alloreactive T cells.[4-7]

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The major histocompatibility complex class I (MHC I)-*Strep*tamer technology is developed for the detection and isolation of human antigen-specific T cells from PBMC. This technique is based on the direct labeling of $CD8^{pos}$ T cells with MHC I-*Strep*tamers which are composed of peptide-loaded MHC I-*Strep*-tag fusion proteins (MHC I-*Strep* proteins) reversibly multimerized on magnetically labeled *Strep*-Tactin (*Strep*-Tactin nanobeads). After the magnetic separation, the MHC I-*Strep*tamers can be dissociated from the positively selected cells by the addition of D-Biotin, a high affinity competitor for the binding sites on *Strep*-Tactin. This isolation technique allows the purification of non-coated, unlabeled antigen-specific T cells under GMP conditions for clinical application in only one day.[8-12]

The feasibility of the MHC I-*Strep*tamer approach for the isolation of cytomegalovirus (CMV) or Epstein-Barr virus (EBV) antigen-specific T cells from seropositive donors was demonstrated in various (pre-)clinical studies.[10,13-16] The achieved high purities of virus-specific T cells in the products and the acceptable numbers of contaminating cells resulted in T-cell products feasible for direct clinical application or further *in vitro* manipulation. The safety and efficacy of the clinical application of donor-derived CMV-specific T-cell products in patients with persistent CMV viremia after alloSCT demonstrated no reports of initiation or aggravation of acute GVHD. Furthermore, in all patients donor-derived CMV epitope-specific T cells became detectable *in vivo* after infusion of the product, suggesting expansion of infused cells. Moreover, the majority of patients experienced a partial or complete response.[14,16]

The MHC I-*Strep*tamer technology has so far been used mainly for the isolation of relatively frequent virus-specific T cells from the memory T-cell compartment of seropositive donors. However, T cells with more rare precursor frequencies in donor PBMC, like virus-specific T cells from seronegative donors, minor histocompatibility antigen (MiHA)-specific T cells or tumor-associated

antigen (TAA)-specific T cells, are also relevant candidates for adoptive T-cell therapy.[17-21] Previous attempts to enrich such low frequent T cells, like human adenovirus (AdV) or TAA-specific T cells, with the MHC I-*Strep*tamer technique resulted in less pure T-cell products.[10,15] An approach to solve this problem is an *in vitro* target antigen-specific expansion induced by stimulation with peptide pools for 12-14 days prior to isolation.[22] Although this resulted in T-cell products with relatively high purities of AdV- or TAA-specific T cells, this strategy is time-consuming, might impair *in vivo* T-cell function and will abrogate the advantage of the MHC I-*Strep*tamer technology to create a selected lymphocyte product with regulatory advantages in contrast to an Advanced Therapy Medicinal Product (ATMP).[15,23]

The T-cell products generated with the MHC I-*Strep*tamer technology that were clinically applied until now contained only a limited number of specificities. Although T cells directed against a single antigen can control viral reactivations, the inclusion of T cells with different target antigen specificities in one product may be preferred for clinical application.[24,25] Freimuller et al compared the simultaneous isolation of low frequent AdV-specific T cells and high frequent EBV-specific T cells versus the isolation of AdV-specific T cells alone. Although the frequencies of AdV-specific T cells in the product were still low after the combined isolation, the addition of EBV-specific MHC I-*Strep*tamers resulted in increased purity of the final T-cell product to levels acceptable for clinical application.[15]

In this study, we aimed to develop a robust and widely applicable GMP-compliant method for the simultaneous isolation of purified T-cell products containing multiple antigen specific T-cell populations from donor PBMC using the MHC I-*Strep*tamer technology. Therefore, we assessed how many T-cell populations can be targeted in one isolation procedure. Besides the isolation of high frequent viral T-cell populations, we also studied the isolation of low frequent viral T-cell populations and TAA-specific T cells. Our data show that the MHC I-*Strep*tamer technology allows the combined isolation of multiple T-cell populations with a wide range of precursor frequencies in donor PBMC, resulting in a pure and safe T-cell product for direct clinical application. However, the expected therapeutic relevance of direct clinical application without *in vitro* expansion of very low frequent T-cell populations is questionable.

MATERIALS AND METHODS

LEUKAPHERESIS PRODUCTS FROM HEALTHY DONORS

Peripheral blood or leukapheresis products were obtained from stem cell donors after approval by the Leiden University Medical Center (LUMC) Institutional Board and written informed consent according to the Declaration of Helsinki. PBMC were collected by the use of Ficoll-Isopaque

separation or red blood cell lysis using an NH_4Cl (8.4 g/L) and $KHCO_3$ (1 g/L) buffer (pH = 7.4) (LUMC Pharmacy, Leiden, The Netherlands). PBMC were used directly (donors M-P) or thawed after cryopreservation in the vapor phase of liquid nitrogen (donors A-L). Donor characteristics (HLA-typing, CMV and EBV serostatus and amount of cells used for experiments) are provided in Supplementary Table 1.

GENERATION OF MHC I-*STREP*TAMERS

MHC I-*Strep*tamers (Juno Therapeutics, Goettingen, Germany) were generated by the incubation of peptide-loaded MHC I-*Strep*-tag fusion proteins (MHC I-*Strep* proteins) with magnetically labeled *Strep*-Tactin (*Strep*-Tactin nanobeads) in phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.4% human serum albumin (HSA, Sanquin Reagents, Amsterdam, The Netherlands) (PBS/HSA buffer) overnight at 4°C, to allow the multimerization of MHC I-*Strep* proteins on the *Strep*-Tactin nanobeads. In accordance to the manufacturer's instruction (Juno Therapeutics), the number of starting cells determined the amount of MHC I-*Strep* proteins and *Strep*-Tactin nanobeads used in the procedure and the ratio between these two components remained constant in all cases. Unbound MHC I-*Strep* proteins were removed by washing the MHC I-*Strep*tamers on LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) placed in the magnetic field of the MidiMACS separator device (Miltenyi Biotec). MHC I-*Strep*tamers were eluted in a volume of 3 ml PBS/HSA buffer. In case multiple MHC I-*Strep*tamers with different target antigen specificities were used in one isolation procedure, this incubation and washing protocol was performed in separate tubes for each individual MHC I-*Strep*tamer to prevent the generation of MHC I-*Strep*tamers with mixed MHC I-*Strep* proteins. In the experiment with empty *Strep*-Tactin nanobeads, the same protocol was applied with the only difference that no MHC I-*Strep* proteins were added to the *Strep*-Tactin nanobeads during the overnight incubation step. Table 1 provides an overview of the peptide-loaded MHC I-*Strep* proteins used in this study.

SMALL SCALE MHC I-*STREP*TAMER ISOLATIONS: OPTIMIZATION OF PROCEDURE

For the optimization of the isolation protocol, different amounts of MHC I-*Strep*tamers were used in isolation procedures. 1x indicates the recommended amount of MHC I-*Strep*tamers for isolations performed with the indicated number of starting cells as described in the manufacturer's instruction. When less or more than the recommended amount of MHC I-*Strep*tamers per amount of starting cells were used in a procedure, the fold reduction or fold increase relative to this recommended amount are indicated (1/16 – 20x).

The isolation procedures were started with $40*10^6$ PBMC of HLA-A*02:01^{neg} donors A-C. In specific experiments, cells of a CMV pp65-NLV/A*02:01 T cell line were added to PBMC in the mentioned frequencies of 0.05% or 1%. Starting cells were incubated with MHC I-*Strep*tamers in equal volumes within experiments, implying different concentrations of MHC-I *Strep*tamers, for 45 minutes on a

MACSmix Tube Rotator (Miltenyi Biotec) at 4°C. Afterwards, the cells with MHC I-*Strep*tamers were centrifuged to eliminate unbound MHC I-*Strep*tamers. The isolation procedures were performed on the MidiMACS Separator using LS columns. MHC I-*Strep*tamers were dissociated from the positively isolated cells using D-Biotin in accordance to the manufacturer's instructions, counted in duplicate and used for fluorescence activated cell sorting (FACS) analysis. The efficacy of the isolation of target-antigen-specific T cells was calculated by dividing the absolute number of CMV pp65-NLV/A*02:01 tetramer^{pos} cells in the positive fractions by the absolute number of CMV pp65-NLV/A*02:01 tetramer^{pos} cells in the positive and negative fraction together.

INTERMEDIATE- AND LARGE- SCALE MHC I-*STREP*TAMER ISOLATIONS: VALIDATION OF PROCEDURE

Based on the results regarding the small scale MHC I-*Strep*tamer isolations and the isolation performed in donor D and E, half of the recommended amount of each individual MHC I-*Strep*tamer was used for the indicated number of starting cells for donor F-P. The intermediate/large scale test runs were performed with 435-2,000*10^{6} PBMC of HLA-A*02:01^{pos} donors as starting material. Starting cells were incubated with the indicated pool of different MHC I-*Strep*tamers for 45 minutes at 4°C. After this incubation, the cell suspensions were washed and centrifuged to remove unbound MHC I-*Strep*tamers. The isolations for donors D-P were performed under GMP conditions on a CliniMACS Plus instrument (Miltenyi Biotec) using a CliniMACS tubing set TS (161-01) with the selection program 'CD34 selection 1'.

IDENTIFICATION OF TARGET ANTIGEN-SPECIFIC T-CELL POPULATIONS IN PRODUCT

After the primary isolation procedures on the CliniMACS, the positive fractions of donors G-P were non-specifically expanded in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Basel, Switzerland) supplemented with 10% human serum, 100 IU/ml interleukin-2 (Chiron, Amsterdam, The Netherlands) and 800 ng/ml phytohemagglutinin (PHA, Oxoid Limited, Hampshire, UK) with five times irradiated (35 Gy), autologous or allogeneic PBMC as feeder cells. After 10-14 days of expansion, the cultures were analyzed by FACS to detect individual target antigen-specific T-cell populations. To investigate the possible presence of antigen specific T-cell populations that were not directly visible in this post-expansion analysis, subsequent enrichments were performed using the MHC I-*Strep*tamer complexes for these specificities and isolation on a MidiMACS Separator using LS Columns, followed by another round of non-specific expansion. This procedure was repeated until all target antigen-specific T-cell populations became visible by direct tetramer staining after expansion of the positive fractions or until 4 consecutive rounds were accomplished.

SAFETY OF MHC I-*STREP*TAMER ISOLATED T-CELL PRODUCTS FOR CLINICAL APPLICATION

The safety of T-cell products for clinical application is determined by the absolute number of T cells with an unknown specificity, that may potentially induce GVHD. Based on our experience

with the administration of unmodified DLI early after T-cell depleted alloSCT and results from a previous clinical study,[26] a limit of $0.1*10^6$ T cells with an unknown specificity per kg bodyweight of the patient is anticipated to be safe and is therefore included as a criteria in the release of T-cell products for clinical application. In this respect, the infusion of $5*10^6$ T cells of unknown specificity is acceptable for a patient over 50 kg. When isolation procedures are started with (maximum) $5*10^9$ donor PBMC, this corresponds with 0.1% of starting cells. Therefore, the limit of T cells with unknown specificity of 0.1% of starting material was applied to all experiments in this study.

FACS ANALYSIS WITH HLA CLASS I TETRAMERS AND MONOCLONAL ANTIBODIES

To determine the composition of the starting material and the fractions after isolation, cells were stained using fluorescein isothiocyanate (FITC)-labeled CD4 and CD14 (Beckton Dickinson (BD) Biosciences, San Jose/San Diego, CA, USA) antibodies, phycoerythrin (PE)-labeled CD56 (BD), peridinin-chlorophyll-protein complex (PerCP)-labeled CD3 and CD8 (BD) antibodies, allophycocyanin (APC)-labeled CD19 (BD) antibodies, phycoerythrin-cyanine 7 (PeCy7)-labeled CD4 and CD33 (BD) antibodies, V500-labeled CD4 (BD) antibodies, APC-H7-labeled CD3 (BD) antibodies and Pacific Blue-labeled CD8 (BD) antibodies. PE- and APC-labeled tetramers were produced as described previously for all target antigen-specific T-cell populations indicated in Table 1.[27] For FACS analysis, cells were stained with monoclonal antibodies for 20 min at 4°C. When tetramers were involved in the staining, cells were incubated with tetramers for 10 min at 37°C prior to monoclonal antibody staining. Cells were analyzed on a FACS Calibur or Canto (BD) and analyzed using FlowJo Software (TreeStar, Ashland, OR, USA) or Diva software (BD), respectively.

Table 1. HLA/peptide-complexes for which MHC I-*Strep* proteins for isolation and tetramers for FACS staining were used.

Protein	Peptide	HLA-type
CMV pp65	**NLV**PMVATV	A*02:01
CMV pp65	**QYD**PVAALF	A*24:02
CMV pp65	**TPR**VTGGGAM	B*07:02
CMV IE-1	**QIK**VRVDMV	B*08:01
EBV BMLF-1	**GLC**TLVAML	A*02:01
EBV LMP2	**PYL**FWLAAI	A*24:02
EBV EBNA-3A	**RPP**IFIRRL	B*07:02
EBV BZLF-1	**RAK**FKQLL	B*08:01
AdV E1A	**LLD**QLIEEV	A*02:01
AdV Hexon	**TDL**GQNLLY	A*01:01
AdV Hexon	**TYF**SLNNKF	A*24:02
AdV Hexon	**KPY**SGTAYNAL	B*07:02
TAA NY-eso-1	**SLL**MWITQV	A*02:01
TAA WT1	**RMF**PNAPYL	A*02:01
TAA RHAMM	**ILS**LELMKL	A*02:01
TAA proteinase-3	**VLQ**ELNVTV	A*02:01
TAA PRAME	**VLD**GLDVLL	A*02:01

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RESULTS

AN INCREASED AMOUNT OF MHC I-*STREP*TAMERS USED IN THE PROCEDURE RESULTS IN A DECREASED PURITY OF TARGET-ANTIGEN-SPECIFIC T CELLS IN THE PRODUCT

For the isolation of multiple T-cell populations in one isolation procedure, PBMC need to be incubated with multiple MHC I-*Strep*tamers containing all corresponding peptide-loaded MHC I-*Strep* proteins. According to the recommendations of the manufacturer, the amount of MHC I-*Strep*tamers used per specificity in a procedure is based on the total number of starting cells (PBMC) and is independent of the precursor frequency of target-antigen-specific T cells in this starting material. When per target antigen specificity the recommended amount of MHC I-*Strep*tamers is used, the starting cells will then consequently be incubated with a proportional fold increase in MHC I-*Strep*tamers in a simultaneous isolation procedure. It was hypothesized that this increase in MHC I-*Strep*tamers may result in an accumulation of contaminating cells in the positive fraction, comprising T cells of unknown specificity that can potentially cause GVHD. To investigate how many different T-cell populations can be targeted within one combined isolation procedure, the maximum tolerable amount of MHC-I *Strep*tamers was determined at which the number of contaminating T cells in the positive fraction was still acceptable, based on the upper limit of T cells of unknown specificity defined in our release criteria for T-cell products for clinical application ($0.1*10^6$/kg, equal to 0.1% of total starting cells when the isolation procedure is started with $5*10^9$ PBMC for a 50 kg patient). To analyze exclusively the effect of the amount of MHC I-*Strep*tamers on the co-isolated amount of contaminating cells, MHC I-*Strep*tamers containing MHC I-*Strep* proteins with HLA-restrictions not expressed by the respective donors (further called irrelevant MHC I-*Strep*tamers; in this experiment EBV EBNA3a RPP/HLA-B*07:02, EBV BZLF-1 RAK/HLA-B*08:01 and ADV Hexon TDL/HLA-A*01:01) were used, as T cells with these HLA/peptide specificities are not supposed to be abundantly present in these donors (donor characteristics provided in Supplementary Table 1). For three donors (donors A-C), six small scale parallel isolation procedures were started with $40*10^6$ PBMC each, and increasing amounts of irrelevant MHC I-S*trep*tamers were added to the cells in equal volumes, ranging from the recommended amount (1x) of MHC I-*Strep*tamers for the isolation of a single T-cell population until twenty times the recommended amount (20x) of MHC I-*Strep*tamers to simulate a simultaneous isolation of twenty different T-cell populations within one isolation procedure. Figure 1A shows that the absolute numbers of total contaminating cells obtained in the positive fractions increased when more irrelevant MHC I-*Strep*tamers were included in the isolation. Over $4*10^5$ total contaminating cells (>1% of the total number of starting cells) ended up in the positive fractions when 16x or 20x irrelevant MHC I-*Strep*tamers were used for donors A and B. The adjustment of the incubation volumes to equalize the final concentration of MHC I-*Strep*tamers in the different parallel isolation procedures had no influence on the isolation outcomes (data not shown). Monocytes and B cells comprised the majority of contaminating cells in the positive fractions (Figure 1B). However, the

number of co-isolated T cells also increased when isolations were performed with more irrelevant MHC I-*Strep*tamers (Figure 1C), outreaching the pre-defined limit of $4*10^4$ contaminating T cells (= 0.1% of total number of starting cells) in the procedures with 16x and 20x irrelevant MHC I-*Strep*tamers for donors A and B. To confirm that cross reactive or allo-HLA restricted T cells targeting the specific HLA/peptides of the irrelevant MHC I-*Strep*tamer complexes did not contribute significantly to the contaminating T-cell populations after isolation, the positive fractions were stained with corresponding tetramers (Supplementary Figure 1; representative example for donor A).

Based on these results, 12x is the maximum amount of MHC I-*Strep*tamers that can be used in a simultaneous isolation procedure with an acceptable number of contaminating cells, comprising T cells of unknown specificity, in the positive fraction. By using the recommended amount of MHC I-*Strep*tamers per targeted T-cell population, this suggests that at maximum 12 different T-cell populations can be simultaneously targeted in one isolation procedure.

To investigate whether the contaminating T cells of unknown specificity end up in the positive fraction due to non-specific interaction of the T cell receptor with the MHC I-*Strep* proteins on the MHC I-*Strep*tamers, isolation procedures were repeated with increasing amounts (0x to 20x) of *Strep*-Tactin nanobeads without multimerized MHC I-*Strep* proteins (further called empty *Strep*-Tactin nanobeads) in donor A. Figure 1D shows that the number of total contaminating cells co-isolated in the positive fraction increased with the addition of more empty *Strep*-Tactin nanobeads to the procedure. Again, monocytes and B cells comprised the majority of contaminating cells. The number of contaminating T cells with an unknown specificity were comparable to the numbers obtained after isolations performed with irrelevant MHC I-*Strep*tamers (Figure 1E). This suggests that the co-isolation of contaminating cells including T cells of unknown specificity is independent of the presence of MHC I-*Strep* proteins on the *Strep*-Tactin nanobeads, and is due to complete non-specific adherence to the *Strep*-Tactin nanobeads.

THE ISOLATION EFFICACY OF A SINGLE TARGET ANTIGEN-SPECIFIC T-CELL POPULATION IS PRESERVED USING HALF THE AMOUNT OF MHC I-*STREP*TAMERS IN THE PROCEDURE

Reducing the amount of MHC I-*Strep*tamers per target antigen-specific T-cell population would allow enrichment of more T-cell populations in one simultaneous isolation procedure without exceeding the pre-defined limit of contaminating T cells of unknown specificity in the positive fraction. Therefore, we investigated whether the utilized amount of MHC I-*Strep*tamers per target antigen specificity could be reduced, without affecting the isolation efficacy of the targeted T-cell populations. As the specific isolation efficacy might be influenced by the precursor frequency of the targeted T-cell population in the starting material, the effect of lowering the amount of isolation reagents was examined in starting material containing two different frequencies of target-antigen-specific T cells

(0.05% and 1%). These frequencies were based on the range of frequencies of circulating memory virus specific T cells naturally occurring in PBMC of seropositive donors.[11,28,29]

As a model, *ex vivo* expanded CMV pp65-NLV/A*02:01-specific T cells were added in frequencies of 0.05% and 1.0% to $40*10^6$ PBMC of three HLA-A*02:01^{neg} donors (donors A-C). Starting cells were incubated with five different amounts of CMV pp65-NLV/A*02:01-specific MHC I-*Strep*tamers, ranging from 1/16x the recommended amount of MHC I-*Strep*tamers (1/16x) until

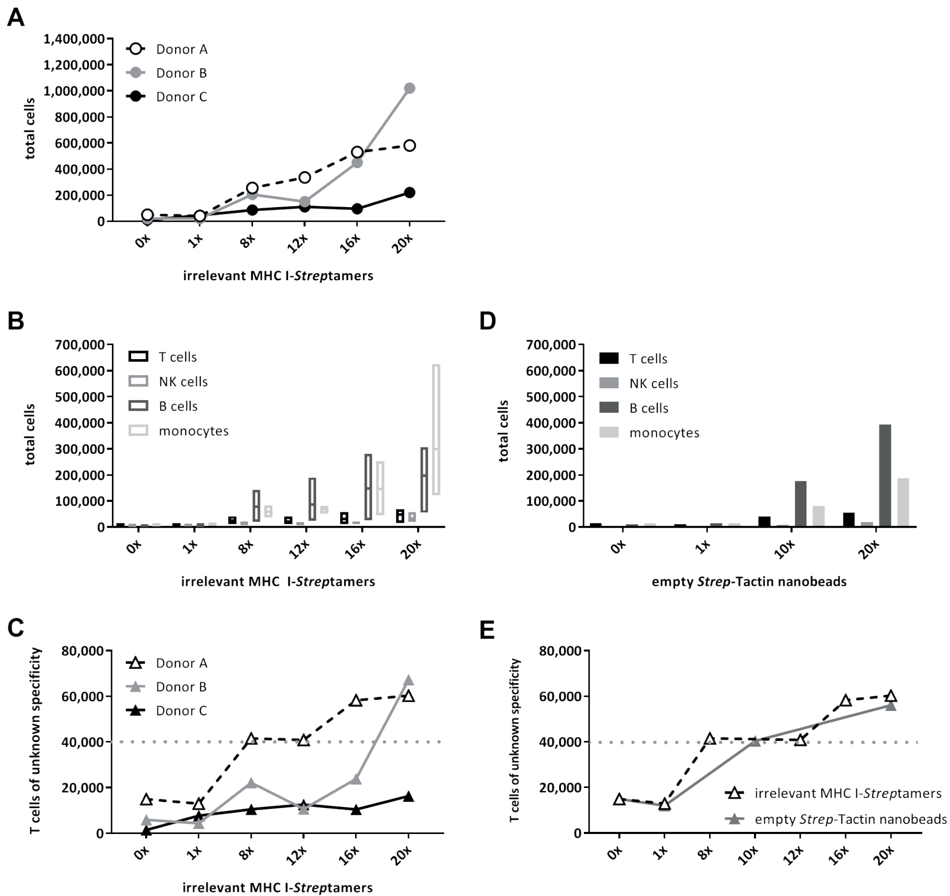

Figure 1. Contaminating cells in positive fractions after isolation procedures performed with different amounts of irrelevant MHC I-*Strep*tamers and empty *Strep*-Tactin nanobeads. **A**, Total cells, **B**, different cell types and, **C**, T cells of unknown specificity in positive fractions after isolations performed with 1x – 20x the recommended amount of irrelevant MHC I-*Strep*tamers for donor A, B and C. **D**, Cell types in positive fractions after isolations performed with different amounts of empty *Strep*-Tactin nanobeads for donor A. **E**, Comparison of the absolute number of T cells with an unknown specificity in positive fractions after isolations performed with irrelevant MHC I-*Strep*tamers versus empty *Strep*-Tactin nanobeads for donor A. Dotted line indicates defined acceptable limit of contaminating T cells (0.1% of starting material).

the recommended amount of MHC I-*Strep*tamers (1x) per T-cell specificity. Figure 2A shows that the absolute numbers of isolated CMV pp65-NLV/A*02:01-specific T cells were comparable for the isolations performed with 1x and 1/2x the amount of MHC I-*Strep*tamers. However, a further decreasing of in the utilized amount of MHC I-*Strep*tamers resulted in a substantial decrease in the number of isolated target-antigen-specific T cells. This was also reflected by the loss of target-antigen-specific T cells to the corresponding negative fractions (Supplementary Figure 2) and the decreasing normalized isolation efficacies of CMV pp65-NLV-A*02:01-specific T cells (Figure 2B), both depicted for the isolations performed with 1% target-antigen-specific T cells in the starting material. These results show that a 50% reduction of the recommended amount of MHC I-*Strep*tamers had no significant influence on the isolation efficacy of a single (*ex vivo* expanded) target antigen-specific T-cell population, allowing the simultaneous isolation of 24 instead of 12 T-cell populations. Therefore, 50% of the recommended amount of MHC I-*Strep*tamers per targeted T-cell population was used for all subsequent experiments.

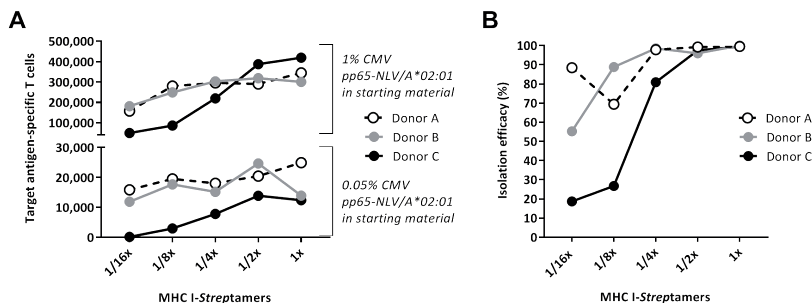

Figure 2. Target-antigen-specific T cells in positive fractions after isolations performed with different amounts of CMV pp65-NLV/A*02:01 specific MHC I-*Strep*tamers. **A**, Absolute numbers of target-antigen-specific T cells in the positive fraction after isolations performed with 1/16x- 1x the recommended amount of CMV pp65-NLV/A*02:01-specific MHC I-*Strep*tamers. CMV pp65-NLV/A*02:01-specific T cells were added in two frequencies (0.05% and 1.0%) to the starting material of HLA-A*02:01^{neg} healthy donors A, B and C. **B**, Isolation efficacy of target-antigen-specific T cells from starting material containing 1% of CMV pp65-NLV/A*02:01-specific T cells.

THE ISOLATION EFFICACY OF A SINGLE T-CELL POPULATION IS NOT HAMPERED BY THE PRESENCE OF MHC I-*STREP*TAMERS WITH OTHER TARGET ANTIGEN SPECIFICITIES IN THE PROCEDURE

For the conversion of the optimal isolation protocol for a single specificity to a protocol for the simultaneous isolation of multiple T-cell populations, we investigated whether the isolation of a single T-cell population is hampered by the presence of MHC I-*Strep*tamers with other target antigen specificities in the same isolation procedure. CMV pp65-NLV/A*02:01-specific T cells

were added in two frequencies (0.05% and 1%) to $40*10^6$ PBMC of three donors (donors A-C). Parallel isolation procedures were performed with CMV pp65-NLV/A*02:01-*Strep*tamers in three conditions: in the absence of other MHC I-*Strep*tamers or in the presence of MHC I-*Strep*tamers for 9x or 19x other T-cell populations (irrelevant isolation complexes; in this experiment EBV EBNA 3a-RPP/B*07:02 and ADV Hexon-TDL/A*01:01), to mimic the situation where 10 or 20 T-cell populations are targeted simultaneously. Here, half the recommended amount of MHC I-*Strep*tamers per targeted T-cell population was used. Figure 3 illustrates that the absolute numbers of CMV pp65-NLV/A*02:01-specific T cells enriched in the positive fractions after isolation were comparable among the three conditions for all three donors and for both starting frequencies of target-antigen-specific T cells. These data illustrate that the efficacy of isolation of a single target antigen-specific T-cell population was not disturbed by the addition of MHC I-*Strep*tamers for other target antigen specificities to the isolation procedure.

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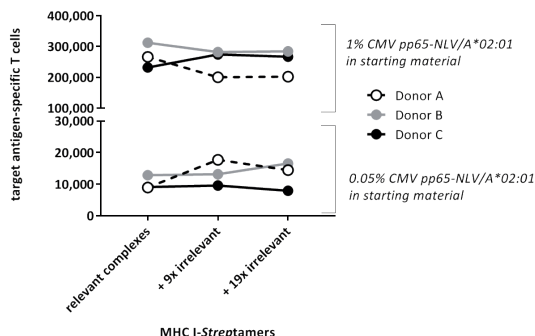

Figure 3. Target-antigen-specific T cells in the positive fractions after isolation procedures performed with only CMV pp65-NLV/A*02:01-specific MHC I-*Strep*tamers or in the presence of 9x or 19x irrelevant MHC I-*Strep*tamers in donor A, B and C.

MULTIPLE T-CELL POPULATIONS CAN BE ENRICHED TO HIGH PURITIES IN ONE SIMULTANEOUS ISOLATION PROCEDURE

Next, we examined whether multiple target antigen-specific T-cell populations from donor PBMC could be enriched in one simultaneous isolation procedure using the MHC I-S*trep*tamer technology to generate T-cell products for clinical purposes. To scale up and adapt the procedure to GMP-compliant conditions, the CliniMACS Plus isolation device with closed tubing set system was used. We also investigated whether besides virus-specific memory T cells, T-cell populations with lower precursor frequencies could be efficiently isolated within the same procedure. As a model, the isolation of TAA-specific T cells from healthy donors was analyzed. To confirm that 0.5x the recommended amount of MHC I-*Strep*tamers per targeted T-cell population was also sufficient for an efficient large scale isolation containing multiple (resting) target antigen-specific T-cell

populations with a wide variety of precursor frequencies in donor PBMC, we compared the usage of 0.5x and 1x the recommended amount of MHC I-*Strep*tamers per targeted T-cell populations in parallel isolations of donor D and E. The isolation of donor F was only performed with 0.5x MHC I-*Strep*tamers. Five intermediate/large scale test runs were performed starting with 435-1,770*10^6 PBMC (donors D-F). Based on the available MHC I-*Strep* proteins (Table 1) and donor HLA-typing, and irrespective of donor viral serostatus, MHC I-*Strep*tamers with 10 or 12 different MHC I-*Strep* proteins were used in the isolation procedures (Table 2). After the procedures, 1.32-5.60*10^6 cells were obtained in the positive fractions, containing 73-91% target-antigen-specific T cells (Table 3). This resulted in purities of target-antigen-specific T cells within the isolated T-cell compartment of 96-100%, indicating contamination with only a marginal number of T cells with an unknown specificity (<<0.1% of starting material). In donor D and E, the isolations performed with 0.5x versus 1x the recommended amount of MHC I-*Strep*tamers resulted in isolation of similar numbers of target-antigen-specific T cells (Table 3 and Figure 4).

Table 2. Frequencies of target antigen-specific T-cell populations in total starting material and total positive fractions after isolations performed with 0.5x or 1x MHC I-*Strep*tamers per target antigen-specific T-cell population in donor D, E and F. nd indicates not detectable; no clear cell population detectable by tetramer staining. - indicates not determined.

		Target-antigen-specific T cells		
	MHC I-Streptamers used in isolation procedure	*In starting material [%]*	*In positive fraction directly after isolation with 0.5x MHC I-Streptamers [%]*	*In positive fraction directly after isolation with 1x MHC I-Streptamers [%]*
Donor D	CMV pp65-NLV/A*02:01	nd	0.28	0.49
CMV^{pos}/EBV^{pos}	CMV pp65-TPR/B*07:02	0.31	60.4	46.5
	CMV IE-1-QIK/B*08:01	nd	0.30	0.43
	EBV BMLF-1-GLC/A*02:01	nd	4.65	4.64
	EBV EBNA-3A-RPP/B*07:02	nd	4.79	3.86
	EBV BLZF-1-RAK/B*08:01	0.1	20.4	17.5
	AdV E1A-LLD/A*02:01	nd	0.16	nd
	AdV Hexon-KPY/B*07:02	nd	nd	nd
	TAA NY-eso-1-SLL/A*02:01	nd	nd	nd
	TAA WT1-RMF/A*02:01	nd	nd	nd
	TAA RHAMM-ILS/A*02:01	nd	nd	nd
	TAA proteinase-3-VLQ/A*02:01	nd	nd	nd
Donor E	CMV pp65-NLV/A*02:01	nd	nd	nd
CMV^{neg}/EBV^{pos}	CMV pp65-TPR/B*07:02	nd	nd	nd
	EBV BMLF-1-GLC/A*02:01	2.15	78.2	74.2
	EBV EBNA-3A-RPP/B*07:02	0.15	2.81	3.17
	AdV E1A-LLD/A*02:01	nd	nd	nd
	AdV Hexon-KPY/B*07:02	nd	nd	nd
	TAA NY-eso-1-SLL/A*02:01	nd	nd	nd
	TAA WT1-RMF/A*02:01	nd	nd	nd
	TAA RHAMM-ILS/A*02:01	nd	nd	nd
	TAA proteinase-3-VLQ/A*02:01	nd	nd	nd

Table 2 continued.

		Target-antigen-specific T cells		
	MHC I-Streptamers used in isolation procedure	*In starting material [%]*	*In positive fraction directly after isolation with 0.5x MHC I-Streptamers [%]*	*In positive fraction directly after isolation with 1x MHC I-Streptamers [%]*
Donor F	CMV pp65-NLV/A*02:01	nd	0.63	-
CMV^{pos}/EBV^{pos}	CMV pp65-TPR/B*07:02	0.64	75.9	-
	CMV pp65-QYD/A*24:02	nd	0.26	-
	EBV BMLF-1-GLC/A*02:01	nd	4.52	-
	EBV EBNA-3A-RPP/B*07:02	nd	3.44	-
	EBV LMP2-PYL/A*24:02	nd	nd	-
	AdV E1A-LLD/A*02:01	nd	nd	-
	AdV Hexon-KPY/B*07:02	nd	nd	-
	AdV Hexon-TYF/A*24:02	nd	0.83	-
	TAA WT1-RMF/A*02:01	nd	nd	-
	TAA RHAMM-ILS/A*02:01	nd	nd	-
	TAA proteinase-3-VLQ/A*02:01	nd	nd	-

To investigate the presence of the individual target antigen-specific T-cell populations, the starting material and positive fractions were analyzed by FACS using specific HLA/peptide tetramers (Table 2). As expected, the virus-specific T-cell populations that were already present at detectable frequencies in the starting material were the main component of the positive fractions. However, also virus-specific T-cell populations that were present at initially undetectable precursor frequencies in the donor PBMC could be enriched to detectable frequencies in the positive fractions, resulting in visible frequencies of almost all CMV- and EBV- specific T-cell populations in seropositive donors D and F. In CMV-seronegative donor E, only the 2 EBV-specific T-cell populations reached visible frequencies after isolation. This results shows that using this approach it is feasible to isolate multiple antigen-specific T-cell populations in one simultaneous isolation procedure with 0.5x the recommended amount of MHC I-*Strep*tamers.

Table 3. Characteristics of starting materials and positive fractions after intermediate scale (donor D and E) and large scale (donor F) MHC I-*Strep*tamer isolations.

	Starting material per isolation [10^6 PBMC]*	*Target-antigen-specific T cells in starting material (within $CD3^{pos}$ cells) [%]*	*Amount of MHC I-Streptamers per targeted T-cell population*	*Total cells in positive fraction [*10^6]*	*Target-antigen-specific T cells in positive fraction (within $CD3^{pos}$ cells) [%]*
Donor D	500	0.41 (0.53)	0.5 x	2.60	91.0 (95.9)
			1x	3.8	73.4 (100)
Donor E	435	2.30 (4.93)	0.5 x	1.94	81.0 (99.2)
			1x	1.32	77.4 (98.5)
Donor F	1770	0.64 (1.02)	0.5 x	5.60	85.6 (97.8)

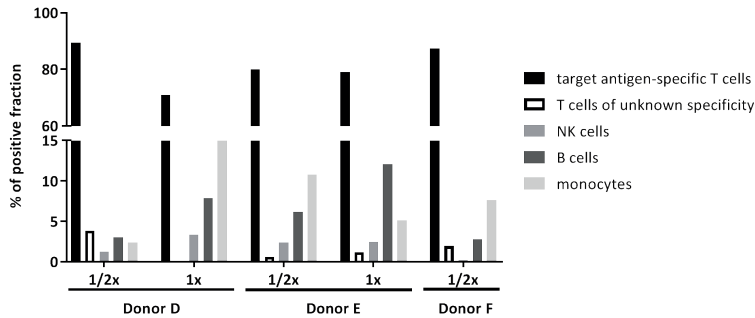

Figure 4. Composition of positive fractions after isolation procedures performed with multiple different MHC I-*Strep*tamers specificities. For donor D and E, isolations performed with 0.5x versus 1x the recommended amount of MHC I-*Strep*tamers per target antigen-specific T-cell population were compared side by side.

TAA-SPECIFIC T CELLS ARE PRESENT AT LOW FREQUENCIES IN THE PRODUCTS AFTER MHC I-*STREP*TAMER ISOLATIONS

T-cell populations that are present at very low frequencies in the starting material might be missed in direct detection with tetramer staining due to the too low frequency in the positive fraction after isolation. To validate whether the MHC I-*Strep*tamer technology allows the reproducible co-isolation of low frequent T-cell populations, procedures were performed targeting exclusively TAA-specific T cells. Intermediate scale isolations were accomplished starting with $500*10^6$ PBMC of six HLA-A*02:01^{pos} donors (donors G–L) using MHC 1-*Strep*tamers for five different TAA. After MACS isolation, the positive fractions containing very low cell numbers were first expanded for 10-14 days. Thereafter, the presence of the five TAA-specific T-cell populations was assessed by tetramer staining. For T-cell populations that were not directly visible in this analysis, their presence was investigated by sequential subsequent MHC-I *Strep*tamer enrichment rounds, each followed by an expansion step. As expected based on the very low (undetectable) frequencies in the starting material, none of the TAA specificities could be detected in the positive fractions after the first isolation round for all donors (Table 4). However, after the second enrichment round, five TAA specificities became visible in frequencies >1% (Figure 5; representative example for donor J). In all donors, one to five TAA specificities were detected after the second enrichment round, further increasing after the third and fourth enrichment rounds (Table 4). These results indicate that even antigen-specific T-cell populations present at very low precursor frequencies in donor PBMC (like TAA-specific T cells) can be enriched using the MHC I-*Strep*tamer technology and that these cells were already present in the positive fraction after the first isolation round. However, to visualize these cells, subsequent sequential enrichment and expansion rounds are required.

To determine whether the isolation of low frequent T-cell populations is hampered by the simultaneous isolation of high frequent T-cell populations within the same isolation procedure, the

Table 4. Target antigen-specific T-cell populations visualized in the positive fractions after intermediate scale (donor G-L) and large scale (donor M-P) MHC I-*Strep*tamer isolations llowed by subsequent enrichment and expansion rounds. PR3, proteinase-3

		After 1st round	After 2nd round	After 3rd round	After 4th round
Isolation of 5 TAA- specific T-cell populations	**Donor G**		TAA NY-eso-1-SLL/A*02:01 TAA WT1-RMF/A*02:01 TAA RHAMM-ILS/A*02:01 TAA PRAME-VLD/A*02:01		
	Donor H		TAA WT1-RMF/A*02:01	TAA RHAMM-ILS/A*02:01 TAA PRAME-VLD/A*02:01	
	Donor I		TAA WT1-RMF/A*02:01	TAA RHAMM-ILS/A*02:01 TAA PRAME-VLD/A*02:01	TAA PR3-VLQ/A*02:01
	Donor J		TAA NY-eso-1-SLL/A*02:01 TAA WT1-RMF/A*02:01 TAA RHAMM-ILS/A*02:01 TAA PR3-VLQ/A*02:01 TAA PRAME-VLD/A*02:01		
	Donor K		TAA RHAMM-ILS/A*02:01	TAA WT1-RMF/A*02:01	TAA PRAME-VLD/A*02:01
	Donor L		TAA WT1-RMF/A*02:01 TAA RHAMM-ILS/A*02:01 TAA PRAME-VLD/A*02:01		
Isolation of 5 TAA- and 3 virus-specific T-cell populations	**Donor M** CMV^{pos}	CMV pp65-NLV/A*02:01 AdV E1A-LLD/A*02:01 EBV BMLF-1-GLC/A*02:01	TAA WT1-RMF/A*02:01 TAA RHAMM-ILS/A*02:01 TAA PR3-VLQ/A*02:01 TAA PRAME-VLD/A*02:01	TAA NY-eso-1-SLL/A*02:01	
	Donor N CMV^{neg}	AdV E1A-LLD/A*02:01 EBV BMLF-1-GLC/A*02:01	TAA WT1-RMF/A*02:01		CMV pp65-NLV/A*02:01 TAA NY-eso-1-SLL/A*02:01 TAA RHAMM-ILS/A*02:01 TAA PR3-VLQ/A*02:01 TAA PRAME-VLD/A*02:01
	Donor O CMV^{pos}	CMV pp65-NLV/A*02:01	AdV E1A-LLD/A*02:01 EBV BMLF-1-GLC/A*02:01	TAA WT1-RMF/A*02:01 TAA RHAMM-ILS/A*02:01 TAA PR3-VLQ/A*02:01 TAA PRAME-VLD/A*02:01	TAA NY-eso-1-SLL/A*02:01
	Donor P CMV^{pos}	CMV pp65-NLV/A*02:01 AdV E1A-LLD/A*02:01 EBV BMLF-1-GLC/A*02:01	TAA WT1-RMF/A*02:01 TAA RHAMM-ILS/A*02:01	TAA NY-eso-1-SLL/A*02:01 TAA PR3-VLQ/A*02:01 TAA PRAME-VLD/A*02:01	

simultaneous isolation of five TAA- and three virus-specific T-cell populations was analyzed in large scale isolation procedures starting with 2*10^9 PBMC of four HLA-A*02:01^{pos} donors (donors M-P). As expected, the pools of target-antigen-specific T cells in the positive fractions after expansion consisted mainly of virus-specific memory T cells, containing a median of 62.2% (range 36.9-73.3%) CMV-specific T cells, 11.4% (range 5.6-27.6%) EBV-specific T cells and 22.4% (range 3.7-50.7%) AdV-specific T cells. The presence of all TAA-specific T-cell populations in the product for clinical application was demonstrated after 2 to 4 subsequent sequential enrichment rounds for all 4 donors (Table 4). These results indicate that the isolation of low frequent T-cell populations is not hampered by the simultaneous isolation of T-cell populations with high frequencies in donor PBMC.

In conclusion, the MHC I-*Strep*tamer isolation technology is feasible for the isolation of multiple T-cell populations from donor PBMC within a GMP-compliant procedure. The isolation of low frequent T-cell populations in donor PBMC, like TAA-specific T cells, can be combined with the isolation of high frequent T-cell populations for the fast generation of highly individualized T-cell products.

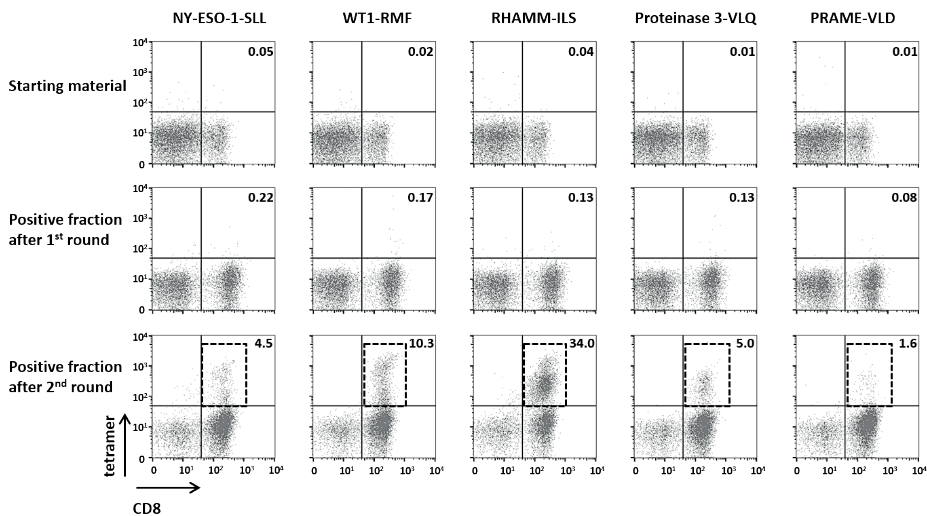

Figure 5. Detection of 5 different HLA-A*02:01 restricted TAA-specific T-cell populations after MHC I-*Strep*tamer isolations. Representative example of FACS plots of the starting material, positive fraction after first and positive fraction after second isolation and expansion round for HLA-A*02:01^{pos} donor J. Living cells are depicted.

DISCUSSION

In this study we have shown that the MHC I-*Strep*tamer isolation technology can be successfully used for the enrichment of multiple T-cell populations from donor PBMC in one simultaneous isolation procedure. By adjusting the amount of MHC I-*Strep*tamers per target antigen-specific T-cell population, up to 24 T-cell populations could be targeted within one simultaneous isolation, resulting in a T-cell product with a high purity of target-antigen-specific T cells and low numbers of contaminating T cells of unknown specificity. Moreover, not only T-cell populations with relatively high precursor frequencies in donor PBMC, but also low frequent T cell could be reproducibly isolated with this approach. Therefore, the MHC I-*Strep*tamer technology is a feasible method for the generation of highly individualized T-cell products for adoptive immunotherapy in only one day.

3

We have shown that TAA-specific T cells can be reproducibly isolated from donor PBMC with MHC I-*Strep*tamers. However, due to the extremely low frequencies of these T cells in the starting material, these cells could not be visualized by direct tetramer staining. After additional expansion and enrichment rounds, these TAA-specific T-cell populations were reproducibly visualized. These results suggest that this technology can also be used for the isolation of other T-cell populations present at low frequencies in donor PBMC, like MiHA-specific T cells from HLA-matched MiHA negative donors and virus-specific T cells from seronegative donors. To boost GVL responses, MHC I-*Strep*tamers for the isolation of donor-derived T cells directed against MiHA selectively expressed on hematopoietic or leukemic cells of the patient can be included in the isolation procedure.[17,18,21,30,31] However, the expected therapeutic relevance of direct clinical application without *in vitro* expansion of very low frequent T-cell populations is questionable.

A limitation of the MHC I-*Strep*tamer technology is that it requires knowledge of immune-dominant antigens (defined peptides) and their HLA-restrictions. Only CD8^{pos} T cells can be targeted since MHC I-*Strep* proteins are only available for antigens presented in HLA class I molecules. Although clinical studies performed with a variety of isolation methods targeting a single CMV or EBV specific CD8^{pos} T-cell population reported promising results regarding T-cell expansion and clinical outcomes as applied in therapeutic settings,[14,16,24,32] CD4^{pos} helper T cells probably contribute to *in vivo* survival, persistence and function of CD8^{pos} T cells.[32-37]

MHC I-*Strep*tamer isolated non-expanded T-cell products will generally not exceed a few million cells due to limitations regarding the size of donor leukapheresis products and the frequencies of target-antigen-specific T cells in this starting material. The expected therapeutic effect of this small number of target-antigen-specific T cells *in vivo* and therefore the clinical utility is dependent on several factors including antigen burden in the patient together with expansion capacity

and persistence ability of transferred T cells *in vivo.*[35] Previous studies describing the adoptive transfer of *in vitro* selected virus-specific T cells isolated from seropositive donors have shown that the adoptive transfer of a minimum of 250 to 5,000 virus specific T cells/kg body weight of the patient can be sufficient for virus control in the therapeutic setting.[15,24,38,39] This suggests that these antigen-experienced T cells have a high proliferative potential and are able to expand rapidly enough to control a high antigen burden at the moment of active virus infections. Using the MHC I-*Strep*tamer technology, these T cell numbers can be easily reached by isolating virus-specific T-cell populations from seropositive donor-derived leukapheresis products. However, for T-cell populations with rare precursor frequencies in donor PBMC, like TAA-specific T cells, comparable numbers will not be realized directly after MHC I-*Strep*tamer isolations. Low frequent T-cell populations derived from the donor's naïve T-cell compartment may require priming by an professional antigen presenting cell in the presence of sufficient co-stimulatory signals. Therefore, the administration of low numbers of these cells may be unsuccessful in the therapeutic setting because time is essential for adequate stimulation and expansion until appropriate cell numbers are reached. An option might be to expand T-cell products after the initial isolation and before clinical application with the aim to transfer a better defined cellular product containing higher numbers of these T cells.

MHC I-*Strep*tamer isolated T-cell products may be applied in prophylactic or pre-emptive settings, when T cells have time to expand and the antigen burden is low. Especially patients treated with a T-cell depleted alloSCT will experience a prolonged period of immune deficiency associated with a high risk for viral complications and disease relapses in the absence of residual patient-derived or transferred donor-derived T cells. Therefore, this patient group might especially benefit from stem cell donor-derived adoptive T-cell prophylaxis to bridge the immune deficient period between T-cell-depleted alloSCT and DLI six months after transplantation. In this setting, T-cell products can be administered as soon as T-cell depleting antibodies (alemtuzumab and/or anti-thymocyte globuline) are eliminated *in vivo,* generally six to eight weeks after alloSCT.[40] Recently initiated phase 1/2 and 3 clinical trials have to reveal whether T cells will survive *in vivo* and diminish the risk of several viral infections or disease relapses (T-Control, EudraCT-No. 2014-003171-39; NCT01077908; NCT01220895). Our study shows that the MHC I-*Strep*tamer technology can be successfully used for the generation of multi-antigen-specific T-cell products for several adoptive immunotherapy applications.

ACKNOWLEDGEMENTS

This work was supported by the European Union's seventh Framework Program (FP/2007-2013) under grant agreement no 601722.

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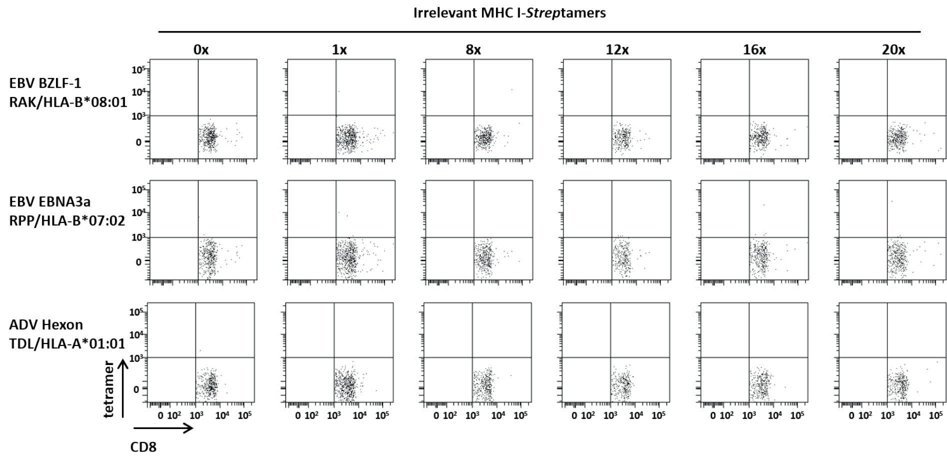
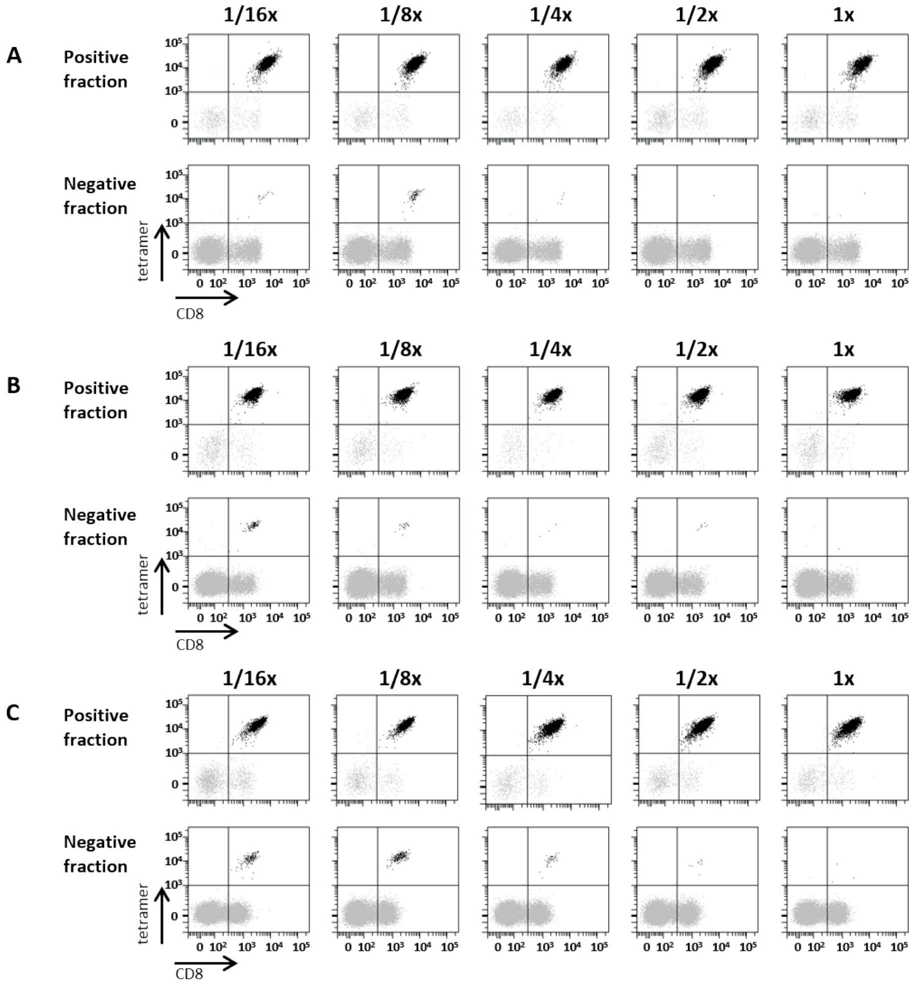
SUPPLEMENTARY MATERIAL

Supplementary Table 1. Donor characteristics. HLA-A and –B typing, CMV and EBV serostatus and number of PBMC used per MHC I-*Strep*tamer isolation.- indicates not determined; neg, negative; pos, positive.

	HLA-A typing	***HLA-B typing***	***CMV serostatus***	***EBV serostatus***	***Number of starting cells per MHC I-Streptamer isolation (*10^6 PBMC)***
Donor A	26 / 68	35 / 38	pos	pos	40
Donor B	11 / 68	35 / 55	pos	pos	40
Donor C	03 / 68	35 / 44	neg	pos	40
Donor D	02 / 29	07 / 08	pos	pos	500
Donor E	02 / 02	07 / 35	neg	pos	435
Donor F	02 / 24	07 / 35	pos	pos	1770
Donor G	02 / 31	13 / 35	pos	pos	500
Donor H	02 / 02	15 / 51	neg	pos	500
Donor I	02 / 68	15 / 35	pos	pos	500
Donor J	02 / 23	15 /44	neg	pos	500
Donor K	02 / 68	07 / 44	pos	pos	500
Donor L	02 / 30	13 / 18	neg	pos	500
Donor M	01 / 02	08 / 40	pos	-	2000
Donor N	01 / 02	51 / 07	neg	-	2000
Donor O	02 / 25	15 / 18	pos	-	2000
Donor P	02 / 26	27 / 44	pos	-	2000



Supplementary Figure 1. FACS analysis of positive fractions after isolations performed with different amounts (0x to 20x) of irrelevant MHC I-*Strep*tamers for donor A. Positive fractions were stained with CD8 and tetramers specific for the viral specificities presented in the MHC I-*Strep*tamers to investigate the isolation of allo-HLA restricted T cells. CD8^{pos}, living cells are depicted.



Supplementary Figure 2. Target-antigen-specific T cells in positive and negative fractions after isolations performed with different amounts of MHC I-*Strep*tamers (1/16x – 1x) in **A**, donor A; **B**, donor B and; **C**, donor C. CD33^{neg}, living cells are depicted; 7,000 events for the positive fractions and 18,000 events for the negative fractions.

3

CHAPTER 4

GENERATION AND INFUSION OF MULTI-ANTIGEN-SPECIFIC T CELLS TO PREVENT COMPLICATIONS EARLY AFTER T-CELL DEPLETED ALLOGENEIC STEM CELL TRANSPLANTATION - A PHASE I/II STUDY

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Leukemia 2020; 34(3): 831-844.

ABSTRACT

Prophylactic infusion of selected donor T cells can be an effective method to restore specific immunity after T-cell-depleted (TCD) allogeneic stem cell transplantation (alloSCT). In this phase I/II study, we aimed to reduce the risk of viral complications and disease relapses by administrating donor-derived CD8^{pos} T cells directed against cytomegalovirus (CMV), Epstein-Barr virus (EBV) and adenovirus antigens, tumor-associated antigens (TAA) and minor histocompatibility antigens (MiHA).

Twenty-seven of 36 screened HLA-A*02:01^{pos} patients and their CMV^{pos} and/or EBV^{pos} donors were included. Using MHC I-*Strep*tamers, 27 T-cell products were generated containing a median of 5.2*10^{6} cells. Twenty-four products were administered without infusion-related complications at a median of 58 days post-alloSCT. No patients developed graft-versus-host-disease during follow-up. Five patients showed disease progression without coinciding expansion of TAA/MiHA-specific T cells. Eight patients experienced CMV and/or EBV reactivations. Four of these reactivations were clinically relevant requiring antiviral treatment, of which two progressed to viral disease. All resolved ultimately. In 2/4 patients with EBV reactivations and 6/8 patients with CMV reactivations, viral loads were followed by expansion of donor-derived virus target-antigen-specific T cells.

In conclusion, generation of multi-antigen-specific T-cell products was feasible, infusions were well tolerated and expansion of target-antigen-specific T cells coinciding viral reactivations was illustrated in the majority of patients.

4

INTRODUCTION

The curative potential of allogeneic stem cell transplantation (alloSCT) to treat hematologic malignancies is based on the induction of a graft-versus-leukemia (GVL) effect mediated by donor T cells.[1-3] However, administration of donor T cells with the graft often results in the induction of graft-versus-host disease (GVHD).[4,5] Therefore, T-cell depleted (TCD) alloSCT and postponed donor lymphocyte infusion (DLI) at 3-6 months after transplantation is applied as a strategy to promote GVL at a moment when the risk of GVHD is significantly reduced.[6-9]

In the interval between the TCD alloSCT and the application of DLI, patients experience a period of immunodeficiency associated with a relatively high incidence of viral reactivations. Especially cytomegalovirus (CMV), Epstein-Barr virus (EBV) and adenovirus (AdV) can cause serious complications with high morbidity and mortality in the absence of virus-specific T-cell immunity.[10-13] Reconstitution of the virus-specific T-cell repertoire in the first months after alloSCT has been demonstrated to be essential to prevent viral disease.[14] Additionally, multiple studies have shown that virus-specific T cells from virus-seropositive donors can be used to treat viral reactivations.[15-24] From a patient-care perspective, prevention of viral reactivations may be favorable over the treatment of persistent viral disease with medication associated with toxicities.[25] TCD alloSCT is furthermore associated with a higher risk of disease relapse. As tumor-associated antigens (TAA) and minor histocompatibility antigens (MiHA) are proposed as targets for GVL, donor-derived T cells directed against these antigens may contribute to the therapeutic anti-leukemia effect.[26-29] Therefore, patients treated with TCD alloSCT may benefit from prophylactic donor-derived adoptive T-cell transfer to bridge this immunodeficient period.[30-34] T-cell products can be administered as soon as T-cell depleting antibodies like alemtuzumab and/or anti-thymocyte globulin are cleared *in vivo*, generally 6-8 weeks after alloSCT.[35]

We and others have previously shown that the major histocompatibility complex class I (MHC I)-*Strep*tamer technology can be used for the generation of T-cell products containing multiple antigen-specific T-cell populations simultaneously isolated from donor peripheral blood mononuclear cells (PBMC) in one day.[24,36,37] With this approach, virus-specific memory T cells can be isolated together with T-cell populations with rare precursor frequencies in donor PBMC, like TAA-, MiHA- and naïve virus-specific T cells.[20,38,39]

In this phase I/II clinical study, we aimed to administer donor-derived multi-antigen-specific T-cell products containing CD8^{pos} T cells directed against CMV, EBV and AdV antigens, TAA and MiHA to patients early after TCD-alloSCT. We investigated the feasibility of patient/donor inclusion and donor-derived T-cell product generation using the MHC I-*Strep*tamer technology, assessed the safety of the early prophylactic infusion, and monitored the clinical outcome and target-antigen-

4

specific immune reconstitution.

MATERIALS AND METHODS

STUDY DESIGN AND PATIENT/DONOR INCLUSION

This single center, phase I/II safety and feasibility study (T-Control, EudraCT-number 2014-003171-39) was approved by the Central Committee on Research Involving Human Subjects (CCMO; TOL number NL 48393.000.14). In- and exclusion criteria are listed in Supplementary Table 1. Briefly, HLA-A*02:01^{pos} patients with one of the indicated hematologic malignancies, that received a TCD-alloSCT (bone marrow- or peripheral blood-derived) from a related or unrelated, EBV- and/or CMV-seropositive donor were eligible. Both myeloablative (MA) and non-myeloablative (NMA) conditioning regimens were allowed, containing *in vivo* alemtuzumab and/or anti-thymocyte globulin-based T-cell depletion in combination with *in vitro* alemtuzumab addition to the graft before graft infusion (Supplementary Table 2).[40] Informed consent was obtained from patients and donors in accordance with the Declaration of Helsinki. For included patients that underwent alloSCT without complications, a donor leukapheresis product was requested 4-6 weeks after alloSCT. PBMC were used for multi-antigen-specific T-cell product generation and the remaining cells were frozen for DLI, regularly applied 6 months after TCD-alloSCT. Patients received their T-cell product on the day of product generation and received institutional routine clinical care during follow-up.

MULTI-ANTIGEN-SPECIFIC T-CELL PRODUCT GENERATION

Multi-antigen-specific T-cell products were generated from alloSCT donor-derived leukapheresis products using MHC I-*Strep*tamer isolation technology (Juno Therapeutics, Munich, Germany).[37] Isolation complexes (MHC I-*Strep*tamers) were generated the day before the actual isolation by the incubation of peptide loaded MHC-I-*Strep*-tag fusion proteins (MHC-I-*Strep* proteins) with magnetically labelled *Strep*-Tactin (*Strep*-Tactin nanobeads) in phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.4% human serum albumin (HSA, Sanquin Reagents, Amsterdam, The Netherlands) overnight at 4°C. The generation of MHC-I-*Strep*tamers was performed per target-antigen in a separate tube, to prevent the production of isolation complexes with mixed MHC-I-*Strep* proteins. Twenty different MHC-I-*Strep* proteins were available for this study (Table 1). MHC-I-*Strep*tamers containing at least the 4 HLA*02:01-restricted viral (2 CMV, 1 EBV and 1 AdV) and 5 HLA*02:01-restricted TAA MHC-I-*Strep* proteins were prepared for every patient. In addition, depending on the HLA-typing of the patient/donor and regardless of donor CMV, EBV and AdV serostatus, MHC-I-*Strep*tamers containing MHC-I-*Strep* proteins in HLA-A*01:01, -A*24:02, -B*07:02 and/or B*08:01 were added. Furthermore, MHC-I-*Strep*tamers containing the MiHA HLA-A*02:01/HA-1H-protein were included in 3 procedures (products A, L

and Y) where a MiHA disparity in the GVL direction was present (donor HA-1H^{neg}, patient HA-1H^{pos}). As TAA and MiHA MHC-I-*Strep* proteins were all HLA-A*02:01-restricted, HLA-A*02:01 positivity of patient/donor couples was an inclusion criteria. The recommended amount of MHC-I-*Strep*tamers per target-antigen was based on the number of starting cells as described in the manufacturer's instruction (Juno Therapeutics). However, based on our experience, we used per target-antigen, MHC-I-*Strep*tamers for half of the number of starting cells.[37] On the day of isolation, $2*10^9$ PBMC were collected from fresh non-mobilized stem cell donor leukapheresis products after red blood cell lysis using an NH_4Cl (8.4 g/L) and $KHCO_3$ (1 g/L) buffer (pH = 7.4; LUMC Pharmacy) (only PBMC for product L were cryopreserved in the vapor phase of liquid nitrogen and thawed for product generation) and were incubated with the indicated pool of washed MHC-I-*Strep*tamers in PBS/HSA for 45 minutes to allow the labelling of target-antigen-specific T cells. Afterwards, the cell suspension was washed to eliminate unbound MHC-I-*Strep*tamers. The isolations were performed under Good Manufacturing Practice (GMP) conditions on a CliniMACS Plus instrument using CliniMACS tubing set TS (161-01) (both Miltenyi Biotec, Bergisch Gladbach, Germany) with the selection program 'CD34 selection 1'. MHC-I-*Strep*tamers were dissociated from the positively isolated cells using D-Biotin in accordance with the manufacturer's instructions (Juno Therapeutics).

Table 1. HLA/peptide-complexes for which MHC-I-Strep proteins for isolation and tetramers for flow cytometry were used.

HLA-restriction	Protein	Peptide
A*02:01	CMV pp65	**NLV**PMVATV
	CMV IE-1	**VLE**ETSVML
	EBV BMLF-1	**GLC**TLVAML
	AdV E1A	**LLD**QLIEEV
	TAA NY-eso-1	**SLL**MWITQV
	TAA WT1	**RMF**PNAPYL
	TAA RHAMM	**ILS**LELMKL
	TAA proteinase-3	**VLQ**ELNVTV
	TAA PRAME	**VLD**GLDVLL
	MiHA HA-1H	**VLH**DDLLEA
A*01:01	CMV pp50	**VTE**HDTLLY
	AdV Hexon	**TDL**GQNLLY
A*24:02	CMV pp65	**QYD**PVAALF
	EBV LMP2	**PYL**FWLAAI
	AdV Hexon	**TYF**SLNNKF
B*07:02	CMV pp65	**TPR**VTGGGAM
	EBV EBNA-3A	**RPP**IFIRRL
	AdV Hexon	**KPY**SGTAYNAL
B*08:01	CMV IE-1	**QIK**VRVDMV
	EBV BZLF-1	**RAK**FKQLL

FLOW CYTOMETRIC ANALYSIS OF STARTING MATERIAL AND T-CELL PRODUCTS AFTER ISOLATION

The composition of the starting material for T-cell product generation and the T-cell product directly after isolation was analyzed by flow cytometry. Cells were stained with fluorescein isothiocyanate (FITC)-labelled CD4 (Beckton Dickinson (BD) Biosciences, San Diego/San Jose, USA; catalog number 555346) and CD14 antibodies (BD, catalog number 555397), phycoerythrin (PE)-labeled CD56 (BD, catalog number 555516) antibodies, peridinin-chlorophyll-protein complex (PerCP)-labelled CD3 (BD, catalog number 345766) and CD8 (BD, catalog number 345774) antibodies, allophycocyanin (APC)-labelled CD19 (BD, catalog number 555415) antibodies in combination with PE- or APC-labelled tetramers. Tetramers were produced as described previously for all target-antigens indicated in Table 1.[41] To minimize the use of cells for product release (only $0.1*10^6$ cells of the product were used), PE- or APC-labelled tetramers for all target-antigen specificities included in the isolation were combined in one FACS sample to assess the total percentage of target-antigen-specific T cells. Cells were measured on a FACS Calibur or Canto (BD) and analyzed using FlowJo Software (TreeStar, Ashland, USA) or Diva Software (BD) respectively.

T-CELL PRODUCT RELEASE SPECIFICATIONS AND PRODUCT ADMINISTRATION

T-cell product release specifications for infusion are mentioned in Table 3. The maximum allowed number of T cells of unknown specificity in the products was based on our experience regarding the application of unmodified DLI early after TCD-alloSCT and previous clinical studies.[23] In addition, products were retrospectively tested for sterility and mycoplasma contamination, and DNA profiling analysis was performed to confirm the identity of the donor material. 95% of cells of successfully generated product were suspended in 100 ml NaCl 0.9% (Fresenius Kabi, Bad Homburg, Germany) supplemented with 4% HSA and intravenously administered at the day of product generation to patients that still met the inclusion criteria.

T-CELL PRODUCT IN-DEPTH ANALYSIS

5% of cells of the products (all cells from the products K and R which were not infused) were *in vitro* expanded for in-depth analysis. Non-specific expansion was performed in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Basel, Switzerland) supplemented with 10% pooled human serum, 100 IU/ml interleukin-2 (Chiron, Amsterdam, The Netherlands) and 800 ng/ml phytohemagglutinin (Oxoid Limited, Hampshire, United Kingdom) with 5x irradiated (35 Gy) autologous PBMC as feeder cells. After expansion of the cells, separate flow cytometry stainings per target-antigen-specificity were performed with PE- or APC-labelled tetramers in combination with FITC-labelled CD4 (BD, catalog number 555346) and PerCP-labelled CD8 antibodies (BD, catalog number 345774) to assess the percentages of individual T-cell populations. Cells were measured on a FACS Calibur or Canto and analyzed using FlowJo Software or Diva Software, respectively.

MONITORING OF CLINICAL EVENTS

Patients were monitored from the moment of T-cell product infusion until 6 months after alloSCT with regard to viral reactivations, GVHD and disease activity. CMV, EBV and AdV DNA loads were assessed weekly until 6 weeks after infusion and every 2 weeks thereafter by quantitative polymerase chain reaction (qPCR) in plasma. GVHD was graded according to modified Glucksberg and Shulman criteria.[42,43] Bone marrow chimerism and disease status were assessed just before, and around 8 and 18 weeks after product infusion.

DEFINITIONS AND TREATMENTS OF VIRAL REACTIVATIONS AND DISEASES

During patient follow-up, relevant viral reactivations for CMV, EBV and AdV were defined as positive DNA loads in two subsequent measurements. Clinically relevant CMV reactivations were defined as CMV DNA loads >log 4, or >log 3 with at least 1 log increase in one week or >log 3 in two consecutive measurements, and were treated with oral valganciclovir 900mg twice daily for 2 weeks or intravenously ganciclovir 5mg/kg twice daily for 2 weeks. Clinically relevant EBV reactivations were defined as EBV DNA load >log 3 and were treated with rituximab gifts of 375mg/m^2. Viral disease was defined as viral reactivation with proven organ involvement.[44]

TARGET-ANTIGEN-SPECIFIC IMMUNE RECONSTITUTION

Peripheral blood samples for immune reconstitution measurements were taken every 2 weeks until 8 weeks after infusion and every 4 weeks thereafter until 6 months after alloSCT. Absolute numbers of circulating T, B and NK cells were determined on fresh blood by flow cytometry. Frequencies of target-antigen-specific T cells were determined on thawed PBMC (all follow-up samples per patient simultaneously) by direct tetramer-staining using flow cytometry. Absolute numbers of target-antigen-specific T cells were calculated by multiplying the percentages of target-antigen-specific T cells (tetramer^{pos} cells within the CD33^{neg} cells), with the absolute numbers of lymphocytes. Chimerism analysis was performed on flow cytometry sorted target-antigen-specific T-cell populations.

FLOW CYTOMETRIC ANALYSIS OF TARGET-ANTIGEN-SPECIFIC IMMUNE RECONSTITUTION

In vivo immune reconstitution was measured after T-cell product infusion using flow cytometry. Absolute numbers of circulating CD4^{pos} T cells (CD45^{pos}CD3^{pos}CD4^{pos}), CD8^{pos} T cells (CD45^{pos}CD3^{pos}CD8^{pos}), B cells (CD45^{pos}CD3^{neg}CD19^{pos}) and NK cells (CD45^{pos}CD3^{neg}CD16/CD56^{pos} cells) were determined as part of routine clinical evaluation on fresh venous blood using BD TruCount Tubes (BD), following the manufacturer's instructions. Samples were stained with APC-labeled CD3 (BD, catalog number 555342), FITC-labeled CD4 (BD, catalog number 555346), PE-labeled CD8 (BD, catalog number 555367), PerCP-labeled CD45 (BD, catalog number 347464) or with FITC-labeled CD3 (BD, catalog number 555339), PE-labeled CD16 (BD, catalog number 561313), APC-labeled CD19 (BD, catalog number 555415), PerCP-labeled CD45 (BD, catalog number 347464) and PE-labeled CD56 (BD, catalog number 555516). Percentages of target-antigen-specific T cells in

thawed follow-up samples were analyzed using allophycocyanin-H7 (APC-H7)-labeled CD4 (BD, catalog number 560158) or CD8 (BD, catalog number 560179) antibodies and phycoerythrin-cyanine 7 (Pe-Cy7)-labeled CD33 (BD, catalog number 333946) antibodies, in combination with FITC-, PE-, PerCP-, APC-, V450- and/or V500-labeled tetramers. Cells were measured on a FACS Canto and analyzed using Diva Software.

CHIMERISM ANALYSIS

Chimerism was evaluated using a short tandem repeat PCR-based protocol, as previously described.[29] PCR analysis was performed with primers specific for patient and donor selected polymorphic short tandem repeats using the AmpFLSTR Profiler Plus ID amplification kit (Applied Biosystems, Waltham, MA, USA) and a GeneAmp 9700 thermocycler (Applied Biosystems) using AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR products were analyzed using the ABI PRISM 3500 Genetic Analyzer and Genemapper V5 analysis software (Applied Biosystems). Sensitivity was set at 2% for all markers.

STATISTICAL ANALYSIS

Donor leukapheresis collection was determined feasible when for the first included 15 patient/donor-couples, in ≥6 cases donor leukapheresis was successfully obtained around 8 weeks after transplantation. T-cell product generation was determined feasible when for the first 15 isolation procedures, ≥6 procedures resulted in T-cell products that met the release specifications (Table 3). Infusion of T-cell products was determined potentially effective when ≥1 target-antigen-specific T-cell populations appeared (>0.1% within total CD8^{pos} cells) or doubled during follow-up compared to the percentage before the infusion, in patients experiencing CMV, EBV and/or AdV reactivation or disease relapse. A sample size of 17 patients who received their T-cell product and were available for monitoring during the follow-up period until 6 months after alloSCT was needed to test the hypothesis of success probability >=0.5 against the null hypothesis of success probability <0.2 (futility), with one-sided alpha=0.05, power=0.8. As we estimated that 40% of the patients who met the baseline inclusion criteria (Supplementary Table 1) at the moment of TCD-alloSCT would not be eligible for T-cell product infusion, we expected to require 30 included patients.

RESULTS

PATIENT AND DONOR INCLUSION

During the inclusion period between October 2014 and January 2016, 36 patients met the baseline criteria for study participation (Figure 1 and Supplementary Table 1). Between alloSCT and the initiation of T-cell product generation, 9 patients were excluded due to patient (n = 8) or donor (n = 1) medical problems, resulting in 27 included patient/donor-couples for this study; patient and

transplantation characteristics are indicated in Table 2 (details in Supplementary Table 3).

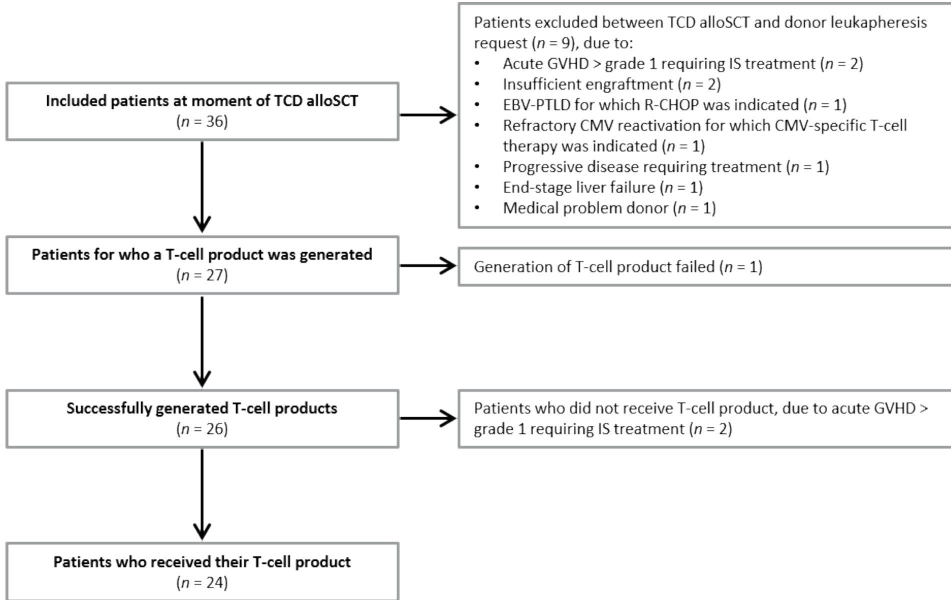

Figure 1. Flow diagram of patient inclusion and multi-antigen-specific T-cell product generation. IS, immunosuppressive treatment; PTLD, post-transplant lymphoproliferative disease.

Table 2. Patient and transplantation characteristics. BM-SCT, bone marrow stem cell transplantation; neg, negative; PB-SCT, peripheral blood stem cell transplantation; pos, positive.

	n=27
Median age, years (range)	55 (25-73)
Gender, n (%)	
Male	17 (63)
Female	10 (37)
Disease, n (%)	
Acute myeloid leukemia	13 (48)
Multiple myeloma	7 (26)
Myelodysplastic syndrome	3 (11)
B-cell lymphoma	2 (7)
Myeloproliferative syndrome	1 (4)
Acute lymphoblastic leukemia	1 (4)
Conditioning intensity, n (%)	
Myeloablative	10 (37)
Non-myeloablative	17 (63)

Table 2 continued.

	n=27
Donor type (HLA-match), n (%)	
Related donor (12/12)	9 (33)
Unrelated donor (at least 9/10)	18 (67)
Graft source, n (%)	
PB-SCT	26 (96)
BM-SCT	1 (4)
CMV serostatus patient/donor, n (%)	
pos/pos	9 (33)
pos/neg	5 (19)
neg/pos	5 (19)
neg/neg	8 (30)
EBV serostatus patient/donor, n (%)	
pos/pos	25 (93)
neg/pos	2 (7)

SUCCESSFUL GENERATION OF MULTI-ANTIGEN-SPECIFIC T-CELL PRODUCTS

Donor leukapheresis products for 27 patients were obtained at a median of 62 days after TCD-alloSCT from unrelated donors (range 53-105 days, *n* = 18) and at a median of 54 days after TCD-alloSCT from related donors (range 50-62 days, *n* = 9). Multi-antigen-specific T-cell products were isolated using the MHC I-*Strep*tamer isolation technology as described before.[37] The products contained a median of $5.2*10^6$ total cells (range $0.4–26.5*10^6$) with a median purity of target-antigen-specific T cells within the T-cell compartment of 79.6% (range 33.5–99.9%) (Table 3, details in Supplementary Table 4). T cells showed predominantly a memory phenotype (median 53.3%, range 8.9-89.1%). The median absolute number of target-antigen-specific T cells was $2.3*10^6$ (range $0.1–25.5*10^6$) (Figure 2A). A correlation was observed between the absolute numbers of target-antigen-specific T cells in the starting materials and in the final products (R^2 = 0.8266; p = <0.0001; Figure 2B). The remaining cells in the products were mainly monocytes, B cells and NK cells, but only a small number of T cells with an unknown specificity that potentially could cause GVHD (median $0.33*10^6$; range $0.0–5.58*10^6$). Based on the release specifications regarding product purity and safety, 26 of 27 generated T-cell products were approved; the first generated product had a purity of target-antigen-specific T cells within the T-cell compartment of only 33.5% and was therefore not approved. These results illustrate the feasibility of multi-antigen-specific T-cell product generation using the MHC I-*Strep*tamer isolation technology.

COMPOSITION OF T-CELL PRODUCTS

To investigate which target-antigen specific T-cell populations were present in the 26 successfully generated products, 5% of cells in the products were expanded *in vitro.* From 23 of 26 products, T cells were successfully expanded allowing subsequent analysis with all separate tetramers for the

specificities included in the product generation. AdV-specific T-cell populations could be observed in all products except product W (Figure 2C). T cells directed against at least one EBV-specificity could be confirmed in all products in line with EBV-seropositivity of all donors. CMV-specific T-cell populations were detected in products derived from all 14 CMV-seropositive donors. As expected and previously illustrated, the presence of CMV-specific T cells in products derived from seronegative donors, and TAA- or MiHA-specific T cells could not be confirmed in the majority of products with tetramer-staining due to their low frequencies.[37] However, in product L and product C, NY-eso-1-specific T cells and WT1-specific T cells were detected, respectively. This surrogate analysis for product composition showed that the products were mainly composed of virus-specific memory T cells and contained T-cell populations directed against at least 2-8 different target-antigens.

Table 3. Release specifications and characteristics of generated multi-antigen-specific T-cell products.

	Release specifications	**T-cell product characteristics (*n*=27)**	
		Median	*Range*
Purity of target-antigen-specific T cells (%)	-	41.7	8.1- 96.3
Purity of target-antigen-specific T cells in CD3^{pos} population (%)	≥ 40	79.6	33.5- 99.9
Absolute numbers of cells (*10^6)	-	5.2	0.4- 26.5
Absolute numbers of target-antigen-specific T cells (*10^6)	0.1- 100	2.3	0.1- 25.5
Absolute numbers of T cells with unknown specificity (*10^6)	≤ 0.1 per kg body-weight of the patient	0.33	0- 5.58

T-CELL PRODUCTS WERE SAFELY ADMINISTERED

Twenty-four patients received their product at the day of product generation at a median of 58 days after alloSCT (range 51-107 days). The infusion of 2 of 26 successfully generated T-cell products was cancelled as patient 12 and 19 experienced progressive acute skin GVHD >grade 1 requiring immunosuppressive treatment. To investigate the safety and toxicity of the administered donor-derived T-cell products, we evaluated the incidence of direct infusion-related complications, acute GVHD and non-relapse mortality until 6 months after alloSCT.

Infusion-related complications. None of the 24 patients that received their product experienced direct infusion-related complications.

GVHD. The 5 patients that received MA conditioning and an unrelated donor graft (patient 6, 7, 20, 22 and 24) were still on low dose immunosuppressive therapy as part of the conditioning regimen at the moment of product infusion. None of the total 24 patients that received their T-cell product developed severe acute GVHD or extensive chronic GVHD. Patients 4 and 20 experienced limited skin GVHD grade 1 at 83 and 16 days after T-cell product administration, respectively.

Patient 4 received topical steroid therapy and patient 20 restarted systemic immunosuppressive medication. Clinical signs of GVHD resolved in both patients.

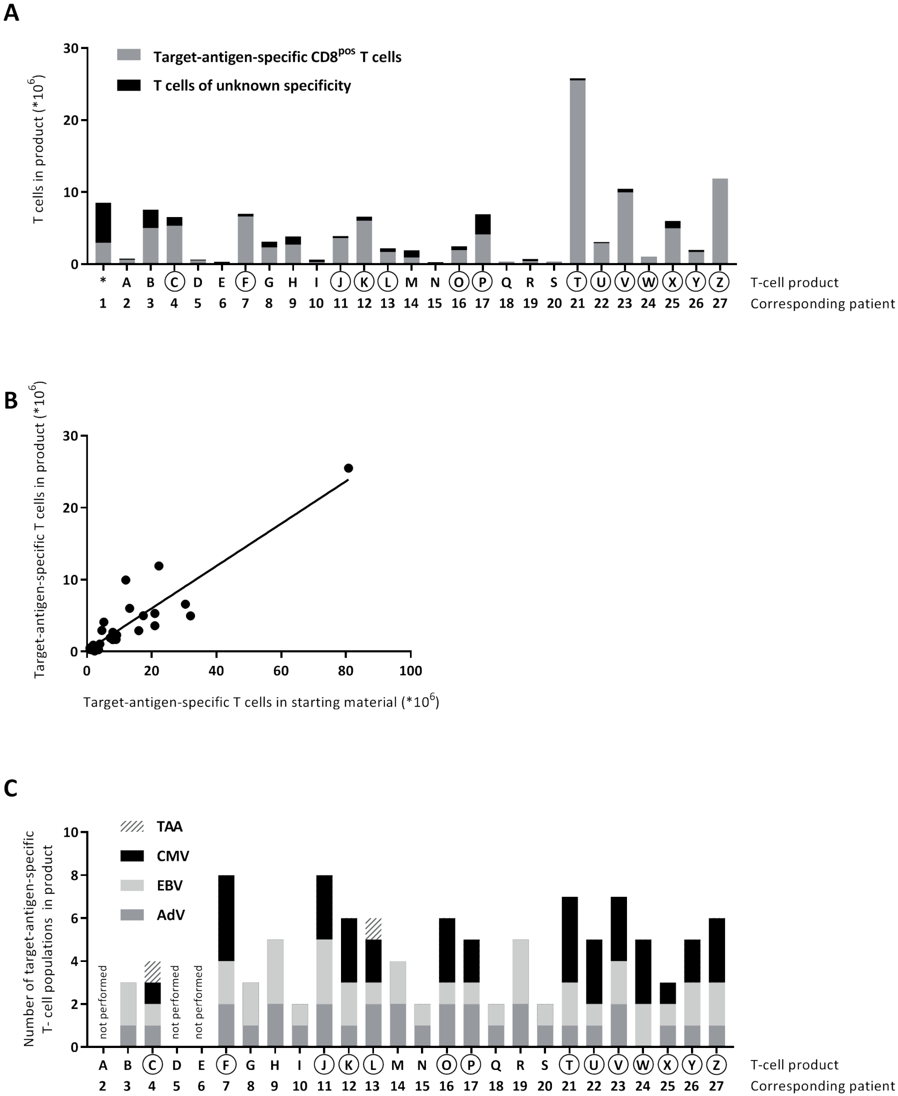

Figure 2. Generated multi-antigen-specific T-cell products**.** All products were derived from EBV-seropositive donors; the donors of the encircled products were also CMV seropositive. **A**, Absolute numbers of target-antigen-specific T cells (gray) and T cells of unknown specificity (black) per product analyzed directly after product generation (*n* = 27). The T-cell product indicated with the asterisk (*) did not meet the release specifications. **B**, Correlation between absolute numbers of target-antigen-specific T cells in starting material and in product after MHC I-*Strep*tamer isolation (*n* = 27). R^2 = 0.8266, *p* = <0.0001. **C**, Number of CMV, EBV, AdV and TAA target-antigen-specific T-cell populations confirmed by tetramer staining during product in-depth analysis (after *in vitro* expansion of 5% of the original products).

Non-relapse mortality. One patient died 44 days after T-cell product infusion due to a non-relapse cause. Patient 7 was admitted to the hospital because of renal and respiratory complaints 28 days after infusion of the T-cell product. A nephrotic syndrome was diagnosed and the patient died 16 days later. No expansion of target-antigen-specific T cells or increase in total $CD8^{pos}$ T cells could be observed *in vivo* during this clinical presentation.

These data illustrate that infusion of the study product early after transplantation did not result in direct infusion-related complications or severe/extensive GVHD during follow-up.

CLINICAL EVENTS AFTER T-CELL PRODUCT INFUSION

To evaluate the clinical events after prophylactic infusion of multi-antigen-specific T cells, we assessed the incidence of clinically relevant CMV, EBV and AdV reactivations and disease progression from the moment of T-cell product infusion until 6 months after alloSCT.

Clinically relevant viral reactivations. Details of the viral reactivations in individual patients are summarized in Table 4. Four patients (patients 2, 11, 24, 27) were diagnosed with a clinically relevant CMV reactivation already before T-cell product infusion and were still on valganciclovir treatment at the moment of T-cell product infusion. During follow-up, 3 patients (patients 8, 22, 27) started antiviral treatment for a clinically relevant CMV reactivation and 1 patient (patient 8) for a clinically relevant EBV reactivation. In patient 27, valganciclovir treatment was reinstalled 30 days after T-cell product infusion due to continuing positive CMV DNA loads. In patient 22, the CMV reactivation progressed to CMV pneumonia within two weeks after product infusion, which resolved after ganciclovir treatment. Patient 8 had an EBV and CMV reactivation at the same time. The CMV reactivation was successfully treated with first-line therapy and the EBV reactivation progressed to EBV-related post-transplant lymphoproliferative disease (EBV-PTLD) 55 days after product infusion. This patient received rituximab, prednisolone and 96 days after product infusion unmodified DLI, which successfully boosted EBV-specific T-cell immunity. No AdV reactivations were detected during follow-up.

Disease progression. Five of 24 patients had progression of their hematologic malignancy. Patients 3, 13 and 17 had relapse of multiple myeloma at 76, 92 and 55 days after T-cell product administration, respectively, and were treated with bortezomib and dexamethason. The MDS in patient 27 progressed to AML 106 days after T-cell product infusion; the patient declined further therapy. Patient 21 received pre-emptive DLI 86 days after product infusion due to increasing mixed-chimerism in bone marrow, with 1-7% patient-derived cells containing 1-2% cells with a morphology suspected for AML.

Taken together, 21 patients remained free of clinically relevant viral reactivations during follow-up.

Two patients developed viral disease and five patients had progression of their primary malignancy.

TARGET-ANTIGEN-SPECIFIC IMMUNE RECONSTITUTION

To investigate the TAA-, MiHA- and virus-specific immune reconstitution after the administration of the T-cell products, we assessed the appearance or expansion of target-antigen-specific T cells in peripheral blood samples with tetramer-staining for all 24 patients. In 18 patients, this immunological monitoring was performed during the complete follow-up period. In patient 7 the follow-up ended prematurely due to death, in patients 3, 13, 17 and 21 the interpretation of the immunological monitoring was complicated by start of relapse treatment, and in patient 8 by therapeutic DLI for EBV-PTLD.

TAA/MiHA. Donor-derived T cells directed against TAA were hypothesized to potentially expand *in vivo* in response to disease relapse with malignant cells expressing TAA. In case of MiHA HA-1 disparity between patient and donor, HA-1H-specific T cells of donor-origin could appear *in vivo* after stimulation with patient-derived malignant (disease relapse/progression) or residual healthy hematopoietic cells (mixed-chimerism).

Five of the 24 patients developed relapse or progression of their primary malignancy and 8 (NMA conditioned) patients had mixed-chimerism detected in bone marrow. In none of the 5 patients with a relapse or progression of their primary malignancy (patients 3, 13, 17, 21, 27), TAA-specific T cells were detectable with tetramer-staining above the detection limit of 0.1% in the peripheral blood. Patient 13 had HA-1 disparity in the right direction with his donor, but appearance of HA-1H-specific T cells could not be observed. Of the 8 patients with mixed-chimerism (patients 2, 5, 10, 11, 14, 15, 16, 25), patient 2 had HA-1 disparity with her donor but HA-1H-specific T cells were not detected during follow-up.

The expansion or appearance of virus target-antigen-specific T cells in peripheral blood were anticipated to be seen in patients that experienced viral reactivations and especially in patients that received their product from virus-seropositive donors.

AdV. In none of the patients, positive AdV DNA loads were detected. In patients 9 and 23, T cells directed against AdV-E1A-LLD/A*02:01, Hexon-KPY/B*07:02, and Hexon-TYF/A*24:02, respectively, appeared or expanded *in vivo* after infusion of the T-cell products (data not shown). T cells directed against these AdV target-antigens were present in the corresponding infused T-cell products.

EBV. Details of the expansion/appearance of EBV target-antigen-specific T cells for individual patients are summarized in Table 4. Four patients experienced EBV reactivations during follow-up. Patients 2, 13 and 27 had reactivations not requiring treatment at 49, 32 and 98 days after T-cell

product infusion, respectively (Figure 3A). In patient 27, donor-derived EBNA-3a-RPP/B*07:02-specific T cells expanded to detectable frequencies. In patient 8, the EBV reactivation progressed rapidly to EBV-PTLD. 12% monoclonal donor-derived B cells with lambda light chains were detectable 55 days after T-cell product infusion in the absence of EBV target-antigen-specific T cells (Figure 3B). During rituximab treatment, expansion of donor-derived BZLF-1-RAK/B*08:01- and BMLF-1-GLC/A*02:01-specific T cells was observed and EBV DNA loads normalized. To investigate whether T cells directed against EBV-antigens other than our target-antigens expanded during the EBV-PTLD, we analyzed the presence of T cells directed against 6 additional HLA-A*01:01-, -A*02:01- and -B*08:01-restricted EBV antigens in follow-up samples obtained 41 and 55 days after T-cell product infusion (Supplementary Table 5), but no EBV-specific T cells were detected at these time points.

CMV. Details of the expansion/appearance of CMV target-antigen-specific T cells for individual patients are summarized in Table 4. Eight patients encountered CMV reactivations during follow-up; all reactivations occurred in CMV-seropositive patients. Patients 2, 8, 10, 13, 22, 24 and 27 had positive CMV DNA loads already at the moment of T-cell product infusion and patient 11 developed positive loads 13 days after infusion.

In the five patients (patients 11, 13, 22, 24, 27) who received products from CMV-seropositive donors, one or more CMV target-antigen-specific T-cell populations of predominantly donor-origin significantly expanded during follow-up (Figure 4A). To analyze the expansion of CMV-specific T cells directed against antigens other than our target-antigens, additional tetramer stainings were performed on follow-up samples of patients 11 and 24. Expansion of CMV-specific T-cell populations not included in the product generation was not observed in these two patients (Supplementary Table 5).

Patients 2, 8 and 10 had CMV-seronegative donors (Figure 4B). In patients 2 and 10, patient-derived pp65-NLV/A*02:01 cells dominated the anti-CMV response after product infusion. In contrast, 3 different CMV target-antigen-specific T-cell populations in patient 8 expanded after product administration and converted within several weeks from patient-origin to full donor-origin. CMV loads in all 8 patients with CMV reactivations normalized within a median of 34 days (range 10-84 days) after product infusion or after the first measured positive CMV DNA load.

Based on the immunological monitoring after multi-antigen-specific T-cell product infusion, we conclude that in 2 of 4 patients with EBV reactivations and in 6 of 8 patients with CMV reactivations, positive viral DNA loads were followed by expansion of virus target-antigen-specific T cells of donor-origin.

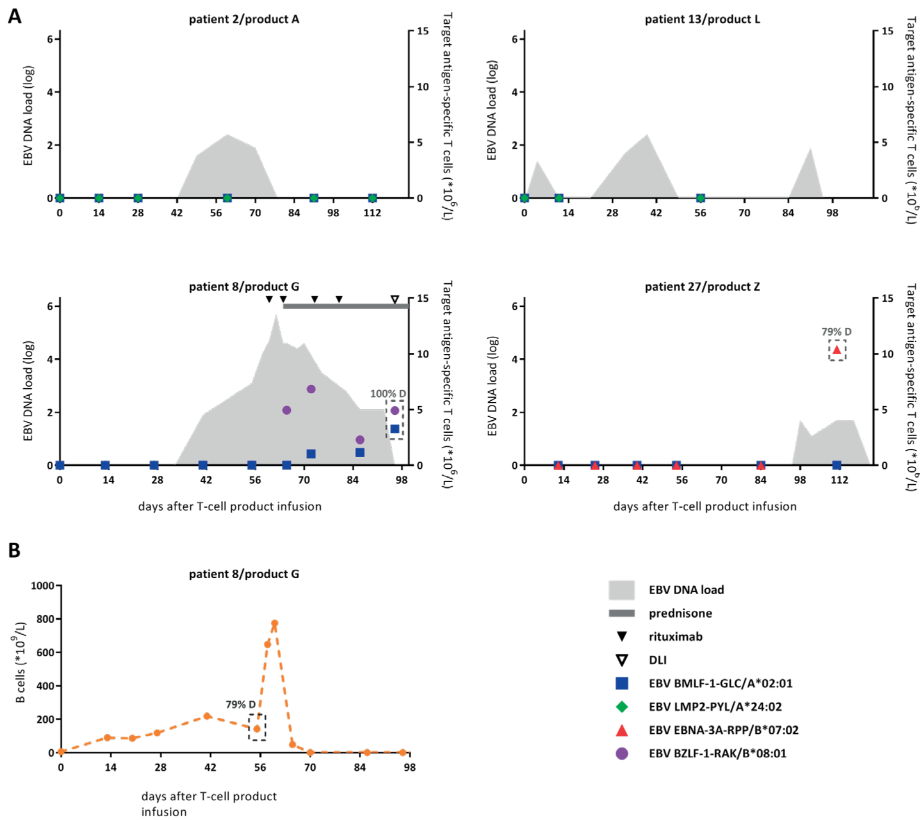

Figure 3. Patients with EBV reactivations during follow-up. **A**, EBV DNA loads and concentration of EBV-target-antigen-specific T cells in the peripheral blood of patients 2, 8, 13 and 27 are illustrated from the moment just before product infusion (day 0) until the end of follow-up. Rituximab and prednisone treatment for EBV-PTLD in patient 8 are indicated. The origin of target-antigen-specific T-cell populations determined by chimerism analysis is indicated in framed boxes (% of cells of donor-origin (D)). **B**, Clinical course of B-cell reconstitution in patient 8 with EBV-PTLD. The origin of the monoclonal B cells determined by chimerism analysis was measured at the time point indicated with the framed box (% of cells of donor-origin (D)).

Table 4. Clinically relevant CMV and EBV reactivations in patients that received a multi-antigen specific T-cell

product. CR, complete remission; DLI, donor lymphocyte infusion; gan, ganciclovir; neg, negative; pos, positive; PR, partial response; rit, rituximab; val, valaciclovir.

CMV

Patient	CMV serostatus patient/donor	Positive CMV DNA load at moment of infusion T-cell product	Anti-CMV treatment at moment of T-cell product infusion	Clinically relevant CMV-reactivation during follow-up	Expansion of donor CMV-specific T-cells during follow-up	CMV outcome (additional therapy)
2	pos/neg	yes	val	yes	no	CR
8	pos/neg	yes	no	yes	yes	CR (+ val)
10	pos/neg	yes	no	yes	no	CR
11	pos/pos	no	val	yes	yes	CR
13	pos/pos	yes	no	yes	yes	CR
22	pos/pos	yes	no	yes	yes	CMV disease, CR (+ gan)
24	pos/pos	yes	val	yes	yes	CR
27	pos/pos	yes	val	yes	yes	PR (+ val)
3 + 5 + 6 + 9 + 18 + 20	neg/neg	no	no	no	no	no CMV infection
4 + 17 + 21 + 23 + 25	neg/pos	no	no	no	no	no CMV infection
7 + 16 + 26	pos/pos	no	no	no	no	no CMV infection
14 + 15	pos/neg	no	no	no	no	no CMV infection

EBV

Patient	EBV serostatus patient/donor	Positive EBV DNA load at moment of infusion T-cell product	Anti-EBV treatment at moment of T-cell product infusion	Clinically relevant EBV-reactivation during follow-up	Expansion of donor EBV-specific T-cells during follow-up	EBV outcome (additional therapy)
2	pos/pos	no	no	yes	no	CR
8	pos/pos	no	no	yes	yes	EBV-PTLD, PR (+ rit and DLI)
13	pos/pos	no	no	yes	no	CR
27	pos/pos	no	no	yes	yes	CR
4 + 9	neg/pos	no	no	no	no	no EBV infection
3 + 5-7 + 10 + 11 + 14-18, 20-26	pos/pos	no	no	no	no	no EBV infection

4

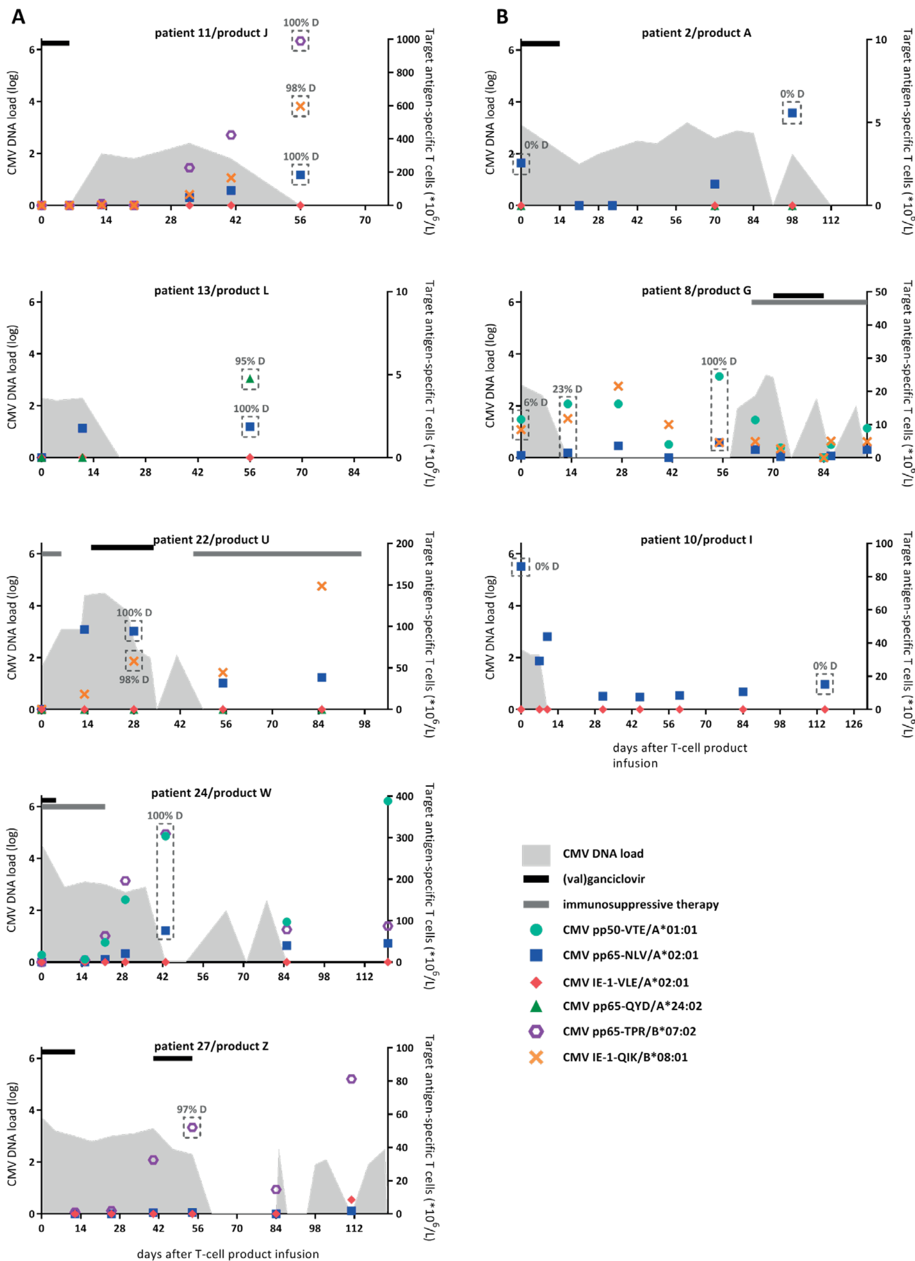

Figure 4. Patients with CMV reactivations during follow-up. CMV DNA loads and concentrations of CMV-target-antigen-specific T cells are indicated from the moment just before product infusion (day 0) until the end of follow-up in the peripheral blood**. A**, Patients 11, 13, 22, 24 and 27 received their T-cell product from CMV-seropositive donors. **B**, Patients 2, 8 and 10 received their T-cell products from CMV-seronegative donors. Systemic immunosuppressive and antiviral therapy are indicated. The origin of target-antigen-specific T-cell populations determined by chimerism analysis is indicated in framed boxes (% of cells of donor-origin (D)). The range in absolute numbers of target-antigen-specific T cells (right Y-axis) in the different patients is rather high.

DISCUSSION

The application of TCD-alloSCT delays immune reconstitution after transplantation and therefore increases the risk of viral reactivations and disease relapses.[9,45,46] However, the avoidance of long-term immunosuppression after TCD-alloSCT creates a platform to apply individualized T-cell therapy to restore specific immunity. In this phase I/II study, we have shown that MHC I-*Strep*tamer-based generation and adoptive transfer of multi-antigen-specific donor T-cell products early after TCD-alloSCT is feasible and safe. Moreover, potential efficacy of this prophylactic infusion is illustrated by expansion of donor-derived target-antigen-specific T cells in patients coinciding viral reactivations and the prevention of viral complications in the majority of patients in the follow-up period after T-cell product infusion.

Donor leukapheresis collection had to be cancelled for only one patient due to donor-related medical problems. The isolation of multi-antigen-specific T-cell products using MHC I-*Strep*tamers led to the successful generation of T-cell products with high purities for 26 of 27 included patients. Indeed, none of the 24 patients that received the study product developed severe acute or extensive chronic GVHD and only two patients experienced limited skin GVHD in the follow-up period, which is in line with previous studies that applied comparable numbers of selected or even unselected donor-derived T cells early after alloSCT in the absence of immunosuppressive GVHD prophylaxis.[22-24,30] As these results exceeded our expectations mentioned during trial design, donor leukapheresis collection, product generation and product infusion were evaluated as feasible and safe.

4

One patient died soon after T-cell product administration due to complications of a nephrotic syndrome. The chronology could suggest a relation with the T-cell product, as complaints and laboratory deviations developed within days after product infusion. As a renal biopsy was contraindicated, provoking factors for the nephrotic syndrome remain uncertain and a relation with the infused T-cell product cannot be fully ruled out. However, coinciding expansion of target-antigen-specific T cells was not observed.

The rationale behind the prophylactic administration of T-cell products was that *ex vivo* isolated T-cells are infused at a moment when the antigen-burden in the patient is low, in order to allow infused T-cells time to expand *in vivo* at the moment that the antigen burden increases during viral reactivation or disease relapse. The infused dose and the *in vivo* persistence and expansion capacity of target-antigen-specific T-cells determine whether appropriate cell numbers are reached to achieve antigen clearance. Due to limitations regarding the size of donor leukapheresis products and the frequencies of target-antigen-specific T-cells within these leukapheresis products, the size of non-expanded T-cell products could not be further increased, but it was anticipated that

virus-specific memory T cells harbor the potential to vigorously expand upon antigen encounter. However, indisputable proof that expansion of donor-derived virus-specific T cells originated from the infused T-cell product and not from T cells that survived T-cell depletion could not been made in the study. Although our study was not designed to investigate efficacy of the study product, several observations suggest a relation between the prophylactic infusion of multi-antigen-specific T-cell products, *in vivo* T-cell reconstitution and clinical outcome. We observed that 21 of 24 patients remained free of clinically relevant viral reactivations during the follow-up period. For 9 of 12 observed reactivations (4 EBV and 8 CMV reactivations), patients received their T-cell product from virus-seropositive donors. All these products contained high percentages of T cells directed against corresponding viral target-antigens and during 7 of 9 reactivations donor-derived target-antigen-specific T cells expanded *in vivo*. In 4 patients with ongoing CMV reactivations at the moment of product infusion in the absence of detectable CMV-specific T cells (patient 13, 22, 24 and 27), expansion of CMV-specific T cells was observed soon after T-cell product infusion. Moreover, *in vivo* expansion of virus-specific T-cell populations directed against CMV- or EBV-antigens other than the antigens included in the study products was not observed in 3 analyzed patients. Therefore, these results suggest that the small numbers of infused virus-specific T cells had a sufficient proliferative potential and expansion capacity to reconstitute viral target-antigen-specific T-cell immunity in these 7 patients encountering viral reactivations, which is in line with previous observations.[17,20,36,38,39] However, administered T cells did not prevent the progression to EBV-PTLD in patient 8, the clinically relevant CMV reactivation in patient 27 and CMV pneumonia in patient 22. Most likely, in patient 8 and 27, the expansion of target-antigen specific T cells to reasonable numbers to combat the viral reactivation required too much time. In patient 22, the CMV pneumonia became apparent within two weeks after T-cell product infusion, which might be explained by aggravation of lung inflammation due to migration of CMV-specific T cells to CMV-infected organs. Furthermore, coinciding expansion of EBV-specific T cells was not observed in two patients (patient 2 and 13) developing EBV reactivations. As viral DNA loads normalized very quickly, we hypothesize that EBV-specific T cells migrated to infected organs or tissue and were therefore not measurable in the peripheral blood. Another option might be that donor- or patient-derived EBV-specific T cells directed against other EBV-epitopes were responsible for the clearance of virus-infected cells.

Potential efficacy of the prophylactic application of virus-specific T cells from seronegative donors was illustrated in CMV-seropositive patient 8. CMV target-antigen-specific T cells converted from predominantly patient-origin before product infusion to 100% donor-origin after product infusion. It is generally known that naïve T-cell populations need time to expand upon priming by professional antigen presenting cells. Direct administration of very low numbers of *ex vivo* isolated naïve T cells may be unsuccessful in the therapeutic setting because this time is not available. Our hypothesis that administration of naïve virus-specific T cells might contribute in a prophylactic

setting, seems to be supported by the observation in this patient. However, in two other patients with CMV reactivations that received products from CMV-seronegative donors, contribution of donor-derived CMV-specific T cells to CMV-immunity was not observed. The potential prophylactic effectivity of our approach can only be truly assessed in a large randomized study.

In the 5 patients with disease progression, we were not able to confirm expansion of TAA- or HA-1H-specific T cells during follow-up. Possible explanations could be that the relapsing malignant cells did not express the TAA/MiHA, that infused target-antigen-specific T cells did not persist until disease progression or that target-antigen-specific T cells were not of sufficient avidity to attack malignant cells.

As the majority of our study patients remained free of CMV, EBV and AdV reactivations, a defined group of patients might benefit from the prophylactic application of MHC I-*Strep*tamer isolated, non-expanded multi-antigen-specific T-cell products. In future studies it should be considered to restrict prophylactic infusion of virus-specific memory T-cells to patients at high-risk for viral reactivations. However, risk stratification for clinically relevant EBV and AdV reactivations is more complex than for CMV, particularly because the incidence for EBV- and especially AdV complications is generally lower than for CMV in the adult alloSCT population.[47-50] Furthermore, the ideal moment of T-cell product infusion needs consideration. In this study, products were administered in a prophylactic setting at a median of 58 days after alloSCT, when seven of 14 CMV-seropositive patients already had detectable CMV DNA loads and four patients were receiving antiviral treatment at the moment of product infusion. These observations indicate that for the prevention of especially clinically relevant CMV reactivations, infusion of the product at an earlier time point after alloSCT may be more optimal.

Another consideration for future trials is the application of virus-specific memory T-cell products in a pre-emptive or therapeutic setting, allowing direct antigen encounter upon adoptive transfer, possibly leading to faster expansion of the infused T cells. Since MHC I-*Strep*tamer isolated non-expanded T-cell products can be generated within one day, patients can receive a personalized product soon after diagnosis of viral infection. Previous studies have shown that *ex vivo* isolated memory T-cell products containing 250-5000 virus-specific T cells/kg body weight of the patient seem sufficient to control viral reactivations in a therapeutic setting.[20,38,39] Otherwise, a short and intensive *in vitro* expansion before infusion can be considered to increase the size of the T-cell product and the chance on virus control at the moment of high antigen burden in the patient.
In conclusion, we have shown that the generation and administration of multi-antigen-specific T-cell products with the MHC I-*Strep*tamer technology is feasible and safe. Moreover, efficacy of prophylactic infusion of these products was suggested by predominantly donor-derived viral immune reconstitution during 8 of 12 viral reactivations. Therefore, this study suggests that the

application of especially virus-specific memory T cells in a prophylactic setting early after TCD-alloSCT can prevent viral complications.

ACKNOWLEDGEMENTS

The authors thank patients, donors and medical staff involved in this clinical trial; Lisette Bogers and Susan Collins for excellent managing of clinical study data; Employees of the Laboratory for Stem Cell Therapy, Laboratory for Specialized Hematology and the Interdivisional GMP Facility of the LUMC for logistical, technical and GMP assistance.

This study was financially supported by the European Union's seventh Framework Program (FP/2007-2013) under grant agreement number 601722 and by Dutch Cancer Society grant UL 2008-4263.

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4

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Patient in- and exclusion criteria at baseline and at the moment of donor leukapheresis request 4-6 weeks after TCD-alloSCT. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CR, complete remission; FL, follicular lymphoma; LPL, lymphoplasmacytic lymphoma; MALT, mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma; MDS, myelodysplastic syndrome; PR, partial remission; WBC, white blood cell count.

	Inclusion criteria	Exclusion criteria
Baseline	18-75 years	Life expectation <6 months

	TCD-alloSCT from a related (12/12 HLA-matched) or unrelated (9/10 or 10/10 matched for HLA-A,-B,-C,-DR and –DQ) donor for one of the following diagnoses:
	• ALL in CR after pre-phase and first induction and consolidation therapy and WBC <$30*10^9$/L in B-ALL or <$100*10^9$/L in T-ALL at initial diagnosis. ALL with t(9;22), t(4;11), complex karyotype or 11q23 abnormalities are excluded.
	• AML in CR, excluding AML with:
	    • Monosomal karyotype
	    • Abn3q26
	    • EVI1 overexpression
	• Multiple myeloma at least in stable PR
	• Non-high grade B-cell lymphoma (B-CLL, MCL, FL, MALT, LPL) at least in stable PR
	• Myeloproliferative syndromes at least in stable PR, excluding CML blastic phase
	• MDS at least in stable PR
		Disease specific treatment foreseen in the first 6 months after TCD-alloSCT

	HLA-A*02:01 positivity	End stage irreversible multi-system organ failure
	Written informed consent of the patient	Pregnant or lactating women
	Availability of stem cell donor 6-8 weeks after TCD-alloSCT, who meets the following inclusion criteria: • HLA-A*02:01 positivity • CMV and/or EBV seropositivity • Written informed consent	Severe psychological disturbance
At moment of donor leukapheresis request (4-6 weeks after TCD-alloSCT)	Stable engraftment of the allogeneic graft (platelets >$20*10^9$/L, granulocytes >$0.5*10^9$/L)	Histologically proven acute GVHD > grade 1 for which immune suppressive treatment is given
		Progressive disease for which therapy is needed
		Use of >20 mg prednisone a day
		Life expectation <12 weeks
		End stage irreversible multi-system organ failure
		Uncontrolled bacterial or fungal infection
		Evidence of graft rejection

Supplementary Table 2. TCD-alloSCT conditioning regimens. T-cell depleting agents with dosages are underlined. BM-SCT, bone-marrow stem cell transplantation; iv, intravenously; MA, myeloablative; NMA, non-myeloablative; PB-SCT, peripheral blood stem cell transplantation; TBI, total body irradiation. *for *in vitro* TCD of the graft, alemtuzumab was added to the graft and incubated for 30 minutes before infusion into the patient.

Donor	Conditioning intensity	Graft source	Agents	Dose	Administration days related to transplantation (day 0)
Related	*MA*	*PB-SCT*	Cyclophosphamide	60 mg/kg iv	-6,-5
			Mesna	60 mg/kg iv	-6,-5,-4
			TBI	9 Gy	-1
			Alemtuzumab added to the graft*	20 mg ‘in the bag’	0
			or		
			Busulfan	3.2 mg/kg iv	-9 until-6
			Cyclophosphamide	60 mg/kg iv	-4,-3
			Mesna	60 mg/kg iv	-4,-3,-2
			Alemtuzumab added to the graft*	20 mg ‘in the bag’	0
		BM-SCT	TBI	9 Gy	-8 or-7
			Cyclophosphamide	60 mg/kg iv	-6,-5
			Mesna	60 mg/kg iv	-6,-5,-4
			Alemtuzumab added to the graft*	20 mg ‘in the bag’	0
	NMA	*PB-SCT*	Fludarabine	50 mg/m^2 oral	-10 until-5
			Busulfan	3.2 mg/kg iv	-7,-6
			Alemtuzumab	15 mg iv	-4,-3
			Alemtuzumab added to the graft*	20 mg ‘in the bag’	0
Unrelated	*MA*	*PB-SCT*	TBI	9 Gy	-8 or-7
			Cyclophosphamide	60 mg/kg iv	-6,-5
			Mesna	60 mg/kg iv	-6,-5,-4
			Alemtuzumab	15 mg iv	-6,-5
			Cyclosporine A	3 mg/kg iv	-1 until +30, then tapered off
			Alemtuzumab added to the graft*	20 mg ‘in the bag’	0
			or		
			Busulfan	3.2 mg/kg iv	-9 until-6
			Cyclophosphamide	60 mg/kg iv	-4,-3
			Mesna	60 mg/kg iv	-4,-3,-2
			Alemtuzumab	15 mg iv	-6,-5
			Cyclosporine A	3 mg/kg iv	-1 until +30, then tapered off
			Alemtuzumab added to the graft*	20 mg ‘in the bag’	0
	NMA	*PB-SCT*	Fludarabine	50 mg/m^2 oral	-10 until-5
			Busulfan	3.2 mg/kg iv	-7,-6
			Alemtuzumab	15 mg iv	-4,-3
			Anti-thymocyte globulin	1 mg/kg iv	-2
			Alemtuzumab added to the graft*	20 mg ‘in the bag’	0
			Prednisolone	1 mg/kg iv or oral	+1 until +10, then tapered off

Supplementary Table 3. Patient and transplantation characteristics. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CR, complete remission; LPL, lymphoplasmacytic lymphoma; MA, myeloablative; MCL, mantle cell lymphoma; MDS, myelodysplastic syndrome; neg, negative; NMA, non-myeloablative; pos, positive. ‡ patient 23 received bone marrow- instead of peripheral blood-derived alloSCT.

Patient	Age at alloSCT, years	Sex	Diagnosis	Graft type	Conditioning regimen	CMV serostatus patient/donor	EBV serostatus patient/donor	HLA-matching	HLA A/B typing for which MHC I-*Strep* proteins were available
1	51	M	AML	related	MA	neg/neg	pos/pos	12/12	A*02:01/B*07:02
2	64	F	MDS	related	NMA	pos/neg	pos/pos	12/12	A*02:01/A*24:02
3	68	M	MM	unrelated	NMA	neg/neg	pos/pos	10/10	A*02:01/B*07:02
4	51	M	MM	related	NMA	neg/pos	neg/pos	12/12	A*02:01
5	65	F	AML	unrelated	NMA	neg/neg	pos/pos	10/10	A*01:01/A*02:01/B*07:02
6	44	M	AML	unrelated	MA	neg/neg	pos/pos	10/10	A*02:01
7	28	M	AML	unrelated	MA	pos/pos	pos/pos	10/10	A*02:01/A*24:02/B*07:02
8	70	M	AML	unrelated	NMA	pos/neg	pos/pos	10/10	A*01:01/A*02:01/B*08:01
9	73	M	AML	unrelated	NMA	neg/neg	neg/pos	10/10	A*02:01/B*07:02/B*08:01
10	68	M	MM	related	NMA	pos/neg	pos/pos	12/12	A*02:01
11	49	F	MM	unrelated	NMA	pos/pos	pos/pos	10/10	A*02:01/B*07:02/B*08:01
12	49	F	MDS	unrelated	MA	pos/pos	pos/pos	10/10	A*01:01/A*02:01/B*08:01
13	70	M	MM	unrelated	NMA	pos/pos	pos/pos	10/10	A*02:01/A*24:02
14	55	M	MM	related	NMA	pos/neg	pos/pos	12/12	A*02:01/B*07:02
15	57	M	MCL	related	NMA	pos/neg	pos/pos	12/12	A*02:01
16	62	M	AML	unrelated	NMA	pos/pos	pos/pos	10/10	A*02:01/A*24:02
17	53	F	MM	unrelated	NMA	neg/pos	pos/pos	9/10	A*02:01/A*24:02
18	65	F	AML	unrelated	NMA	neg/neg	pos/pos	10/10	A*02:01
19	52	F	AML	unrelated	MA	neg/neg	pos/pos	10/10	A*02:01/A*24:02/B*08:01
20	57	F	AML	unrelated	MA	neg/neg	pos/pos	10/10	A*02:01
21	49	M	AML	related	MA	neg/pos	pos/pos	12/12	A*01:01/A*02:01/B*08:01
22	26	M	AML	unrelated	MA	pos/pos	pos/pos	10/10	A*02:01/A*24:02/B*08:01
23	45	F	AML	related ‡	MA	neg/pos	pos/pos	12/12	A*02:01/A*24:02/B*08:01
24	30	M	B-ALL	unrelated	MA	pos/pos	pos/pos	9/10	A*01:01/A*02:01/B*07:02
25	64	F	Myelofibrosis	related	NMA	neg/pos	pos/pos	12/12	A*02:01
26	40	M	LPL	unrelated	NMA	pos/pos	pos/pos	10/10	A*02:01/B*07:02
27	70	M	MDS	unrelated	NMA	pos/pos	pos/pos	10/10	A*02:01/B*07:02

Supplementary Table 4. Characteristics of generated multi-antigen-specific T-cell products. ‡ HA-1H-specific MHC I-*Strep*tamer included in isolation procedure.

Product	Product generated for patient	Number of different MHC I-*Strep* proteins used in T-cell product generation	Purity of target-antigen-specific T cells within $CD3^{pos}$ cell in starting material (%)	Total cells in product ($*10^6$)	Purity of target-antigen-specific T cells within $CD3^{pos}$ cells in product (%)	Target-antigen-specific T cells in product ($*10^6$)	Product infusion (days after TCD-alloSCT)
*	1	12	0.38	23.00	33.5	2.93	Product not infused
A	2	13‡	0.36	3.60	71.8	0.56	57
B	3	12	1.39	12.00	66.1	5.00	63
C	4	9	2.76	10.00	81.2	5.30	54
D	5	14	0.07	1.50	79.6	0.50	55
E	6	9	0.20	0.40	81.2	0.10	85
F	7	15	2.38	7.40	94.3	6.60	57
G	8	13	0.86	3.60	74.2	2.30	57
H	9	14	0.76	7.56	69.4	2.68	70
I	10	9	0.16	2.10	46.8	0.29	51
J	11	14	1.74	5.20	92.3	3.60	107
K	12	13	0.94	9.90	90.9	6.00	Product not infused
L	13	13‡	0.94	7.20	77.9	1.70	79
M	14	12	0.19	5.10	46.0	0.90	61
N	15	9	0.15	1.68	51.9	0.14	53
O	16	12	0.57	6.80	77.4	1.92	58
P	17	12	0.48	15.00	59.2	4.10	71
Q	18	9	0.35	1.80	78.0	0.26	73
R	19	14	0.25	1.20	59.7	0.43	Product not infused
S	20	9	0.08	0.65	90.0	0.28	85
T	21	13	6.63	26.50	98.8	25.5	63
U	22	14	1.46	3.28	94.5	2.90	77
V	23	14	0.93	11.50	94.9	9.94	61
W	24	14	0.33	1.30	99.9	1.05	56
X	25	9	2.56	6.40	83.2	4.97	52
Y	26	13‡	0.55	2.25	84.7	1.66	65
Z	27	12	1.66	12.75	99.9	11.90	58

Supplementary Table 5. Tetramers directed against other viral antigens than the target-antigens included in the multi-antigen-specific T-cell product generation, were used for additional flow cytometric analysis on peripheral blood follow-up samples of patient 8, 11 and 24.

	HLA-restriction	Protein	Peptide	Days after T-cell product infusion: virus-specific T cells (*10^6/L)	
Patient 8 EBV-PTLD	A*02:01	EBV LMP2	**CLG**GLLTMV	day 41: 0	day 55: 0
	A*02:01	EBV LMP2	**FLY**ALALLL	day 41: 0	day 55: 0
	A*02:01	EBV EBNA-3C	**LLD**FVRFMGV	day 41: 0	day 55: 0
	A*02:01	EBV BRLF-1	**YVL**DHLIVV	day 41: 0	day 55: 0
	B*08:01	EBV EBNA-3A	**FLR**GRAYGL	day 41: 0	day 55: 0
	B*08:01	EBV EBNA-3A	**QAK**WRLQTL	day 41: 0	day 55: 0
Patient 11 CMV reactivation	B*07:02	CMV pp65	**RPH**ERNGFTVL	day 0: 0	day 56: 0
	B*08:01	CMV IE-1	**ELR**RKMMYM	day 0: 0	day 56: 0
	B*08:01	CMV pp65	**ERK**HRHLPV	day 0: 0	day 56: 0
Patient 24 CMV reactivation	A*01:01	CMV pp65	**YSE**HPTFTSQY	day 0: 1.8	day 43: 0
	B*07:02	CMV pp65	**RPH**ERNGFTVL	day 0: 15.9	day 43: 0

4

CHAPTER 5

A MINORITY OF T CELLS RECOGNIZING TUMOR-ASSOCIATED ANTIGENS PRESENTED IN SELF-HLA CAN PROVOKE ANTITUMOR REACTIVITY

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Blood 2020; 136(4): 455-467.

ABSTRACT

Tumor-associated antigens (TAA) are monomorphic self-antigens that are proposed as targets for immunotherapeutic approaches to treat malignancies. We investigated whether T cells with sufficient avidity to recognize naturally overexpressed self-antigens in the context of self-HLA can be found in the T-cell repertoire of healthy donors. Minor histocompatibility antigen (MiHA)-specific T cells were used as a model, as the influence of thymic selection on the T-cell repertoire directed against MiHA can be studied in both self (MiHA^{pos} donors) and non-self (MiHA^{neg} donors) backgrounds.

T-cell clones directed against the HLA*02:01-restricted MiHA HA-1H were isolated from HA-1H^{neg}/HLA-A*02:01^{pos} and HA-1H^{pos}/HLA-A*02:01^{pos} donors. Of the 16 unique HA-1H-specific T-cell clones, five T-cell clones derived from HA-1H^{neg}/HLA-A*02:01^{pos} donors and one T-cell clone derived from an HA-1H^{pos}/HLA-A*02:01^{pos} donor showed reactivity against HA-1H^{pos} target cells. In addition, in total, 663 T-cell clones (containing at least 91 unique clones expressing different T-cell receptors) directed against HLA*02:01-restricted peptides of TAA WT1-RMF, RHAMM-ILS, proteinase-3-VLQ, PRAME-VLD and NY-eso-1-SLL were isolated from HLA-A*02:01^{pos} donors. Only 3 PRAME-VLD- and one NY-eso-1-SLL-specific T-cell clone provoked IFN-γ production and/or cytolysis upon stimulation with HLA-A*02:01^{pos} malignant cell lines (but not primary malignant samples) naturally overexpressing the TAA.

These results illustrate that self-HLA-restricted T cells specific for self-antigens like MiHA in MiHA^{pos} donors and TAA are present in peripheral blood of healthy individuals. However, clinical efficacy would require highly effective *in vivo* priming by peptide vaccination in the presence of proper adjuvants or *in vitro* expansion of the low numbers of self-antigen-specific T cells of sufficient avidity to recognize endogenously processed antigen.

INTRODUCTION

Tumor-associated antigens (TAA) are proposed as targets for immunotherapeutic approaches to treat malignancies.[1-6] Most identified TAA belong to the group of nonmutated monomorphic self-antigens such as differentiation antigens, aberrantly expressed antigens (eg, WT1, RHAMM, proteinase-3) and cancer-germline antigens (also known as cancer-testis antigens; eg, PRAME, NY-eso-1). Observations have suggested that T cells recognizing these self-antigens may contribute to antitumor reactivity after vaccination strategies or HLA-matched allogeneic hematopoietic stem cell transplantation (alloSCT). A relation has been proposed between expansion of TAA-specific T cells in the peripheral blood of patients after HLA-matched alloSCT and better relapse-free survival. Moreover, relapses have been observed in patients in the absence of TAA-specific T cells.[7-12] These observations have generated interest in manipulating TAA-directed immunity to enhance antitumor reactivity.

Multiple phase 1/2 vaccination studies targeting TAA in patients with hematologic malignancies have been performed.[13-22] In a minority of vaccinated patients, clinical responses coincided with increased frequencies of TAA-specific T cells in peripheral blood. However, a causative relation between induction of high-avidity TAA-specific T cells and clinical effect has not been proven.[23] *In vitro* studies have shown that T cells binding TAA-specific tetramers are regularly found in patients with hematologic malignancies and healthy donors.[24-31] This observation suggests a role for TAA-specific T cells in the process of immune surveillance, whereby the immune system is supposed to identify and eliminate (pre)malignant cells.[32,33] Functional analysis showed that TAA-specific T cells of both patients and donors were capable of recognizing target cells exogenously loaded with TAA peptides. However, it remains uncertain whether these T cells are capable of recognizing endogenously processed antigen.

Because TAA are self-antigens with low or restricted tissue-specific expression in non-malignant cells, negative thymic selection is supposed to prevent autoimmunity by eliminating T cells with high avidity for self-antigens presented in self-HLA from the T-cell repertoire.[34,35] However, dysregulated overexpression of TAA in malignant cells may allow the immune system to discriminate these cells from their healthy counterparts, supporting the potential value of TAA in immunotherapy.[36-39] Minor Histocompatibility Antigens (MiHA) are peptides derived from polymorphic genes that can be recognized as foreign by donor T cells when there is a disparity with the patient. After HLA-matched alloSCT, alloreactive T-cell responses directed against MiHA play a crucial role in the induction of a graft-versus-leukemia (GVL) effect and graft-versus-host disease (GVHD).[40-42] As expected, these functional MiHA-specific T-cell responses have exclusively been described in MiHA^{pos} patients receiving grafts from MiHA^{neg} donors, because high-avidity MiHA-specific T cells are supposed to pass thymic selection only in MiHA^{neg} donors.[43-46] Since MiHA are considered

self-antigens in MiHA^{pos} donors, it is expected that the MiHA-specific T-cell repertoire in MiHA^{pos} donors is shaped in a comparable way as the TAA-specific T-cell repertoire in healthy individuals.

To elucidate the antitumor potential of TAA-specific T cells, we investigated the self-HLA-restricted TAA-specific T-cell repertoire in healthy individuals. First, we assessed the influence of thymic selection on the shaping of the T-cell repertoire against self-antigens presented in self-HLA, using MiHA-specific T cells as a model. The polymorphic feature of MiHA allowed study of the T-cell repertoire directed against the same antigen in both a self (MiHA^{pos} donors) and non-self (MiHA^{neg} donors) background, providing a proven positive control of high-avidity MiHA-specific T-cell responses. Because the hematopoiesis-restricted MiHA HA-1H has a balanced population frequency, this was an ideal antigen for our model.[47] Second, we tested the functional reactivity of T cells recognizing TAA presented in self-HLA and determined whether TAA-specific T cells that are able to recognize endogenously processed antigen are present in the T-cell repertoire of healthy individuals.

MATERIALS AND METHODS

ISOLATION OF HA-1H-SPECIFIC AND TAA-SPECIFIC T-CELL CLONES

This study was conducted after approval was granted by the Leiden University Medical Center (LUMC) Institutional Board. Leukapheresis products and peripheral blood were obtained from healthy donors and patients after written informed consent according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells were isolated by Ficoll-Isopaque separation or red blood cell lysis using an NH_4Cl (8.4 g/L) and $KHCO_3$ (1 g/L) buffer (pH = 7.4) (LUMC Pharmacy, Leiden, The Netherlands) and cryopreserved in liquid nitrogen until further use.

TAA-specific T-cell clones were isolated from 18 HLA-A*02:01^{pos} donors, and HA-1H-specific T-cell clones from 3 HA-1H^{neg}/HLA-A*02:01^{pos} donors and 4 HA-1H^{pos}/HLA-A*02:01^{pos} donors using the MHC I-*Strep*tamer isolation technology as previously described.[48] In short, isolation complexes (MHC I-*Strep*tamers) were generated per T-cell specificity by incubation of peptide-loaded MHC I-*Strep*-tag fusion proteins (MHC I-*Strep* proteins; Table 1) with magnetically labelled *Strep*-Tactin (*Strep*-Tactin nanobeads) to allow multimerization of MHC-*Strep* proteins on *Strep*-Tactin nanobeads. Half the amount of MHC I-*Strep*tamers per specificity were used for the indicated amount of starting cells. The pool of MHC I-*Strep*tamers was incubated with 0.5-2*10^9 donor PBMC to allow TAA- or HA-1H-specific T cells to bind the isolation complexes. MHC I-*Strep*tamer-bound cells were isolated using a CliniMACS Plus instrument or MidiMACS separator device (Miltenyi Biotec, Bergisch Gladbach, Germany). MHC I-*Strep*tamers were dissociated from the

positively isolated cells using D-Biotin. Cells were non-specifically expanded for 10-14 days and analyzed by FACS. Subsequent MHC I-*Strep*tamer isolation and expansion rounds of positively isolated cells were repeated until all TAA- or HA-1H-specific T-cell populations became visible by direct tetramer staining or until four rounds were accomplished.

Table 1. HLA-A*02:01-restricted TAA and the MiHA included in this study. MHC I-*Strep*tamers for isolation and tetramers for flow cytometry analysis were available for the indicated peptides. Peptide binding affinities for HLA-A*02:01 were predicted using NetMHC Version 4.0. SB are predicted strong binders, WB are predicted weak binders.

	Protein	Peptide	Peptide binding affinity for HLA-A*02:01 (nM)	
	WT1	**RMF**PNAPYL	7.14	SB
	RHAMM	**ILS**LELMKL	160.52	WB
TAA	Proteinase-3	**VLQ**ELNVTV	12.33	SB
	PRAME	**VLD**GLDVLL	51.09	WB
	NY-eso-1	**SLL**MWITQV	6.13	WB
MiHA	HA-1	**VLH**DDLLEA	28.61	SB

Three previously identified high-avidity CD8^{pos} HA-1H-specific T-cell clones were included in the indicated T-cell reactivity assays as positive controls. These T-cell clones were isolated *ex vivo* from peripheral blood of three different HA-1H^{pos}/HLA-A*02:01^{pos} patients with a profound GVL response after DLI given for disease relapse (patient 1 and 2: chronic myeloid leukemia (CML); patient 3: multiple myeloma (MM)) after alloSCT from an HA-1H^{neg}/HLA-A*02:01^{pos} donor, as previously described.[49] In short, T cells isolated from post-DLI peripheral blood samples at the time of clinical response to DLI were stimulated with malignant cells isolated from the patient before alloSCT. After 16h of culture, interferon gamma (IFN-γ) producing T cells were sorted, single cell per well plated and non-specifically expanded in ‘T-cell medium’, consisting of Iscove’s Modified Dulbecco’s Medium (IMDM; Lonza, Basel, Switzerland) containing 5% pooled human serum, 5% heat-inactivated fetal bovine serum (FBS; Gibco, Thermo Fischer Scientific, Waltham, MA, USA), 3 mM L-glutamine (Lonza), 100 U/ml penicillin/streptomycin (Lonza) and 100 IU/ml interleukin-2 (Chiron, Amsterdam, The Netherlands). T-cell clones that were CD8^{pos}/ tetramer^{pos} and exerted cytolytic activity against HLA-A*02:01^{pos}/HA-1H^{pos} target cells were selected.

CELL LINES, PRIMARY SAMPLES AND CULTURE CONDITIONS

T cells were cultured in ‘T-cell medium’. T cells were non-specifically expanded after MHC I-*Strep*tamer isolation and single cell FACS sorting and were thereafter restimulated at a 2-3 week interval with culture medium containing irradiated (35 Gy) autologous or allogeneic PBMC and 0.5*10^{6} allogeneic Epstein-Barr virus (EBV)-transformed lymphoblastoid B-cell lines (EBV-LCL) as

feeder cells, and 800 ng phytohemagglutinin (PHA, Oxoid Microbiology Products, Thermo Fisher Scientific). All T-cell reactivity assays were performed 11-18 days after restimulation of T-cell clones in 'test medium' containing IMDM supplemented with 10% human serum, 3 mM L-glutamine, 100IU/ml penicillin/streptomycin and 25 IU/ml IL-2.

The TAP-deficient T2 cell line, CML cell line K562, acute myeloid leukemia (AML) cell line THP-1 and MM cell lines RPMI 8226 and U266 were cultured in IMDM supplemented with 10% FBS. MM cell line OPM-2 was cultured in Roswell Park Memorial Institute 1640 (RPMI 1640; Gibco, Thermo Fisher Scientific) medium supplemented with 20% FBS. Acute lymphoblastic leukemia (ALL) cell lines were generated and cultured as previously described.[50] HLA-A*02:01^{neg} cell lines K562, OPM-2 and RPMI 8226 were transduced with a retroviral vector encoding HLA-A*02:01 as previously described.[51] Stable EBV-LCL were generated from two HLA-A*02:01^{pos} donors using standard procedures. EBV-LCL were cultured in IMDM supplemented with 10% FBS, 3 mM L-glutamine and 100 U/ml penicillin/streptomycin.

Primary bone-marrow and peripheral blood AML, ALL and MM samples were thawed and kept overnight in 'T-cell medium'. On the day of the functional test, samples were analysed by FACS to determine the percentage of blast cells. Only primary samples containing >65% blasts were included in the functional tests.

RETROVIRAL TRANSDUCTION OF EBV-LCL WITH TAA

To induce artificial endogenous overexpression of TAA in cell lines, 2 EBV-LCL were transduced with a retroviral vector encoding the full protein sequences of NY-eso-1, WT1 or PRAME. For NY-eso-1, the protein (RefSeq NM_139250) was sequenced from Mel518 melanoma cell line and cloned into the retroviral MP71 vector, containing the truncated human nerve growth factor receptor (NGFR) selection marker gene linked by an IRES sequence. For WT1, a commercially available codon optimized construct was used (pLZRS-IRES-NGFR; GeneArt, Thermo Fischer Scientific). For PRAME, we used the pcDNA3.1 expression vector encoding wild-type human PRAME (pLZRS-IRES-NGFR; RefSeq HSU65011).[28] EBV-LCL were transduced with retroviral vector supernatants, as previously described.[51] Cells highly expressing the marker-gene NGFR were FACS-sorted to generate a pure population of transduced cells.

FUNCTIONAL REACTIVITY OF T-CELL CLONES

For stimulation assays, 2500 T cells were incubated with 15 000 stimulator cells (responder-to-stimulator (R:S) ratio, 1:6) in 384-well flat-bottom plates. After 18 hours, supernatants were harvested, and IFN-γ release was measured by using a standard enzyme-linked immunosorbent assay (ELISA; Sanquin, Amsterdam, The Netherlands; and R&D Systems, Minneapolis, MN). For peptide titrations, stimulator cells were incubated with different concentrations of peptide for 1

hour at 37°C before the addition of T cells.

T-cell-mediated cytotoxicity was determined using standard ^{51}chromium release assays. Target cells were labelled with 100 µCi ^{51}chromium (PerkinElmer, Waltham, MA) and, if indicated, loaded with peptide for 1 hour at 37°C, washed, and counted. A total of 2500 ^{51}chromium labelled-target cells were incubated with T cells at different effector-to-target ratios (E:T) in 96-well culture plates. After 4 hours of incubation, supernatants were harvested, transferred to Luma plates (PerkinElmer) and ^{51}chromium release (cpm) was measured on a 2450 Microbeta2 plate counter (PerkinElmer). Maximal release was induced by 1% Triton (MilliporeSigma, Burlington, MA). The percentages of specific lysis were calculated with the following formula: [experimental release–spontaneous release]/[maximum release–spontaneous release)] x 100%. As positive control for HLA-A*02:01 expression and stimulatory capacity of malignant cell lines and primary malignant cells, an allo-HLA-A*02:01-reactive CD8^{pos} T-cell clone was included in all experiments.

BINDING AFFINITIES OF PEPTIDES TO HLA-A*02:01

Binding affinities of HA-1H and TAA peptides were predicted in nanoMolar using NetMHC version 4.0.[52] The binding level (strong binder (SB) versus weak binder (WB)) is based on the %Rank, which is the rank of the predicted affinity compared to a set of 400.000 random natural peptides. SB are defined as having %Rank <0.5, while WB have %Rank <2.

TETRAMER STAINING

Positive fractions after MHC I-*Strep*tamer isolation and T-cell clones were checked for TAA and/or HA-1H tetramer positivity by flow cytometry. Phycoerythrin (PE)- and allophycocyanin (APC)-labeled tetramers were produced for all specificities indicated in Table 1 as described previously.[53,54] For FACS analysis, cells were incubated with tetramers for 10 minutes at 4°C followed by incubation with fluorescein isothiocyanate (FITC)-labeled CD4 and peridinin-chlorophyll-protein complex (PerCP)-labeled CD8 antibodies (Beckton Dickinson (BD) Biosciences, Franklin Lakes, NJ, USA) for 20 minutes at 4°C. To compare the intensity of tetramer staining among T-cell clones, 50.000 cells per T-cell clone were stained with equal concentrations of a titrated tetramer. Cells were analyzed on a FACS Calibur or Canto (BD) and analyzed using FlowJo Software (TreeStar, Ashland, OR, USA) or Diva software (BD), respectively.

ANALYSIS OF RELATIVE TAA EXPRESSION BY QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

TAA expression in cell lines and primary samples was analyzed by using quantitative reverse transcription polymerase chain reaction (RT-qPCR). The mRNA expression of WT1, PRAME and NY-eso-1 was quantified in different malignant cell lines and two EBV-LCL transduced with retroviral vectors encoding the full protein sequences of the corresponding TAA. Total RNA was isolated using

micro scale RNAqueous isolation kit and treated with DNAse I for 30 min at 37°C (Thermo Fischer). cDNA synthesis was performed using M-MLV Reverse Transcriptase and Oligo(dT) primers (Thermo Fischer). RT-qPCR was performed using commercially available Taqman Gene Expression assays (WT1: Hs01103751_m1; PRAME: Hs01022301_m1; CTAG1A/CTAG1B (NY-eso-1): Hs00265824_m1) and universal Master Mix II , no UNG (Thermo Fischer) in accordance to the manufacturer's instructions. Amplification was measured in triplicate in real-time on the LightCycler 480 and data were analysed using LightCycler 480 software (Roche, Basel, Switzerland). Data was normalized using two reference genes: ACTB (alias: β-actin; Hs99999903_m1)) and GAPDH (Hs99999905_m1). Expression relative to the housekeeping genes was calculated using 2^-ΔcT. The expression of the codon optimized construct of WT1 could not be detected by RT-qPCR as a suitable primer for this unknown protein sequence was not available.

T-CELL RECEPTOR BETA VARIABLE GENE ANALYSIS AND CDR3 SEQUENCING

The T-cell receptor beta variable (TRBV) gene usage of HA-1H-specific and TAA-specific T-cell clones was determined using the IOTest Beta Mark Kit for TRBV repertoire analysis by flow cytometry (Immunotech SAS, a Beckman Coulter Company, Marseille, France) and indicated following the IMGT nomenclature.[55] Because this kit has a coverage of 70% of the normal human TRBV genes, T-cell clones of which the TRBV gene could not be determined by this strategy were categorized as 'TRBV?'. T-cell clones with the same antigen-specificity, derived from the same donor, using the same TRBV gene and performing equally in the functional screenings, were considered identical T-cell clones. In case the result of the functional screening varied in a group of T-cell clones, sequencing of the CDR3-region of the T-cell receptor beta (TRB) chain (including the variable chain (V), diversity chain (D) and joining chain (J)) was performed on selected T-cell clones using anchoring reverse transcription of immunoreceptor sequences and amplification by nested polymerase chain reaction (ARTISAN PCR) adapted for T-cell receptors (TCR), as previously described.[56-58] Briefly, mRNA was purified from T-cell clones using the Dynabead mRNA DIRECT Kit (Thermo Fischer Scientific, Waltham, MA, USA) and mixed with TRBC1 and TRBC2 constant region-specific primers and anchored template-switching oligonucleotides. TRB cDNA was synthesized using SMARTscribe reverse transcriptase and amplified by Phusion Flash (Thermo Fischer Scientific) using a forward anchor-specific primer and a nested reverse primer specific for the constant region of TRB: 5'-TGT GGG AGA TCT CTG CTT CTG-3'. Sanger sequencing was performed using the same reverse primer at the Leiden Genome Technology Center and T-cell clones sequences were analyzed by IMGT/V-QUEST.

RESULTS

COMPARISON OF T-CELL CLONES SPECIFIC FOR HA-1H ISOLATED FROM HA-1H^{NEG}/HLA-A*02:01^{POS} AND HA-1H^{POS}/HLA-A*02:01^{POS} DONORS

To investigate the effect of thymic selection on shaping of the T-cell repertoire directed against a self-antigen presented in self-HLA, MiHA-specific T-cell clones were used as a model. Because MiHA are derived from polymorphic genes, the genetic background of an individual determines whether the MiHA is considered a non-self (MiHA^{neg} donor) or self-antigen (MiHA^{pos} donor). By investigating in parallel the functional reactivity of MiHA-specific T-cell clones isolated from MiHA^{neg} versus MiHA^{pos} donors, the shaping of the immune repertoire by thymic selection can be investigated.

MiHA HA-1H-specific CD8^{pos} T-cell populations were enriched from PBMC of three HA-1H^{neg}/HLA-A*02:01^{pos} and four HA-1H^{pos}/HLA-A*02:01^{pos} donors using the MHC I-*Strep*tamer technology and cloned by single cell fluorescence-activated cell sorting and expanded (Supplementary Figure 1). Only CD8^{pos} T-cell clones that stained clearly with the HA-1H-tetramer and not with an irrelevant tetramer were included in further analyses. The numbers of unique T-cell clones were estimated by analysis of TRBV gene usage and subsequent sequencing of the TRB-CDR3 region for a selection of T-cell clones. HA-1H-specific T-cell clones were functionally screened by measuring cytokine release after stimulation with TAP-deficient T2 cells exogenously loaded with a titration of HA-1H peptide. Based on this analysis, T-cell clones were classified as high avidity (recognition of $\leq 10^{-10}$M peptide), intermediate avidity (recognition of 10^{-9}M or 10^{-8}M peptide), low avidity (recognition of 10^{-7}M to 10^{-5}M peptide) or not functional (no recognition of 10^{-5}M peptide). As positive controls for high-avidity T-cell clones, 3 previously identified high-avidity CD8^{pos} HA-1H-specific T-cell clones derived from an *in vivo* GVL response were included.[49]

A total of 31 (≥8 unique) and 60 (≥8 unique) HA-1H-specific T-cell clones were generated from HA-1H^{neg}/HLA-A*02:01^{pos} and HA-1H^{pos}/HLA-A*02:01^{pos} donors, respectively (Supplementary Table 1). The functional screening confirmed that the three T-cell clones isolated from *in vivo* GVL responses were indeed of high avidity, and all used the same TRBV7-9*01 gene (Figure 1A). Among the 8 HA-1H T-cell clones isolated from HA-1H^{neg}/HLA-A*02:01^{pos} donors, 2 clones performed similar to the T-cell clones derived from in vivo GVL responses (Figure 1B, red curves) but had different TRB-CDR3 regions, with use of gene TRBV5-1*01 or TRBV5-6*01. The other six T-cell clones all required higher peptide concentrations for profound IFN-γ production: three T-cell clones were classified as intermediate-avidity clones (green curves), whereas the remaining 3 clones were of low avidity (blue curves). Among the HA-1H-specific T-cell clones derived from HA-1H^{pos}/HLA-A*02:01^{pos} donors, only one T-cell clone was found to be of intermediate avidity (Figure 1C, green curve). The other seven T-cell clones exhibited low functional avidity (blue curves) or were not functional (gray curves). As illustrated in Figure 1D, only very subtle differences in intensities of tetramer staining were observed

between high-, intermediate- and low-avidity HA-1H-specific T-cell clones. High-avidity HA-1H-specific T-cell clones displayed overall a slightly higher tetramer staining compared to intermediate- and low-avidity HA-1H-specific T-cell clones. However, the intensity of tetramer staining of high-avidity HA-1H-specific T-cell clones isolated from patient 1 and patient 3 was comparable to several intermediate- and low-avidity HA-1H-specific T-cell clones.

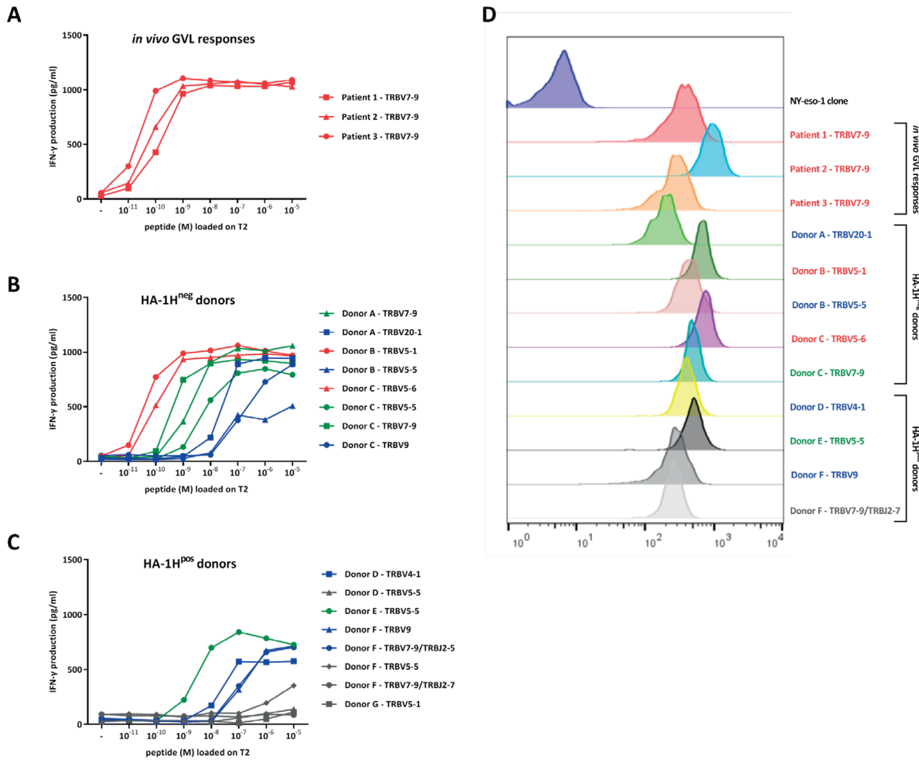

Figure 1. Functional screening and tetramer staining of CD8^{pos} HA-1H-specific T-cell clones. HA-1H-specific T-cell clones were stimulated with HLA-A*02:01^{pos} TAP-deficient T2 cells exogenously loaded with a titration of the HA-1H peptide. IFN-γ production was assessed in a standard ELISA (R:S ratio, 1:6). Clones in red were classified as high avidity, clones in green as intermediate avidity, clones in blue as low avidity and clones in grey as not functional. Results of representative unique clones are depicted. **A**, High-avidity HA-1H-specific T-cell clones isolated from 3 patients during an *in vivo* GVL response after alloSCT. **B**, HA-1H-specific T-cell clones isolated from 3 HA-1H^{neg}/HLA-A*02:01^{pos} healthy donors. **C**, HA-1H-specific T-cell clones isolated from 4 HA-1H^{pos}/HLA*02:01^{pos} healthy donors. **D**, Intensity of HA-1H tetramer staining for a selection of obtained HA-1H-specific T-cell clones. An NY-eso-1-SLL-specific T-cell clone was included as negative control.

These results show that HA-1H-specific T-cell clones showing proper staining with HA-1H-tetramers and using a variety of TRBV genes can be isolated from both HA-1H^{neg}/HLA-A*02:01^{pos} and HA-1H^{pos}/HLA-A*02:01^{pos} donors. As expected, high-avidity HA-1H-specific T-cell clones were only

isolated from HA-1H^{neg}/HLA-A*02:01^{pos} donors, illustrating that the process of thymic selection has eliminated high-avidity T-cell clones in HA-1H^{pos}/HLA-A*02:01^{pos} donors. However, T-cell clones of intermediate avidity are apparently allowed in the T-cell repertoire of both HA-1H^{neg}/HLA-A*02:01^{pos} and HA-1H^{pos}/HLA-A*02:01^{pos} donors. Furthermore, the intensity of tetramer staining was found to be not predictive for the functional reactivity of every HA-1H-specific T-cell clone.

RECOGNITION OF ENDOGENOUSLY PROCESSED ANTIGEN BY HA-1H-SPECIFIC T-CELL CLONES DERIVED FROM HA-1H^{NEG}/HLA-A*02:01^{POS} AND HA-1H^{POS}/HLA-A*02:01^{POS} DONORS

We next investigated whether the functional classification of HA-1H-specific T-cell clones derived from HA-1H^{neg}/HLA-A*02:01^{pos} and HA-1H^{pos}/HLA-A*02:01^{pos} donors, based on their recognition of exogenously loaded HA-1H peptide, was indicative of their capacity to recognize endogenously processed and presented antigen. As positive control for HLA-A*02:01 expression and stimulatory capacity of the target cells, an allo-HLA-A*02:01-restricted T-cell clone was included in all functional tests.

The HA-1H-specific T-cell clones that were classified as low avidity or not functional exhibited no reactivity against HA-1H^{pos}/HLA-A*02:01^{pos} EBV-LCL (data not shown). As expected, the 3 high-avidity T-cell clones derived from *in vivo* GVL responses and the 2 high-avidity T-cell clones derived from HA-1H^{neg}/HLA-A*02:01^{pos} donors clearly specifically recognized the HA-1H^{pos}/HLA-A*02:01^{pos} EBV-LCL, illustrated by high concentrations of IFN- γ production (Figure 2A) and up to 70% of specific target cell lysis (Figure 2B). Of the 3 intermediate-avidity T-cell clones derived from HA-1H^{neg} donors, only T-cell clone ‘Donor A/TRBV7-9’ showed marginal reactivity against one of the HA-1H^{pos}/HLA-A*02:01^{pos} EBV-LCL, whereas the other two T-cell clones were not responsive. The intermediate-avidity T-cell clone derived from an HA-1H^{pos} donor did not exhibit IFN- γ production and only very marginal cytotoxic activity against HA-1H^{pos}/HLA-A*02:01^{pos} EBV-LCL.

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To investigate whether the high- and/or intermediate-avidity HA-1H-specific T-cell clones were able to recognize HA-1H^{pos}/HLA-A*02:01^{pos} malignant cells, these T-cell clones were tested against primary acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) samples containing 65 to 100% blasts. Again, the 3 high-avidity T-cell clones derived from *in vivo* GVL responses and the 2 high-avidity T-cell clones derived from HA-1H^{neg}/HLA-A*02:01^{pos} donors exhibited profound IFN-γ production (Figure 2C) and cytolytic activity (Figure 2D) against HA-1H^{pos}/HLA-A*02:01^{pos} malignant cells. Of the intermediate-avidity T-cell clones derived from HA-1H^{neg}/HLA-A*02:01^{pos} donors, T-cell clones ‘Donor C/TRBV7-9’ and ‘Donor C/TRBV5-5’ surprisingly exhibited minimal/moderate cytolytic activity against the HA-1H^{pos}/HLA-A*02:01^{pos} primary malignant samples with no coinciding IFN-γ production, whereas these T-cell clones were not reactive against the HA-1H^{pos}/HLA-A*02:01^{pos} EBV-LCL. A similar phenomenon was observed for T-cell clone ‘Donor E/TRBV5-5’ derived from an HA-1H^{pos}/HLA-A*02:01^{pos} donor.

These results show that the HA-1H-specific T-cell repertoire of HA-1H^{neg}/HLA-A*02:01^{pos} donors contains high- and intermediate-avidity T-cell clones that are able to recognize endogenously processed and presented peptide. HA-1H-specific clones derived from HA-1H^{pos}/HLA-A*02:01^{pos} donors classified as intermediate avidity showed only limited HA-1H-specific reactivity.

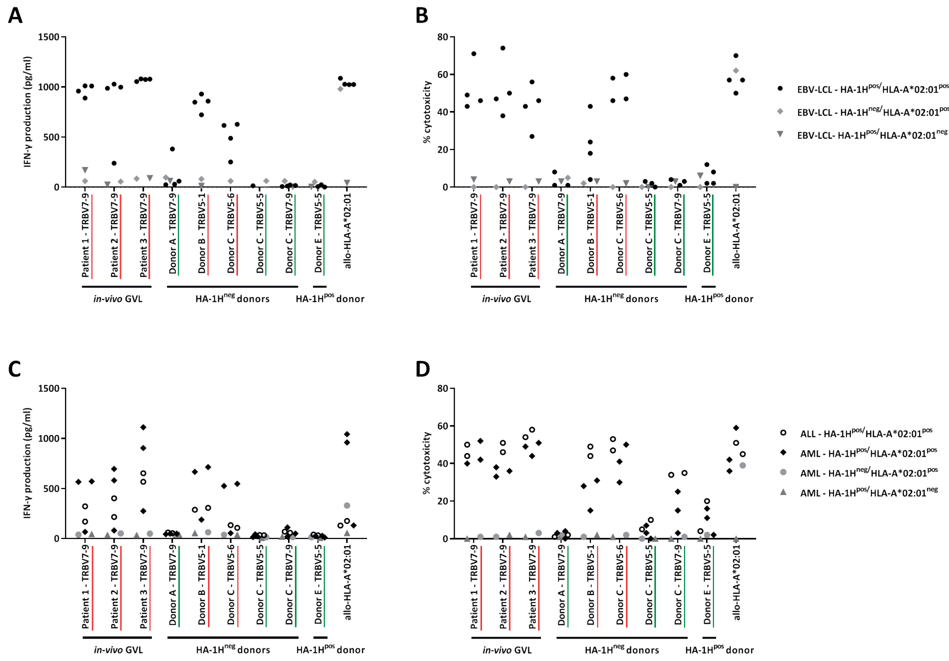

Figure 2. Recognition of endogenously processed HA-1H by high- and intermediate-avidity HA-1H-specific T-cell clones. T-cell clones underlined in red were classified as high avidity and clones underlined in green as intermediate avidity based on the functional screening with exogenously loaded peptide (Figure 1A, 1B and 1C). Results of representative unique clones are depicted. **A**, IFN-γ production by HA-1H-specific T-cell clones after stimulation with an EBV-LCL panel containing 4 HA-1H^{pos}/HLA*02:01^{pos} targets, 1 HA-1H^{pos}/HLA*02:01^{neg} and 1 HA-1H^{neg}/HLA*02:01^{pos} targets (R:S ratio 1:6). A representative example of 3 experiments is shown. **B**, Cytotoxic activity of HA-1H-specific T-cell clones against the same EBV-LCL panel. Cytotoxicity was measured in a standard ^{51}chromium-release assay (E:T ratio, 10:1). Symbols represent median percentages of lysis of triplicates. **C**, IFN-γ production by HA-1H-specific T-cell clones after stimulation with a panel of primary malignant samples, containing 2 HA-1H^{pos} /HLA*02:01^{pos} ALL samples, 3 HA-1H^{pos} /HLA*02:01^{pos} AML samples, 1 HA-1-H^{pos}/HLA*02:01^{neg} AML sample and 1 HA-1H^{neg}/HLA*02:01^{pos} AML sample (R:S ratio, 1:6). **D**, Cytotoxic activity of HA-1H-specific T-cell clones against the same panel of primary malignant samples. Cytotoxicity was measured in a standard ^{51}chromium-release assay (E:T ratio, 10:1). Symbols represent median percentages of lysis of triplicates.

THE FUNCTIONAL REACTIVITY OF SELF-HLA-RESTRICTED TAA-SPECIFIC T-CELL CLONES RESEMBLED THE FUNCTIONAL REACTIVITY OF HA-1H-SPECIFIC T-CELL CLONES DERIVED FROM HA-1H^{POS}/HLA-A*02:01^{POS} DONORS

Because TAA are self-antigens, the shaping of the TAA-specific T-cell repertoire in HLA-A*02:01^{pos} healthy donors by thymic selection is expected to resemble the selection seen for the HA-1H-specific T-cell repertoire in HA-1H^{pos}/HLA-A*02:01^{pos} donors. To investigate the repertoire of T cells recognizing TAA in the context of self-HLA in healthy donors, CD8^{pos} T-cell clones directed against the HLA-A*02:01-restricted peptides of WT1-RMF, RHAMM-ILS, proteinase-3-VLQ, PRAME-VLD and NY-eso-1-SLL were isolated from PBMC of HLA-A*02:01^{pos} donors (Table 1). All CD8^{pos}/tetramer^{pos} T-cell clones were functionally screened for antigen-specific reactivity measured by cytokine release after stimulation with T2 cells exogenously loaded with a titration of the respective peptide; they were accordingly classified as high avidity, intermediate avidity, low avidity or not functional. Their TRBV gene usage was assessed using flow cytometry. T-cell clones with the same antigen specificity, derived from the same donor, using the same TRBV gene and performing equally in the functional screening, were categorized as identical T-cell clones. T-cell clones from the same donor using the same TRBV gene but displaying variable reactivity in the functional screenings were sequenced for their TRB-CDR3 region to identify different unique T-cell clones.

A total of 663 TAA-specific T-cell clones were isolated from 18 donors, among which 301 WT1-RMF-specific T-cell clones from 17 donors, 129 RHAMM-ILS-specific T-cell clones from 9 donors, 36 proteinase-3-VLQ-specific T-cell clones from 4 donors, 114 PRAME-VLD-specific T-cell clones from 9 donors and 83 NY-eso-1-SLL-specific T-cell clones from 6 donors. All CD8^{pos} TAA-specific T-cell clones stained only with the relevant tetramers and not with irrelevant HLA-A*02:01-restricted tetramers (representative examples in Supplementary Figure 2A). Within the 663 T-cell clones, a minimum of 91 unique T-cell clones were identified, including 33 WT1-RMF, 17 RHAMM-ILS, 7 proteinase-3-VLQ, 16 PRAME-VLD and 18 NY-eso-1-SLL-specific T-cell clones (Figure 3A; Supplementary Tables 2-6, respectively). The numbers of isolated unique TAA-specific T-cell clones per specificity did not correlate with the strength of the predicted binding affinity of the peptide to the HLA-A*02:01 molecule (Table 1). The functional screening revealed that no high-avidity T-cell clones were isolated from any of the 18 donors for any of the five TAA specificities. Sixty unique T-cell clones (66%) were shown to be not functional (Figure 3B), 17 unique T-cell clones (19%) were classified as low avidity and 1 WT1-RMF clone (1%) displayed profound reactivity against T2 cells without loading of WT1-RMF peptide, indicating cross-reactivity. However, 13 unique T-cell clones (14%) were categorized as intermediate avidity, including 5 WT1-RMF, 7 PRAME-VLD, and 1 NY-eso-1-SLL T-cell clones (Figure 3C). These T-cell clones showed no reactivity against irrelevant HLA-A*02:01-restricted peptides (Supplementary Figure 2B). The intensity of tetramer staining did not correlate with the functional avidity of the TAA-specific T-cell clones (data not shown).

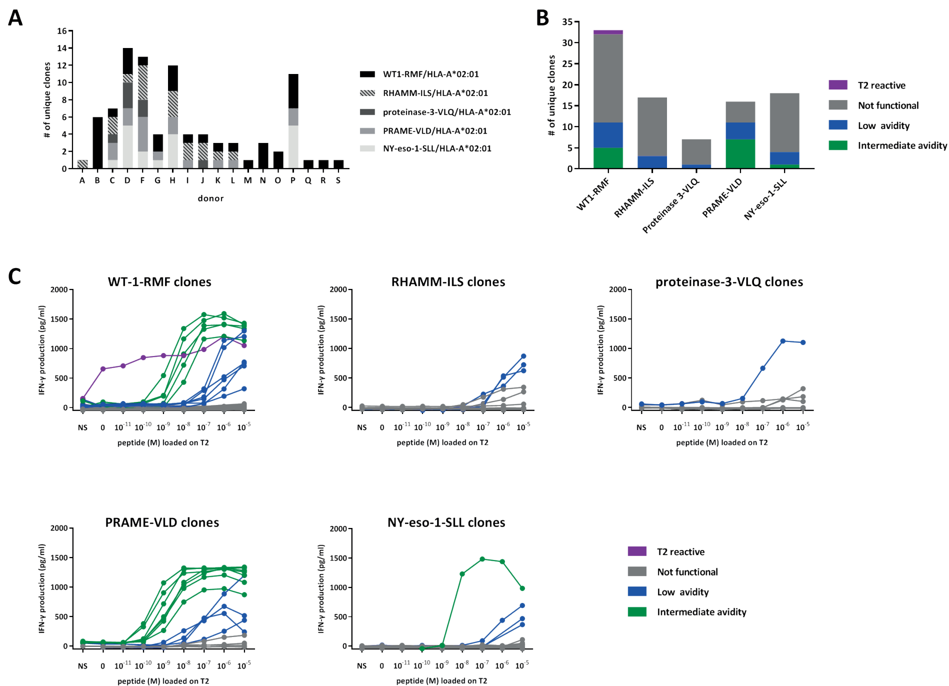

Figure 3. Functional screening of CD8^{pos} TAA-specific T-cell clones derived from HLA-A*02:01^{pos} donors. **A**, Minimal numbers of unique T-cell clones per TAA-specificity isolated from 18 donors. **B**, Summary of classification of unique TAA-specific T-cell clones based on the IFN-γ production after overnight stimulation with TAP-deficient T2 cells exogenously loaded with a titration of the respective peptide (R:S ratio, 1:6). **C**, Results of this functional screening per TAA specificity. Clones depicted in green were classified as intermediate avidity, clones depicted in blue as low avidity, clones depicted in gray as not functional, and clones depicted in purple as T2-reactive T-cell clones. Representative examples of unique clones are depicted. NS, not stimulated.

These results show that the majority of self-HLA-restricted TAA-specific T-cell clones in the repertoire of healthy donors were not functional or of low avidity and that only 13 of 91 unique T-cell clones were of intermediate avidity. These findings are in line with our observations for HA-1H-specific T-cell clones derived from HA-1H^{pos}/HLA-A*02:01^{pos} donors.

A MINORITY OF INTERMEDIATE-AVIDITY TAA-SPECIFIC T-CELL CLONES IS ABLE TO RECOGNIZE CELL LINES NATURALLY OVEREXPRESSING TAA

In contrast to MiHA, TAA are expected to be overexpressed in malignant cells compared to healthy cells. This TAA-overexpression might influence the threshold for TAA-specific T-cell activation and recognition of endogenously processed antigen. We therefore investigated whether WT1-RMF, PRAME-VLD and NY-eso-1-SLL-specific T-cell clones of intermediate avidity were able to recognize endogenously processed antigen. Two HLA-A*02:01^{pos} EBV-LCL were transduced with a retroviral

vector encoding the full protein sequence of WT1, PRAME or NY-eso-1 to induce overexpression of the respective TAA. Furthermore, tumor cell lines known for their natural TAA overexpression and several primary ALL and AML samples were included. Relative TAA expression in these targets compared to household genes was quantified by RT-qPCR. Both cytokine production and cytotoxic capacity of T-cell clones was assessed.

Of the 5 intermediate-avidity WT1-RMF-specific T-cell clones, three T-cell clones ('Donor G/TRBV11-1', 'Donor M/TRBV7-9' and 'Donor R/TRBV28') produced high levels of IFN-γ and one T-cell clone ('Donor B/TRBV28') produced low levels of IFN-γ upon stimulation with EBV-LCL transduced with WT1 and not EBV-LCL transduced with the mock vector; stimulation with peptide-loaded stimulator cells, however, provoked profound IFN-γ production (Figure 4A). Different malignant cell lines and primary malignant samples with limited relative WT1 expression, did not provoke significant IFN-γ production (Figure 5A). A similar reactivity pattern was seen when the cytotoxic activity of WT1-specific T-cell clones was assessed, showing limited lysis of EBV-LCL transduced with WT1 and profound lysis of peptide-loaded target cells (Figure 4B).

Three of the 7 PRAME-VLD-specific T-cell clones of intermediate avidity ('Donor H/TRBV20-1', 'Donor K/TRBV20-1' and 'Donor L/TRBV20-1') demonstrated moderate IFN-γ production after stimulation with the EBV-LCL transduced with PRAME and high IFN-γ production upon stimulation with PRAME-overexpressing malignant cell line K562 or peptide-loaded stimulator cells (Figure 5B and Figure 6A). PRAME-VLD-specific T-cell clones showed only limited cytotoxic activity against the cell-line targets, whereas none of the tested primary AML and ALL samples were recognized (Figure 6B), despite moderate PRAME expression. Peptide-loaded target cells were properly killed.

The single intermediate-avidity NY-eso-1-SLL-specific T-cell clone 'Donor D/TRBV9' clearly recognized EBV-LCL transduced with NY-eso-1 and multiple myeloma (MM) cell lines OPM-2 and U266 transduced HLA-A*02:01 that endogenously overexpressed NY-eso-1 (Figure 5C and Figure 7A), but not the primary MM sample which showed NY-eso-1 expression comparable to the EBV-LCL transduced with NY-eso-1. Cytolytic capacity of the NY-eso-1-SLL-specific T-cell clone was illustrated by profound lysis of NY-eso-1^{pos}/HLA-A*02:01^{pos} cell lines to an extent comparable to that of the allo-HLA-A*02:01-specific T-cell clone, even at low effector-to-target ratios (Figure 7B). However, no lysis of the primary MM sample could be shown despite proper recognition by the allo-HLA-A*02:01-specific T-cell clone.

These results show that of the 13 intermediate-avidity TAA-specific T-cell clones, 3 PRAME-VLD-specific T-cell clones and 1 NY-eso-1-SLL-specific T-cell clone exhibited IFN-γ production upon stimulation with malignant cell lines naturally overexpressing the corresponding TAA. Only for the NY-eso-1-SLL-specific T-cell clone was clear cytotoxic activity against malignant cell lines shown.

However, primary malignant samples displaying proper TAA gene expression were not recognized by any of the intermediate-avidity TAA-specific T-cell clones.

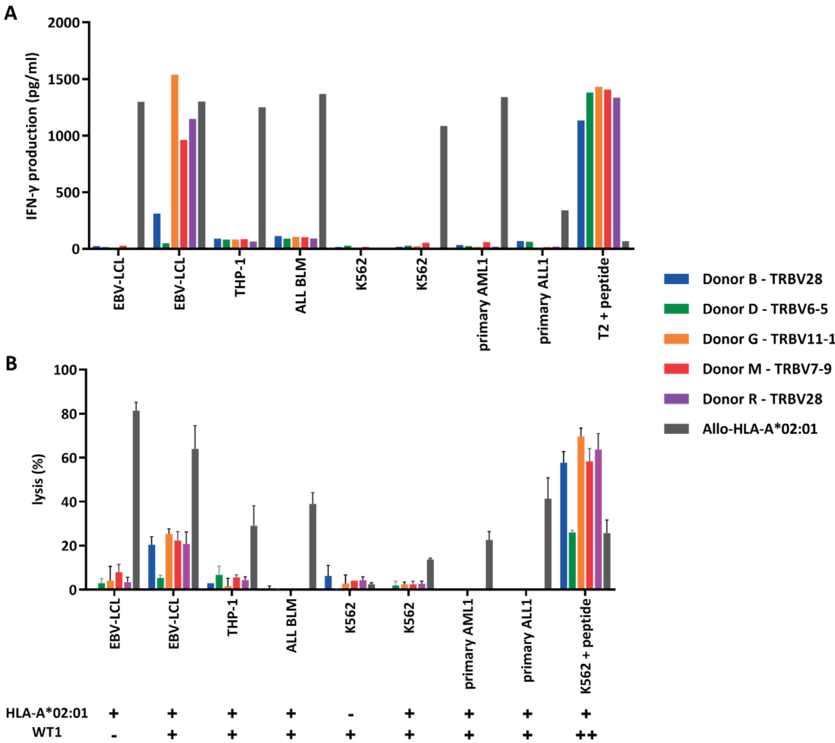

Figure 4. Recognition of endogenously processed WT1 by intermediate-avidity WT1-RMF-specific T-cell clones. The 5 WT1-RMF-specific T-cell clones of intermediate avidity were tested against EBV-LCL transduced with the empty vector (mock) or with the vector encoding the full protein sequence of WT1, AML cell line THP-1, ALL cell line BLM, chronic myeloid leukemia cell line K562 wild-type and transduced with HLA-A*02:01, 5 primary AML samples, and 3 primary ALL samples (2 representative primary samples with relatively high WT1 expression are depicted). An allo-HLA-A*02:01-reactive T-cell clone was included as positive control for HLA-A*02:01 expression and costimulatory capacity of the target cells. T2 or K562 transduced with HLA-A*02:01 and loaded with peptide were included as positive control for functional reactivity of clones. The HLA-A*02:01 and TAA expression of the target cells is indicated as negative (-) or positive (+) below the x-axis. **A**, IFN-γ production was measured by ELISA after overnight stimulation (R:S ratio, 1:6). Representative example of 3 experiments is shown. **B**, Cytotoxic capacity was measured in a standard ^{51}chromium-release assay (E:T ratio, 10:1). Mean of triplicates with standard deviation is depicted.

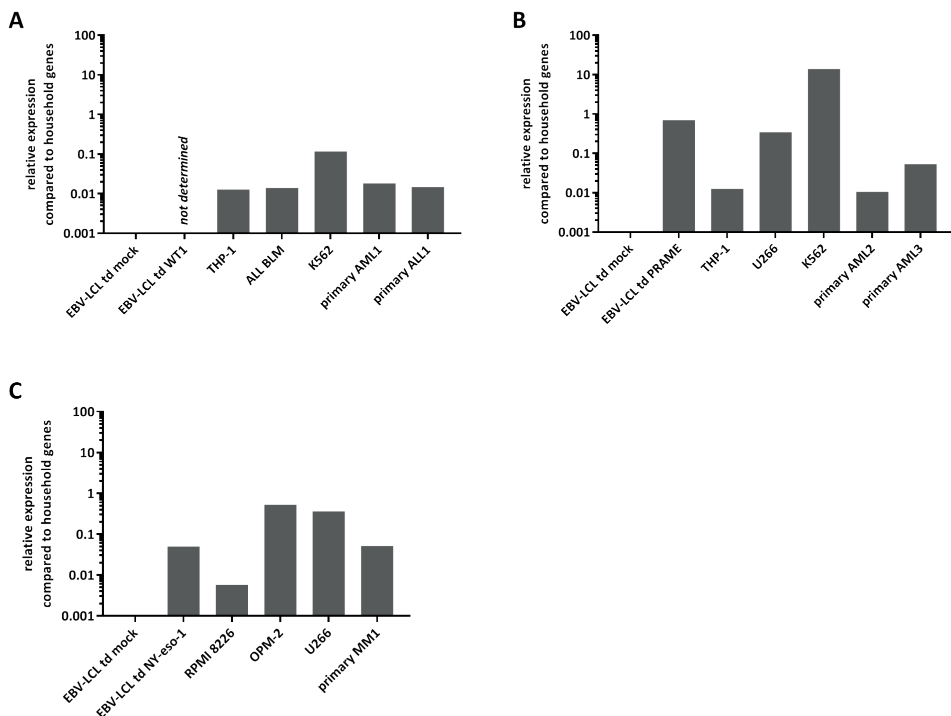

Figure 5. Relative expression of TAA in different target cell lines and primary samples measured by RT-qPCR. The expression of the indicated TAA is calculated relative to expression of two household genes (ACTB and GAPDH). A relative expression of 1 means equal expression compared to the average expression of these household genes. **A**, The expression of WT1 could not be determined in EBV-LCL transduced with the retroviral vector encoding the codon optimized protein sequence of WT1, as a suitable primer was not available. Expression of WT1 in EBV-LCL transduced mock, THP-1, ALL BLM and K562 cell line and a representative primary AML and ALL sample are depicted. **B**, Expression of PRAME in EBV-LCL transduced mock and PRAME, THP-1, U266, K562 cell line and 2 representative primary AML samples are depicted. **C**, Expression of NY-eso-1 in EBV-LCL transduced mock and NY-eso-1, MM cell lines RPMI 8226, OPM-2 and U266, and 1 representative primary MM sample are depicted.

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Figure 6. Functional reactivity of intermediate-avidity PRAME-VLD-specific T-cell clones against targets endogenously overexpressing PRAME. The 7 PRAME-VLD-specific T-cell clones of intermediate avidity were tested against EBV-LCL transduced with the empty vector (mock) or with the vector encoding the full protein sequence of PRAME, MM cell line U266, AML cell line THP-1, chronic myeloid leukemia cell line K562 wildtype or transduced with HLA-A*02:01, 6 primary AML samples and, 2 primary ALL samples (2 representative primary samples with relatively high WT1 expression are shown). An allo-HLA-A*02:01-reactive T-cell clone was included as positive control for HLA-A*02:01 expression and costimulatory capacity of the target cells. T2 or K562 transduced with HLA-A*02:01 and loaded with peptide were included as positive control for functional reactivity of clones. The HLA-A*02:01 and TAA expression of the targets is indicated as negative (-) or positive (+) below the x-axis. **A**, IFN-γ production was measured by ELISA after overnight stimulation (R:S ratio, 1:6). Representative example of 3 experiments is shown. **B**, Cytotoxic capacity was measured in a standard ^{51}chromium-release assay (E:T ratio, 10:1). Mean of triplicates with standard deviation is depicted.

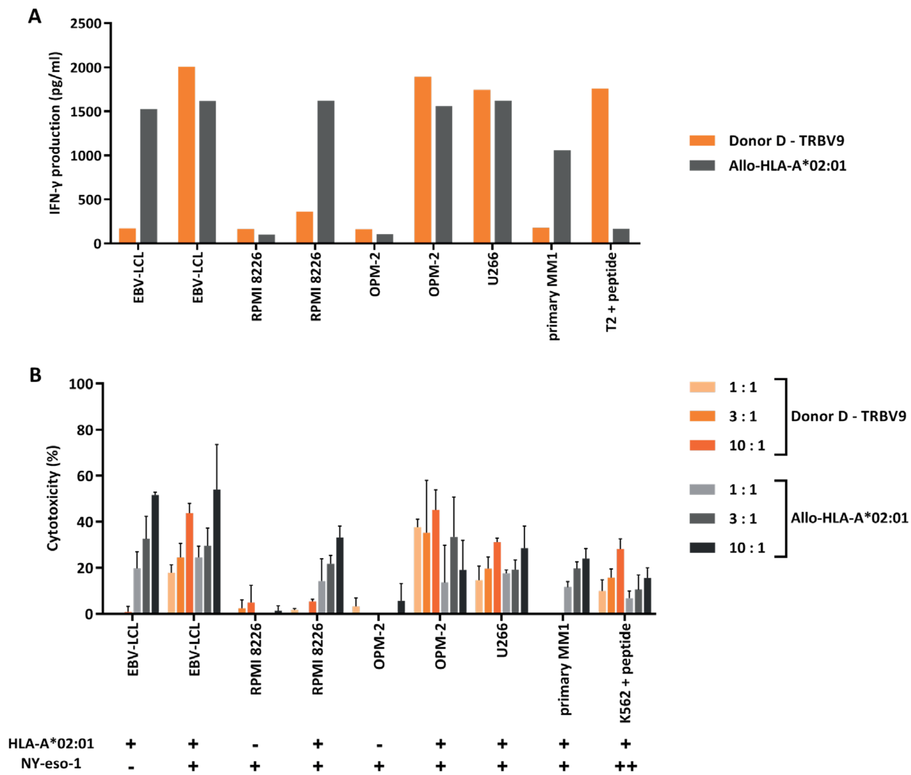

Figure 7. Functional reactivity of the NY-eso-1-SLL-specific T-cell clone of intermediate avidity against cell lines endogenously overexpressing NY-eso-1. NY-eso-1-SLL clone 'Donor D-TRBV9' was tested against EBV-LCL transduced with the empty vector (mock) or with the vector encoding the full protein sequence of NY-eso-1, MM cell lines RPMI 8226 transduced with the empty vector (mock) or with HLA-A*02:01, MM cell line OPM-2 transduced with the empty vector (mock) or with HLA-A*02:01, MM cell line U266 and a primary MM sample. An allo-HLA-A*02:01-reactive T-cell clone was included as positive control for HLA-A*02:01 expression and costimulatory capacity of the targets. T2 or K562 transduced with HLA-A*02:01 and loaded with peptide were included as positive control. The HLA-A*02:01 and TAA expression of the targets is indicated as negative (-) or positive (+) below the x-axis. **A**, IFN-γ production was measured by ELISA after overnight stimulation (R:S ratio, 1:6). Representative example of 3 experiments is shown. **B**, Cytotoxic capacity was measured in a standard ^{51}chromium-release assay at different E:T ratios. Mean of triplicates with standard deviation is depicted.

DISCUSSION

The current study investigated whether significant antitumor responses by self-HLA-restricted TAA-specific T cells isolated from the autologous or HLA-matched T-cell repertoire are likely to be induced in the setting of immunotherapeutic approaches. We described the role of thymic selection on shaping of the T-cell repertoire directed against self-antigens presented in self-HLA using HA-1H-specific T-cell clones isolated from HA-1H^{neg}/HLA-A*02:01^{pos} versus HA-1H^{pos}/HLA-A*02:01^{pos} donors as a model. Furthermore, HLA-A*02:01-restricted TAA-specific T cells were isolated from the T-cell repertoire of HLA-A*02:01^{pos} healthy donors, and it was shown that only a small minority of isolated TAA-specific T-cell clones were able to recognize malignant cell lines expressing endogenously processed TAA but not primary malignant samples. These results illustrate that the induction of a profound antitumor response by T cells recognizing TAA presented in self-HLA is possible, although very exceptional.

We compared the functionality of MiHA HA-1H-specific T-cell clones isolated from HA-1H^{neg}/HLA-A*02:01^{pos} versus HA-1H^{pos}/HLA-A*02:01^{pos} donors. Overall, the capacity of HA-1H-specific T-cell clones to recognize stimulator cells exogenously loaded with low concentrations of the peptide appeared indicative for their reactivity against stimulator cells presenting endogenously processed antigen. As expected, high-avidity HA-1H-specific T-cell clones that recognized HA-1H^{pos}/HLA-A*02:01^{pos} primary malignant samples to the same extent as HA-1H-specific T-cell clones derived from an *in vivo* immune response were only obtained from HA-1H^{neg}/HLA-A*02:01^{pos} donors and not from HA-1H^{pos}/HLA-A*02:01^{pos} donors. However, one HA-1H-specific T-cell clone isolated from a HA-1H^{pos}/HLA-A*02:01^{pos} donor showed limited cytolytic activity against HA-1H^{pos}/HLA-A*02:01^{pos} targets, indicating that self-HLA-restricted T-cell clones with an intermediate avidity that are able to recognize endogenously processed self-antigens can pass thymic selection and become part of the T-cell repertoire. Interestingly, TCR sequencing revealed that the TCR of the two high-avidity HA-1H-specific T-cell clones isolated from HA-1H^{neg}/HLA-A*02:01^{pos} donors used the TRBV5-1*01 and TRBV5-6*01 gene, respectively. Previous studies reported that both *ex vivo* generated and *in vivo* selected HA-1H-specific T-cells preferentially use the TRBV7-9 gene for their TCR configuration, in combination with a variety of TRBJ genes.[44,59-63]

The similar model was used to test 663 T-cell clones (containing at least 91 unique T-cell clones expressing different TCR) directed against HLA*A*02:01-restricted TAA WT1-RMF, RHAMM-ILS, proteinase-3-VLQ, PRAME-VLD, and NY-eso-1-SLL isolated from several HLA-A*02:01^{pos} healthy donors. In agreement with our findings regarding HA-1H-specific T-cell clones derived from HA-1H^{pos}/HLA-A*02:01^{pos} donors, we found no self-HLA-restricted TAA-specific T-cell clones classified as high avidity based on recognition of stimulator cells exogenously loaded with the relevant peptide. This observation confirmed the role of negative thymic selection for TAA-specific

T-cells restricted to self-HLA. We identified many different low-avidity or nonfunctional HA-1H- and TAA-specific T-cell clones that clearly stained with the corresponding tetramer. In line with previous research, the intensity of tetramer staining did not completely correlate with functional reactivity.[29] These observations might explain the discrepancy between the *in vivo* appearance of tetramer^{pos} TAA-specific T cells seen in TAA peptide vaccination studies and the lack of convincing coinciding antitumor reactivity in the majority of studied patients.[16,23,64] Our findings show that identifying tetramer^{pos} TAA-specific T-cell responses *in vivo* does not prove antitumor effect but may be the result of expansion of clonotypes with relatively low functional avidity without the ability to induce clinically relevant immune reactions.

The extensive pool of isolated TAA-specific T-cell clones contained only a limited number of intermediate-avidity T-cell clones. These T-cell clones were specific for WT1-RMF, PRAME-VLD, or NY-eso-1-SLL, whereas for proteinase-3-VLQ- and RHAMM-ILS only low-avidity T-cell clones were identified in this study. An explanation for this discrepancy can be found in the overall lower numbers of isolated proteinase-3-VLQ- and RHAMM-ILS-specific T-cell clones compared to WT-1-RMF-, PRAME-VLD-, and NY-eso-1-SLL-specific T-cell clones, reducing the chance of catching an intermediate-avidity T-cell clone. The numbers of isolated T-cell clones per TAA specificity did not correlate with the predicted binding affinities of the TAA peptides for HLA-A*02:01, as WT1-RMF and proteinase-3-VLQ are predicted strong binders, whereas RHAMM-ILS, PRAME-VLD and NY-eso-1-SLL are predicted weak binders. Therefore, the capacity of the specific TAA-peptides to compete for presentation in HLA-A*02:01 was not predictive for the numbers of isolated T-cell clones for the different specificities. Differences in expression levels between TAA in medullary thymic epithelial cells can affect the threshold for negative thymic selection of TAA-specific T cells, thereby influencing the avidity of T cells present in the T-cell repertoire.[65] For TAA-specific T cells that pass thymic selection, we hypothesized that TAA-overexpressing targets can positively influence their activation threshold, resulting in activation of not only high-avidity but also intermediate-avidity T cells, while preserving tolerance against healthy cells. Although all 13 intermediate-avidity T-cell clones were comparable in the functional tests against peptide-loaded target cells, only 3 PRAME-VLD- and 1 NY-eso-1-SLL-specific T-cell clone exhibited reactivity against malignant cell lines. An explanation may be found in the extent of relative TAA overexpression in the tested malignant cell lines. The TAA expression on the RNA level of PRAME in K562 and NY-eso-1 in U266 was found to be higher than the expression of the household genes.[10,28,66] In contrast, the relative expression of WT1 in K562 was only 0.12-fold the expression of the household genes and even lower in the tested primary malignant samples, as also described previously.[36] In line with this, the intermediate-avidity PRAME-VLD-specific T-cell clones were not able to recognize primary malignant leukemic samples that exhibited much lower RNA expression levels of PRAME than K562 cells. However, the NY-eso-1-SLL T-cell clone of intermediate avidity was not able to recognize the tested MM sample, although the NY-eso-1 expression on the RNA level of these

primary malignant cells were comparable to the expression in the EBV-LCL transduced with NY-eso-1, which were clearly recognized. These observations suggest that in addition to relatively high TAA expression on the RNA level of malignant cells, additional factors (eg, processing and peptide presentation) most likely dictate proper recognition of primary malignant cells. The gene expression on the RNA level might not always correlate to the actual protein expression on the cell surface, as intracellular translation and protein degradation are not taken into account.[67] In addition, because malignant cells have been shown to be a heterogenous population of cells with distinct gene expression profiles, TAA expression on the RNA level might differ among malignant cells.[66,68] This might result in recognition or killing of only a selection of malignant cells with high TAA expression.

Clinically relevant immune responses targeting cancer-germline antigens such as PRAME and NY-eso-1 have been reported in patients suffering from cancer-germline-expressing hematologic cancers.[69] Our data show that the induction of functional TAA-specific T-cell responses from the unmanipulated autologous or HLA-matched T-cell repertoire might be possible but is difficult due to the low frequencies of TAA-specific T-cells that have the potential to recognize naturally overexpressed antigen in malignant cells. Therefore, a very profound immune response has to be induced to enhance the chance of a functionally significant and clinically relevant TAA-specific T-cell response. The addition of highly effective adjuvants to peptide vaccination strategies targeting TAA may increase the efficiency of priming of TAA-specific T cells of sufficient functional avidity to recognize endogenously processed antigens presented on malignant cells. *In vitro* isolation and expansion of high-avidity TAA-specific T cells may be another strategy to utilize the potential of these TAA-specific T cells that seem to be present only at very low frequencies. Although these approaches have shown to be safe and feasible in phase 1/2 studies, convincing evidence regarding the induction of high-avidity epitope-specific T-cell responses after TAA-specific peptide vaccination or adoptive transfer of TAA-specific T-cells in patients with hematologic malignancies has not been reported thus far; strategies may require further improvement, however.[23,64,70-73] Multiple recently initiated trials focusing on TAA are expected to provide additional insight regarding the potential of autologous TAA-specific T cells for the induction of clinically relevant antitumor responses in patients with hematologic malignancies.[74-78]

In conclusion, we have shown that self-HLA-restricted TAA-specific T cells can be easily isolated from peripheral blood of healthy individuals and that only exceptional T-cell clones are capable of recognizing naturally overexpressed antigen in malignant cell lines but not primary malignant cells. Therefore, classification of functional TAA-specific T cells by only high tetramer staining and peptide specificity leads to the overestimation of relevant avidity of these T cells.

ACKNOWLEDGEMENTS

The authors thank the employees of the Laboratory for Specialized Hematology and the Flowcytometry Core Facility, Kees van Bergen and Renate Hagedoorn (Leiden University Medical Center, Leiden, The Netherlands) for providing expert technical assistance. This study was financially supported by the European Union's seventh Framework Program (FP/2007-2013) grant 601722 and by the Dutch Cancer Society grant UL 2008-4263.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. HA-1H clones isolated from HA-1H^{neg}/HLA-A*02:01^{pos} and HA-1H^{pos}/HLA-A*02:01^{pos} healthy donors. Three HA-1H clones that were *in vivo* selected based on their ability to induce GVL after HLA-matched alloSCT and DLI were included in all functional assays as positive controls. Per row, clones are depicted that used the same TRBV-gene (determined using the IOTest Beta Mark Kit for flow cytometry) and performed equally in the functional screening; these clones were considered as identical clones. For clones that were additionally sequenced for their TRB-CDR3 region, the joining gene usage (TRBJ) is indicated with the number of sequenced clones between brackets.

	Donor	Number of clones	TRBV gene usage	TRBJ gene usage (number of clones sequenced)	Classification based on functional screening
HA-1H^{neg} donors	Donor A	1	TRBV20-01*02	TRBJ2-1*01 (1)	Low avidity
		12	TRBV7-9*01	TRBJ1-2*01 (3)	Intermediate avidity
	Donor B	1	TRBV5-5*01	TRBJ1-1*01 (1)	Low avidity
		5	TRBV5-1*01	TRBJ1-2*01 (5)	High avidity
	Donor C	1	TRBV9*01	TRBJ2-5*01 (1)	Low avidity
		4	TRBV5-6*01	TRBJ2-2*01 (4)	High avidity
		4	TRBV5-5*02	TRBJ2-2*01 (4)	Intermediate avidity
		3	TRBV7-9*01	TRBJ2-6*01 (3)	Intermediate avidity
HA-1H^{pos} donors	Donor D	2	TRBV5-5*02	TRBJ1-3*01 (2)	Not functional
		22	TRBV4-1*01	TRBJ1-5*01 (1)	Low avidity
	Donor E	5	TRBV5-5*01	TRBJ1-5*01 (2)	Intermediate avidity
	Donor F	2	TRBV9	-	Low avidity
		2	TRBV5-5*02	TRBJ1-1*01 (1)	Not functional
		11	TRBV7-9*03	TRBJ2-7*01 (6)	Not functional
		1	TRBV7-9*03	TRBJ2-5*01 (1)	Low avidity
	Donor G	15	TRBV5-1	-	Not functional
Positive controls: Patient HA-1H^{pos} Donor HA-1H^{neg}	Patient 1	1	TRBV7-9*01	TRBJ2-1*01 (1)	High avidity
	Patient 2	1	TRBV7-9*01	TRBJ2-1*01 (1)	High avidity
	Patient 3	1	TRBV7-9*01	TRBJ2-1*01 (1)	High avidity

Supplementary Table 2. WT1-RMF-specific T-cell clones derived from HLA-A*02:01pos healthy donors. Per row, T-cell clones are depicted that used the same TRBV gene and performed equally in the functional screening; these clones were considered as identical clones. TRBV-gene usage for all 301 obtained WT-1-RMF-specific T-cell clones was determined using the IOTest Beta Mark Kit for flow cytometry. For clones that were additionally sequenced for their TRB-CDR3 region, the joining gene usage (TRBJ) is indicated with the number of sequenced clones between brackets.

	Number of clones	TRBV gene usage	TRBJ gene usage (number of clones sequenced)	Classification based on functional screening
Donor B	3	TRBV28*01	TRBJ1-4*01 (2)	Intermediate avidity
	9	TRBV10-3*02	TRBJ2-7*01 (1)	Low avidity
	4	TRBV6-6	-	Not functional
	3	TRBV18*01	TRBJ2-5*01 (2)	T2 reactive
	1	TRBV11-2	-	Low avidity
	16	TRBV6-1*01	TRBJ2-7*01 (1)	Not functional
Donor C	4	TRBV?	-	Not functional
Donor D	1	TRBV5-1	-	Low avidity
	13	TRBV6-5*01	-	Intermediate avidity
	3	TRBV7-2*01	TRBJ2-3*01 (1)	Low avidity
Donor F	1	TRBV?	-	Not functional
Donor G	3	TRBV10-2*01	TRBJ2-1*01 (3)	Not functional
	14	TRBV11-1*01	TRBJ1-1*01 (4)	Intermediate avidity
Donor H	4	TRBV28	-	Not functional
	3	TRBV6	-	Not functional
	2	TRBV?	-	Not functional
Donor I	8	TRBV6	-	Not functional
Donor J	4	TRBV?	-	Not functional
Donor K	27	TRBV4-1*01	TRBJ2-5*01 (1)	Low avidity
Donor L	16	TRBV28*01	TRBJ2-7*01 (1)	Low avidity
Donor M	55	TRBV7-9*03	TRBJ2-5*01 (3)	Intermediate avidity
Donor N	1	TRBV9	-	Not functional
	1	TRBV2	-	Not functional
	14	TRBV?	-	Not functional
Donor O	1	TRBV27	-	Not functional
	9	TRBV?	-	Not functional
Donor P	2	TRBV20-1	-	Not functional
	1	TRBV12	-	Not functional
	8	TRBV27	-	Not functional
	4	TRBV19	-	Not functional
Donor Q	45	TRBV16*01	TRBJ2-1*01 (2)	Not functional
Donor R	20	TRBV28*01	TRBJ2-7*01 (1)	Intermediate avidity
Donor S	1	TRBV28	-	Not functional

Supplementary Table 3. RHAMM-ILS-specific T-cell clones derived from HLA-A*02:01^{pos} healthy donors. Per row, T-cell clones are depicted that used the same TRBV gene and performed equally in the functional screening; these clones were considered as identical clones. TRBV-gene usage for all 129 obtained RHAMM-ILS-specific T-cell clones was determined using the IOTest Beta Mark Kit for flow cytometry.

	Number of clones	TRBV gene usage	TRBJ gene usage (number of clones sequenced)	Classification based on functional screening
Donor A	16	TRBV?	-	Low avidity
Donor C	4	TRBV5-6	-	Not functional
	1	TRBV?	-	Not functional
Donor D	10	TRBV?	-	Not functional
Donor F	1	TRBV3-1	-	Not functional
	3	TRBV6	-	Not functional
	6	TRBV30	-	Not functional
	1	TRBV?	-	Not functional
Donor H	2	TRBV4	-	Not functional
	9	TRBV6	-	Low avidity
	23	TRBV?	-	Not functional
Donor I	1	TRBV12	-	Not functional
	1	TRBV?	-	Not functional
Donor J	9	TRBV30	-	Low avidity
	2	TRBV?	-	Not functional
Donor K	21	TRBV?	-	Not functional
Donor L	19	TRBV?	-	Not functional

Supplementary Table 4. Proteinase-3-VLQ-specific T-cell clones derived from HLA-A*02:01^{pos} healthy donors. Per row, T-cell clones are depicted that used the same TRBV gene and performed equally in the functional screening; these clones were considered as identical clones. TRBV-gene usage for all 36 obtained proteinase-3-VLQ-specific T-cell clones was determined using the IOTest Beta Mark Kit for flow cytometry. For clones that were additionally sequenced for their TRB-CDR3 region, the joining gene usage (TRBJ) is indicated with the number of sequenced clones between brackets.

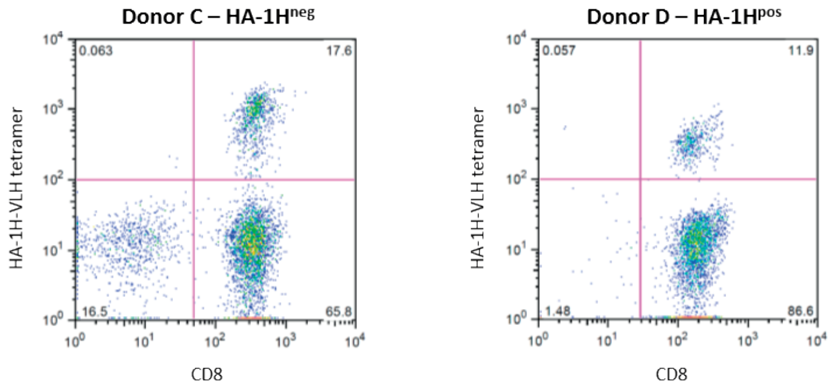
	Number of clones	TRBV gene usage	TRBJ gene usage (number of clones sequenced)	Classification based on functional screening
Donor C	1	TRBV13	-	Not functional
Donor D	6	TRBV11-2*01	TRBJ1-3*01 (4)	Low avidity
	1	TRBV11-2*01	TRBJ2-7*01 (1)	Not functional
	8	TRBV?	-	Not functional
Donor F	4	TRBV19	-	Not functional
	1	TRBV?	-	Not functional
Donor J	15	TRBV?	-	Not functional

Supplementary Table 5. PRAME-VLD-specific T-cell clones derived from HLA-A*02:01^{pos} healthy donors. Per row, T-cell clones are depicted that used the same TRBV gene and performed equally in the functional screening; these clones were considered as identical clones. TRBV-gene usage for all 114 obtained PRAME-VLD-specific T-cell clones was determined using the IOTest Beta Mark Kit for flow cytometry. For clones that were additionally sequenced for their TRB-CDR3 region, the joining gene usage (TRBJ) is indicated with the number of sequenced clones between brackets.

	Number of clones	TRBV gene usage	TRBJ gene usage (number of clones sequenced)	Classification based on functional screening
Donor C	4	TRBV9*01	TRBJ1-1*01 (1)	Not functional
	3	TRBV29-1	-	Low avidity
Donor D	1	TRBV?	-	Low avidity
	10	TRBV7-9*03	TRBJ1-4*01 (2)	Not functional
Donor F	3	TRBV9*01	TRBJ2-1*01 (1)	Intermediate avidity
	1	TRVB20-1*05	TRVJ2-7*01 (1)	Low avidity
	5	TRVB20-1*02	TRVJ2-2*01 (1)	Intermediate avidity
	1	TRBV4-3	-	Not functional
Donor G	14	TRBV9*01	TRBJ1-1*01 (6)	Intermediate avidity
Donor H	12	TRBV9*01	TRBJ2-3*01 (2)	Not functional
	6	TRBV20-1*05	TRBJ2-4*01 (2)	Intermediate avidity
Donor I	15	TRBV 10-3	-	Not functional
Donor K	16	TRBV20-1*02	TRBJ2-2*01 (3)	Intermediate avidity
Donor L	14	TRBV20-1*02	TRBJ2-2*01 (2)	Intermediate avidity
Donor P	6	TRBV20-1*01	TRBJ1-2*01 (6)	Low avidity
	3	TRBV4-1*01	TRBJ2-7*01 (3)	Intermediate avidity

Supplementary Table 6. NY-eso-1-SLL-specific T-cell clones derived from HLA-A*02:01^{pos} healthy donors. Per row, T-cell clones are depicted that used the same TRBV gene and performed equally in the functional screening; these clones were considered as identical clones. TRBV-gene usage for all 83 obtained NY-eso-1-SLL-specific T-cell clones was determined using the IOTest Beta Mark Kit for flow cytometry. For clones that were additionally sequenced for their TRB-CDR3 region, the joining gene usage (TRBJ) is indicated with the number of sequenced clones between brackets.

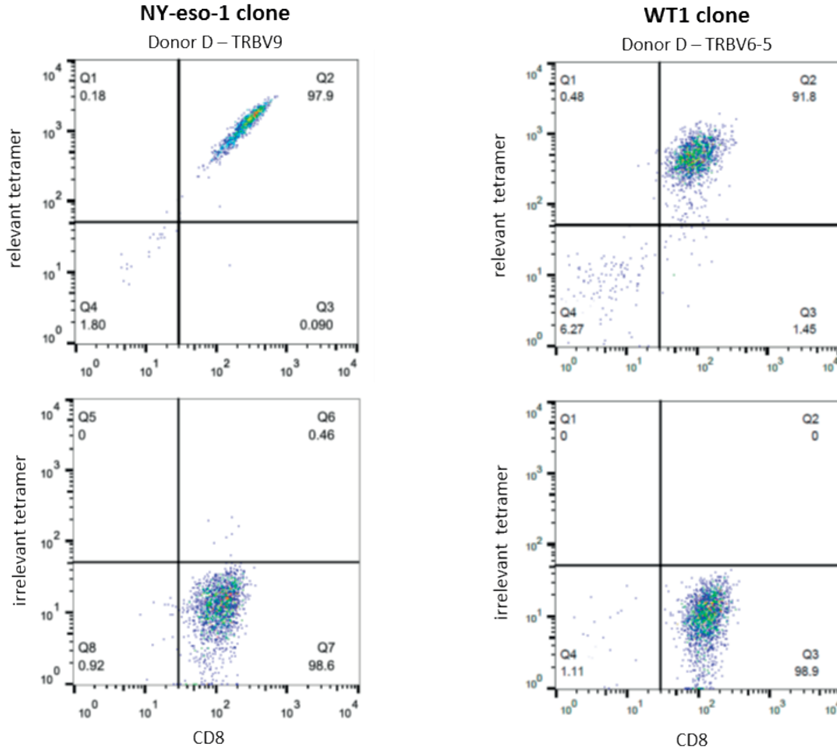
	Number of clones	TRBV gene usage	TRBJ gene usage (number of clones sequenced)	Classification based on functional screening
Donor C	2	TRBV6	-	Not functional
Donor D	12	TRBV9*01	TRBJ1-1*01 (5)	Intermediate avidity
	20	TRBV20-1	-	Not functional
	2	TRBV28	-	Not functional
	2	TRBV6	-	Not functional
	3	TRBV?	-	Not functional
Donor F	1	TRBV4	-	Not functional
	2	TRBV19	-	Not functional
Donor G	3	TRBV?	-	Low avidity
Donor H	1	TRBV29-1	-	Not functional
	1	TRBV4	-	Not functional
	5	TRBV27	-	Not functional
	4	TRBV?	-	Not functional
Donor P	1	TRBV9	-	Not functional
	9	TRBV29-1*01	TRBJ2-3*01 (2)	Low avidity
	4	TRBV5-5	-	Not functional
	7	TRBV3-1	-	Low avidity
	4	TRBV6	-	Not functional



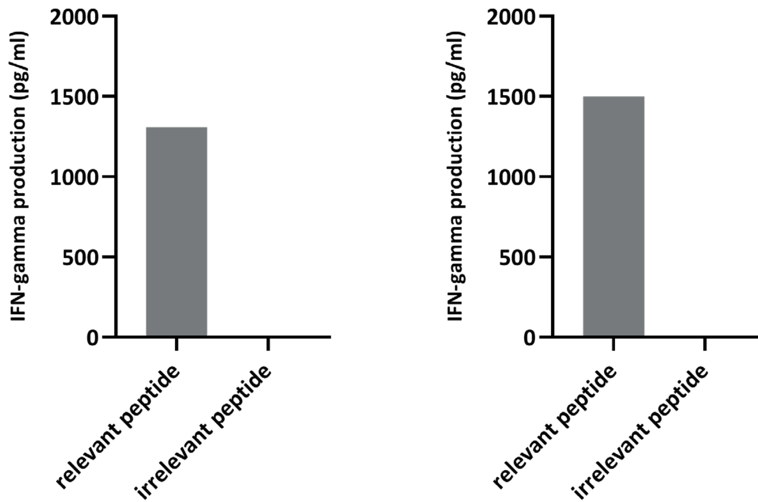
Supplementary Figure 1. HA-1H-specific T-cell population were isolated from PBMC of HA-1H^{neg} and HA-1H^{pos} donors. After several isolation and expansion rounds, HA-1H-specific T-cell populations became detectable with direct tetramer and anti-CD8 antibody staining.

5

A



B



Supplementary Figure 2. TAA-specific T-cell clones only stained with relevant tetramers and only showed functionality against targets loaded with the relevant peptides. **A**, Representative NY-eso-1 clone and WT1 clone stained with the relevant tetramers and an irrelevant tetramer containing another HLA-A*02:01-restricted peptide. **B**, The same NY-eso-1 clone and WT1 clone tested against T2 cells loaded with the relevant and an irrelevant HLA-A*02:01-restricted peptide (10^{-5}M).

5

CHAPTER 6

SUMMARY AND GENERAL DISCUSSION

SUMMARY

The aim of allogeneic stem cell transplantation (alloSCT) is to persistently eradicate malignant cells of the patient, while at the same time a healthy hematopoiesis is restored. Before an alloSCT can be applied, patients receive conditioning which may include chemotherapy and irradiation to eradicate malignant cells and allow engraftment of the donor stem cells into the bone marrow of the patient. In contrast to autologous SCT, the curative potential of alloSCT is not achieved by the conditioning regimen, but is based on the induction of a graft-versus-leukemia (GVL) effect mediated by donor-derived T cells. These T cells of donor origin have the potential to eliminate hematopoietic cells, including malignant cells of the patient, by the recognition of antigens expressed by hematopoietic or malignant patient cells. However, T cells of donor origin transferred with the graft can also induce graft-versus-host disease (GVHD) when antigens on healthy tissue cells of the patients are recognized. Therefore, the holy grail in the field of allogeneic stem cell transplantation is to find the right balance between the induction of a GVL effect and the occurrence of GVHD. Several steps in the application of alloSCT can be manipulated with the aim to skew this balance to the favorable side.

To reduce the risk of GVHD, it is preferred to match donor and patient for HLA as much as possible. However, even after HLA-matched alloSCT, the early administration of donor T cells after transplantation induces a large risk for the initiation of GVHD. Therefore, several strategies have been explored to deplete the graft from T cells before infusion into the patient. T-cell depleting (TCD) approaches include the physical purification of beneficial graft components or physical depletion of harmful T cells. In addition, antibodies like alemtuzumab (ALT) and anti-thymocyte globulin (ATG) have been used for *in vivo* and/or *in vitro* TCD. The Leiden University Medical Center has gained many years of experience using ALT, a humanized monoclonal antibody directed against CD52 (Campath-1H), for *in vivo* and *in vitro* TCD. However, the exact effect of ALT addition 'to the bag' *(in vitro)* has not been explored before. In **chapter 2** we characterized the effect of 'to the bag' ALT addition on the composition of 60 allogeneic stem cell grafts before infusion into the patient. Since the process of TCD is continued *in vivo* after graft infusion, the depletion efficiency of lymphocyte subsets, including T-cell subsets, by ALT *in vitro* is expected to be a good predictor for the total TCD-effect of ALT. We showed that *in vitro* incubation of grafts with 20 mg ALT resulted in a significant compositional change of grafts already before infusion into the patient. Among lymphocyte subsets, T-cell receptor (TCR) alpha/beta (α/β) T cells were depleted most efficiently, compared to gamma/delta T cells, B cells and NK cells. Within the TCR α/β T cells, $CD4^{pos}$ T cells in the graft were depleted more efficiently than $CD8^{pos}$ T cells, and naïve and regulatory T cells were depleted more efficiently than effector and memory T cells. These differences in observed depletion efficiencies among T-cell subsets were in line with their reported CD52-expression levels. The composition of the grafts at the moment of infusion was not predictive for reconstitution of

T cells at 3 or 6 weeks after transplantation, confirming that the process of TCD is continued *in vivo* after graft infusion. Moreover, we reported a very low incidence of acute GVHD within 12 weeks post-transplantation, which implies that both myeloablative as well as non-myeloablative conditioning regimens containing *in vitro* ALT-based TCD of the grafts resulted in efficient GVHD prophylaxis. In this chapter, we have shown that *in vitro* ALT-based TCD does not simply delete all T cells, but induces a balanced depletion of naïve and regulatory T cells, thereby reducing the risk of GVHD induction while partially sparing antiviral immunity mediated by memory T cells. Therefore, we concluded that *in vitro* ALT addition to allogeneic stem cell grafts is an easy, safe and generally applicable method for efficient and balanced TCD.

Effective TCD decreases the risk of GVHD and allows the restoration of damaged tissues after the conditioning regimens. However, by reducing or eliminating T cells from the graft, the curative GVL effect and viral immunity can be reduced as well. To (re)introduce the GVL reactivity and to promote viral immune reconstitution after TCD alloSCT, the concept of donor lymphocyte infusions (DLI) has been developed. In this approach, unselected lymphocytes from the stem cell donor are administered with the aim to induce durable remission of persistent or relapsed disease, as well as to boost viral immunity. In this approach, timing, clinical setting and dosing of DLI determine whether GVL and viral immune reconstitution can be stimulated while ensuring the risk of GVHD remains acceptable. A longer time interval between alloSCT and DLI has been associated with lower intensity and risk of GVHD and allows infusion of higher doses of DLI. In general, DLI is applied at 3 to 6 months post-transplantation.

The advantage of T-cell depleted allogeneic stem cell transplantation is that in most patients no prophylactic immunosuppression is required. This creates the opportunity to apply adoptive T-cell therapy at an earlier point in time after alloSCT than regular DLI, to specifically boost viral and/or antitumor immunity. The main challenge in the generation of T-cell products for infusion early after alloSCT, is to minimize the risk of the induction of GVHD. Ideally, T-cell products need to contain only T cells with a defined specificity and no contaminating T cells of unknown specificity as these cells can potentially cause GVHD. To boost virus-specific T-cell responses, T cells directed against several viral antigens presented in different HLA molecules can be isolated from the peripheral blood of preferably seropositive donors. For the specific induction of GVL, antigens that are exclusively expressed by malignant cells or hematopoietic cells of the patients, need to be targeted. Genetic differences between patient and donor that give rise to polymorphic peptides presented in HLA molecules on patient cells that are recognized by donor T cells are called minor histocompatibility antigens (MiHA). Strong alloreactive immune responses directed against MiHA have been observed after HLA-matched alloSCT. Therefore, MiHA exclusively expressed by hematopoietic cells of the patient are powerful targets for the specific induction of GVL. In addition, tumor-associated antigens (TAA) are proposed as targets for the initiation of antitumor responses.

The MHC I-*Strep*tamer isolation technology has previously been used for the detection and isolation of single T-cell populations with expected high frequencies in the peripheral blood. As this technology has shown to be feasible and safe for the isolation and infusion of T-cell products directed against a single antigen, we broadened its application to the isolation and infusion of multi-antigen specific T-cell products. In **chapter 3**, the feasibility of the simultaneous isolation of multiple antigen-specific T-cell populations from donor peripheral blood mononuclear cells (PBMC) in one procedure using MHC I-*Strep*tamers was proved. By adjusting the amount of MHC I-*Strep*tamer isolation complexes per target antigen specificity, up to 24 different T-cell populations can simultaneously be isolated for the generation of one T-cell product. At the same time, the co-isolation of potentially harmful contaminating T cells remained below our safety limit, ensuring a low risk of GVHD post-infusion. Furthermore, we showed that not only T-cell populations with a high frequency in donor leukapheresis products like virus-specific T cells from seropositive donors can be isolated, but also T-cell populations with very low frequencies like virus-specific T cells from seronegative donors or MiHA- and TAA-specific T cells, can be isolated in the same procedure. Isolation of T-cell populations of high frequency did not have a negative impact on the isolation of low frequent T-cell populations from donor peripheral blood mononuclear cells (PBMC), or the other way around. These findings defined optimal technical conditions to isolate personalized multi-antigen specific T-cell products for direct clinical application.

In **chapter 4**, we reported the results of the clinical application of multi-antigen specific T-cell products for the prevention of viral reactivations and disease relapses early after TCD alloSCT in a phase I/II study. We showed that the MHC I-*Strep*tamer-based generation of donor-derived T-cell products targeting cytomegalovirus (CMV)-, Epstein-Barr virus (EBV)- and adenovirus (AdV)-antigens, as well as TAA and MiHA is feasible, since for 26 of 27 included patients we successfully generated a T-cell product with a high purity of target-antigen-specific T cells and low numbers of contaminating T cells of unknown specificity. None of the patients who received the study product developed infusion-related complications or severe acute or chronic GVHD, implying that the administration of the T-cell products was safe. During follow-up, potential efficacy of this prophylactic infusion was illustrated by the expansion of donor-derived target-antigen-specific T cells in patients coinciding viral reactivations and the prevention of viral complications in the majority of patients within 12 weeks after T-cell product infusion. So far, expansion of TAA- or HA-1H-specific T cells could not be detected in the 5 patients that suffered from disease progression. Overall, this study suggested that the application of especially virus-specific memory T cells can prevent viral complications in a prophylactic setting early after TCD-alloSCT.

Although TAA are proposed as target antigens for the induction of antitumor responses after vaccination strategies or HLA-matched alloSCT, a causative relation between induction of high-avidity TAA-specific T cells *in vivo* and clinical effect has not been proven so far. Theoretically,

TAA are self-antigens with low or restricted tissue-specific expression in non-malignant cells. Therefore, negative thymic selection is supposed to prevent auto-immunity by eliminating T cells with high avidity for self-antigens presented in self-HLA from the T-cell repertoire. In **chapter 5**, we showed that thymic selection has a major influence on shaping of the T-cell repertoire directed against self-antigens presented in self-HLA. In our model of T-cell clones directed against the hematopoiesis-restricted MiHA HA-1H isolated from HA-1H^{neg}/HLA-A*02:01^{pos} versus HA-1H^{pos}/HLA-A*02:01^{pos} donors, we were able to illustrate that high-avidity HA-1H-specific T-cell clones were only present in the T-cell repertoire of HA-1H^{neg}/HLA-A*02:01^{pos} donors, suggesting that thymic selection has eliminated HA-1H-specific T-cell clones of high avidity from the T-cell repertoire of HA-1H^{pos}/HLA-A*02:01^{pos} donors. Furthermore, we isolated >600 TAA-specific T-cell clones directed against the HLA-A*02:01-restricted peptides of the TAA NY-eso-1-SLL, WT1-RMF, RHAMM-ILS, proteinase-3-VLQ and PRAME-VLD from 18 HLA-A*02:01^{pos} healthy donors and showed that only a small minority of isolated TAA-specific T-cell clones were able to recognize malignant cell lines expressing endogenously processed TAA, but not primary malignant samples. These results illustrate that although the induction of an antitumor response by T cells recognizing TAA presented in self-HLA is possible, it is very exceptional.

In summary, we studied the manipulation of donor-derived T cells at different stages during the process of HLA-matched allogeneic stem cell transplantation with the aim to reduce complications early after alloSCT. We showed that ALT can be successfully used for *in vitro* differential T-cell depletion of grafts, resulting in a very low incidence of acute GVHD within 12 weeks after transplantation. After alloSCT, the MHC I-*Strep*tamer technology can be used for the isolation of high- and low-frequent beneficial T-cell populations from donor leukapheresis products, creating the opportunity to generate personalized T-cell products to prevent or treat especially viral complications in patients after alloSCT. Furthermore, we concluded that the induction of clinically relevant antitumor responses by TAA-specific T cells from the autologous or HLA-matched T-cell repertoire is possible, but very exceptional, due to the very low frequency of high-avidity TAA-specific T cells in the peripheral blood of healthy individuals.

GENERAL DISCUSSION

IN VITRO ALEMTUZUMAB ADDITION TO ALLOGENEIC STEM CELL GRAFTS IS AN EFFICIENT STRATEGY FOR T-CELL DEPLETION

GVHD is still one of the dominant complications after alloSCT, associated with high morbidity and mortality. Donor-derived T cells transfused with the graft that recognize healthy tissue cells of the patient can elicit harmful alloreactive T-cell responses affecting the patient's skin, gut, liver and/or lungs. The frequency of donor-derived T cells in the graft has been clearly associated with the risk of GVHD. To reduce the risk of GVHD early after alloSCT, several strategies have been explored to *in vitro* manipulate the graft with the aim to reduce the frequency of harmful donor-derived T cells, better known as TCD. Excessive TCD can be obtained by the physical positive isolation of CD34^{pos} cells, which creates a purified graft of stem and progenitor cells with only a marginal number of contaminating T cells.[1,2] An advantage of this strategy is that it results in a very low incidence of acute and chronic GVHD in the absence of immunosuppressive therapy, but at the cost of an increased risk of graft rejection, disease relapse and opportunistic viral infections.[3] As a compromise, the application of limited T-cell addback to CD34^{pos} selected T-cell grafts has shown to support early T-cell reconstitution and to partly preserve protective immunity after alloSCT.[4] However, besides beneficial T cells, also harmful T cells can be reintroduced by T-cell addback, because only the number of T cells is defined, but not their specificity. In order to deplete potentially harmful T cells from the graft more selectively, depletion of TCR α/β T cells has been investigated mainly in haplo-identical alloSCT. In this approach, γ/δ T cells in the graft are preserved, which are not involved in GVHD development but can contribute to antiviral immunity early after alloSCT.[5-8] However, the incidence of viral complications remains a concern. In **chapter 2**, we showed that the addition of the CD52-targeting antibody ALT (also known as Campath-1H) 'to the bag' as part of the conditioning regimen of the patient, is another very efficient method to deplete T cells from the graft: it is a far more easy, faster and less expensive method compared to previous mentioned methods. This strategy results in a very low incidence of acute GVHD in the first 12 weeks after alloSCT, comparable to TCD achieved by CD34^{pos} cell selection. Although the different TCD-strategies have not been compared side by side in a randomized control trial, the reported incidence of viral complications after ALT-based TCD is thought to be significantly lower compared to CD34^{pos} cell selection.[9-12] This can be explained by the differential depletion of T cells observed in our study. This differential *in vitro* depletion of lymphocytes and T-cell subsets in the grafts was in line with the reported CD52-expression on their cell surface.[13] TCR α/β T cells were depleted more efficiently than TCR γ/δ T cells, CD4^{pos} T cells more efficiently than CD8^{pos} T cells, and naïve and regulatory T cells more efficiently than memory and effector T cells. Because naïve T cells are the main initiators of GVHD, the effective depletion of naïve TCR α/β T cells is presumably responsible for the decreased risk of GVHD post-transplantation. In addition, an inevitable result of the intense depletion of naïve T cells from the graft is the abrogation of alloreactive antitumor

T-cell responses. Therefore, the infusion of donor-derived T cells at a later point in time after alloSCT will still be necessary to prevent disease relapse.

Because memory and effector TCR α/β T cells are not completely depleted due to their moderate CD52-expression, antiviral immunity is partially conserved. In contrast, regulatory T cells have been intensively depleted by *in vitro* ALT addition to grafts. It was previously suggested that high absolute numbers of regulatory T cells in grafts can have a protective effect on GVHD occurrence.[14-16] Besides, extensive depletion of regulatory T cells from grafts was reported to have an overall negative effect on outcome due to their dampening effect on alloreactive T-cell responses.[17] However, in the absence of high frequencies of donor-derived naïve T cells that potentially can initiate alloreactive T-cell responses resulting in GVHD, the efficient depletion of regulatory T cells is not expected to be harmful. A potential disadvantage of regulatory TCD might be that the suppression of T-cell mediated autoimmunity is abrogated.[18] Although autoimmune reactivity is frequently observed in patients treated with this specific transplantation regimen, autoimmune phenomena have not been observed in the first 12 weeks after ALT-based TCD alloSCT in our study.[19]

Based on our observations, ALT addition 'to the bag' is appreciated as a widely applicable, easy and fast strategy for TCD in alloSCT patients, resulting in a very low incidence of GVHD and partial preservation of antiviral immunity due to differential depletion of T-cell subsets.

MHC I-*STREP*TAMERS FOR THE SUCCESSFUL ISOLATION OF SPECIFIC T-CELL POPULATIONS FROM DONOR PERIPHERAL BLOOD MONONUCLEAR CELLS

The adoptive transfer of selected T-cell populations with exclusively beneficial effects is desirable early after TCD alloSCT, especially to prevent or treat viral complications. Prerequisites for the broad application of selective T-cell therapy is a feasible isolation method for the generation of T-cell products that can be used for clinical application, besides the knowledge of targetable antigens. For the *ex vivo* isolation of virus-specific T-cell populations for direct clinical application without *in vitro* expansion, several methods are available. In this thesis, the MHC I-*Strep*tamer isolation technique was extensively studied. In **chapter 3**, technical conditions were optimized to enable the simultaneous *ex vivo* isolation of up to 24 different T-cell populations in one isolation procedure. Furthermore, personalized MHC I-*Strep*tamer isolated T-cell products were generated and infused immediately in 27 patients included in the phase I/II clinical study described in **chapter 4**. Another widely used isolation technique is based on the *in vitro* stimulation of PBMC using viral (overlapping) peptides, followed by isolation of activated T cells based on the secretion of cytokines (eg, interferon gamma (IFN-γ)) or the expression of activation markers (eg, CD137).[20-24] This technique equally enables the fast generation of T-cell products within 1 or 2 days. However, in comparison to this isolation approach, the MHC I-*Strep*tamer technology has demonstrated to

have several advantages and limitations.

A limitation of the MHC I-*Strep*tamer technology is that it requires exact knowledge of antigens and their HLA restrictions. Furthermore, isolation of specific T-cell populations is restricted to the availability of MHC I-*Strep*tamers, which is limited to defined immune-dominant antigens restricted to common HLA-alleles. Because MHC I-*Strep*tamers are (so far) only available for antigens presented in HLA class I molecules, only CD8^{pos} antigen-specific T cells can be isolated using this technology. Although clinical studies performed with a variety of isolation methods targeting only CMV- or EBV-specific CD8^{pos} T-cell populations showed promising results regarding T-cell expansion and clinical outcome in the therapeutic setting,[25-27] CD4^{pos} helper T cells are expected to contribute to *in vivo* survival, persistence and functioning of CD8^{pos} T cells.[28-30] In contrast, the IFN-γ/CD137-based isolation of T cells after the stimulation of PBMC with overlapping peptides will activate both CD4^{pos} and CD8^{pos} T cells and will most likely result in a T-cell product containing T cells targeting a broad repertoire of epitopes, restricted to a variety of HLA-alleles. Because the exact specificities of isolated T cells are unknown, a detailed analysis of the exact composition of reactivities in the T-cell product cannot be assessed after IFN-γ/CD137-based T-cell isolation. This can complicate T-cell product release and *in vivo* follow-up of infused T cells. Using the MHC I-*Strep*tamer technology, the specificities of the targeted T-cell populations are defined beforehand, which makes analysis of product composition, including the frequencies of T cells with unknown specificity, and follow-up *in vivo* after infusion very easy by using direct tetramer staining. In **chapter 3** and **4**, we have shown that MHC I-*Strep*tamer isolation resulted in very pure T-cell products with a minimal number of T cells of unknown specificity, ensuring a very low risk of GVHD after product infusion.

In **chapter 3**, we demonstrated that not only T-cell populations with a relatively high precursor frequency in donor PBMC, but also very low-frequent T-cell populations can be reproducibly isolated using MHC I-*Strep*tamers. At the same time, the isolation efficiency of low-frequent T-cell populations was not affected by the simultaneous isolation of T-cell populations with high frequencies in donor PBMC, or vice-versa. This was illustrated by the simultaneous isolation of virus-specific T cells from seropositive donors and the isolation of TAA-, MiHA-, and virus-specific T cells from seronegative donors, which are expected to derive from the donor's naïve T-cell repertoire. The isolation of T-cell populations from the naïve T-cell repertoire cannot be achieved using *in vitro* peptide stimulation followed by direct IFN-γ/CD137-based T-cell isolation and is therefore a major advantage of MHC I-*Strep*tamers. This observation can be explained by the fact that MHC I-*Strep*tamer isolation is based on T-cell receptor specificity and is independent of kinetics of cytokine production or activation marker expression after T-cell stimulation. Indeed, naïve T cells require priming by professional antigen-presenting cells in the presence of sufficient co-stimulatory signals, which takes time and results in different kinetics of cytokine production and activation marker expression compared to

antigen-experienced T cells like memory and effector T cells.[31] Therefore the simultaneous isolation of both memory, effector and naïve T cells is more complicated using the IFN-γ/CD137-based isolation of T cells. Besides T-cell differentiation status, also T-cell specificity has shown to influence cytokine production and activation marker expression. CMV- and EBV-specific T cells show a rapid and brief IFN-γ production following activation, but AdV- and influenza virus-specific T cells show a more prolonged and moderate IFN-γ production.[21] Despite these kinetic differences, simultaneous isolation of multi-virus specific T-cell populations using IFN-γ-based isolation has been described, although the isolation efficiency can be questioned.

Overall, the MHC I-*Strep*tamer technology was shown to be an easy and fast isolation technique for the generation of multi-antigen specific T-cell products targeting both high- and low-frequent T-cell populations in donor PBMC. The choice of the isolation technique is mainly dependent on the targeted T-cell populations, the need for detailed product release and the necessity of T-cell expansion *in vivo* during follow-up.

ADOPTIVE T-CELL THERAPY TO BOOST VIRAL IMMUNITY

As shown in **chapter 2,** *in vitro* ALT-based TCD of the graft results in a differential depletion of T-cell subsets, partly sparing antiviral immunity mediated by memory T cells. Although the incidence of CMV disease after ALT-based TCD of grafts is limited and comparable with non-TCD alloSCT strategies, patients receiving TCD grafts do have an increased chance of developing reactivations of latent viruses, responsible for significant morbidity.[11,32-36] The most important viruses causing serious complications after alloSCT are CMV, EBV and AdV. Despite advances in pharmacotherapeutic approaches, (long-term) use of antiviral medication is limited by toxic side effects, development of resistant variants, lack of persistent efficacy and inability to provide long-term protection.[37-39] Therefore, immunotherapeutic strategies to accelerate reconstitution of virus-specific immunity after alloSCT remain a powerful alternative to conventional drugs. As long-term immunosuppression is not indicated after TCD alloSCT, this transplantation strategy is an ideal platform for the application of adoptive T-cell therapy to reduce (viral) complications early after TCD alloSCT. As soon as TCD antibodies like ALT and ATG are eliminated *in vivo*, adoptive T-cell therapy can be applied to bridge the immune-deficient period between TCD alloSCT and immune reconstitution derived from transplanted donor stem cells or adoptively transferred regular DLI at 3-6 months after transplantation.

Adoptive T-cell therapy for viral infections can be applied either in a prophylactic, pre-emptive or therapeutic setting. Ideally, virus-specific T-cell products are created that are able to successfully treat every patient suffering from viral disease. Under these circumstances, only patients that are diagnosed with viral disease receive a personalized T-cell product that efficiently restores viral immunity and cures viral disease. For this approach, the T-cell products need to be available

immediately after diagnosis and need to contain a high number of memory virus-specific T cells that are able to immediately attack virus-infected cells *in vivo*. This therapeutic strategy should be efficient and cost-effective, because the generation and infusion of T-cell products is limited to patients that actually need it. Unfortunately, this outlined holy grail for the treatment of viral disease after alloSCT is not realistic, because T-cell products cannot always be generated (in time) and effectiveness is not guaranteed. Therefore, other strategies to prevent viral disease are investigated, including the prophylactic or pre-emptive administration of virus-specific T-cell products in patients after TCD-alloSCT.

<u>Prophylactic or pre-emptive infusion of virus-specific T-cell products from seropositive donors</u>

In the prophylactic setting, the intention is to prevent viral complications by the infusion of virus-specific T cells at the moment a patient has no detectable viral DNA loads (yet). This approach has been described in **chapter 4**, where we clinically applied multi-antigen specific T-cell products in a prophylactic setting to prevent viral complications of CMV, EBV and AdV at an early point of time after TCD alloSCT. In the pre-emptive setting, patients are monitored intensively using PCR-directed surveillance of viral DNA loads to identify patients at an early stage of viral reactivation development. At the moment viral DNA loads are detectable, a personalized virus-specific T-cell product can be generated and infused to prevent progression of the viral reactivation to viral disease. An advantage of the prophylactic setting is that the generation and administration of T-cell products can be planned beforehand, which makes logistics easier compared to the application in the pre-emptive setting, when products need to be generated as soon as possible after viral DNA loads become detectable. Although MHC I-*Strep*tamer isolated T-cell products can be technically generated within one day, both donor leukapheresis products as starting material and experienced technicians need to be available. The donor leukapheresis product can be obtained upfront, before the granulocyte-colony stimulating factor (G-CSF)-mobilized graft is obtained, and stored, or collected fresh from the donor as soon as possible after viral DNA load detection in the patient after transplantation. Logistically, the latter can be a challenge, especially in case of an unrelated donor.

An important consideration in the application of adoptive T-cell therapy is the moment of infusion of T-cell products. In the pre-emptive setting, infusions preferably take place immediately after viral DNA load detection. In the prophylactic setting, T cells are ideally infused at the moment the antigen burden in the patient is (still) low, in order to give the relatively small number of *ex vivo* isolated T cells time to expand *in vivo* at the moment viral DNA loads rise. In the clinical study described in this thesis, T-cell products were administered at a median of 58 days after TCD alloSCT. At the moment of infusion, already half of CMV-seropositive patients had detectable CMV DNA loads and some patients already received antiviral treatment at the moment of product infusion. Therefore, adoptive transfer of T cells at an earlier point in time after alloSCT might

be more optimal to prevent CMV and other viral reactivations. In antibody-based TCD alloSCT, the moment of infusion is constrained by circulating T-cell depleting antibodies like ATG and ALT early after the transplantation. The estimated half-life time of ATG is 6 days and in patients who received a relatively high dose of ATG during conditioning (6 mg/kg), it has shown to take a median of 15 days to achieve a serum concentration below 1 µg/ml, the commonly accepted minimum therapeutic level.[40,41] ALT has been shown to remain detectable until 6-8 weeks after transplantation and was shown to prevent survival of CD52^{pos} T cells at a plasma concentration above 0.7 µg/ml.[12,42] Administration of T cells before 6-8 weeks after TCD alloSCT is therefore ineffective, because infused T cells will be eliminated by ALT and/or ATG. On the other hand, an extended interval between the infusion of virus-specific T cells and a viral reactivation requires a prolonged persistence and survival of infused T cells *in vivo*, which might abrogate the chance to elicit a profound T-cell response at a later point in time after TCD alloSCT.

In a pre-emptive approach, only for patients with detectable viral DNA loads a personalized T-cell product is generated and infused. Therefore only patients that actually need this adoptive therapy will receive it. The application of a prophylactic intervention implies that some patients will be exposed to expensive, unnecessary therapy, which provides a risk of side effects. However, none of the patients in our study developed infusion-related complications or severe acute or chronic GVHD, and the majority of included patients did not develop a CMV, EBV or AdV reactivation during the follow-up period until 12 weeks post-alloSCT. Whether these observations were associated with efficacy of our approach or that they were unrelated to the infusion of our T-cell product is impossible to prove. It might be an option to identify patients at high risk for viral reactivations or primary infections in advance and only provide these patients with a prophylactic T-cell product, in order to reduce adverse risks and costs. Risk stratification for CMV-related complications after alloSCT is relatively straight forward, since CMV reactivations are common and the large majority is restricted to CMV-seropositive patients and, to a lesser extent, seronegative patients receiving a graft from a seropositive donor.[43] Therefore, these categories of patients are likely to benefit from a prophylactic infusion of CMV-specific T cells derived from their seropositive transplant donors. For EBV and AdV (and other viruses like herpes simplex virus, (para-)influenza virus, BK virus) risk stratification is more complex, since EBV and AdV-related complications have a lower incidence in the (adult) transplantation population compared to CMV and risk factors are not that clear.[35] Therefore, it is much harder to predict beforehand which patients would benefit from a prophylactic T-cell product containing EBV- or AdV-specific T cells or T cells specific for other viruses.

Although the effect of adoptively transferred virus-specific T cells from seropositive donors after alloSCT was investigated in several clinical studies and although the results hinted to clinical efficiency, none of the approaches has yet become the standard of care. The reason for this limited translation into daily clinical practice is the lack of randomized, placebo-controlled, prospective

trials investigating efficacy of this immunotherapeutic approach. To investigate whether adoptive transfer of virus-specific T cells in a pre-emptive setting can be integrated into routine clinical practice, a multinational, randomized, placebo-controlled phase III clinical study (TRACE) has recently been initiated. The aim of this study is to examine whether patients with refractory viral infections after alloSCT can be cured with the infusion of a multi-virus specific T-cell product from their virus-seropositive stem cell donor.

Adoptive virus-specific T-cell therapy for patients with a seronegative donor

The development of adoptive immunotherapy using virus-specific T cells has mainly focused on the generation of virus-specific T-cell products from seropositive stem cell donors for adoptive transfer to their alloSCT recipient. Because the prevalence of CMV and EBV seropositivity in the Western population is about 46% and 90% at adult age, respectively, a considerable percentage of alloSCT recipients are transplanted with a CMV-seronegative donor.[44,45] Especially CMV-seropositive patients transplanted with a graft from a seronegative donor are at highest risk for the development of CMV disease. Previously, the isolation of virus-specific T cells from seronegative donors has been appreciated as complicated. However, we have shown that MHC I-*Strep*tamers have the ability to isolate virus-specific T cells from PBMC of seronegative donors, although their frequencies in the T-cell products were very low. Therefore, the infusion of T-cell products derived from seronegative donors in a pre-emptive setting is expected to be unsuccessful, because time is essential for adequate priming and expansion until appropriate cell numbers are reached to fight the virus-infected cells. In one of our study patients, we observed potential efficacy of the prophylactic infusion of virus-specific T cells from a seronegative donor, since CMV target-antigen-specific T cells converted from predominantly patient-origin before product infusion to 100% donor-origin after product infusion. Although the potential prophylactic effectivity of the infusion of virus-specific T cells from seronegative donors can only be truly assessed in a large randomized study, this approach is expensive, time-consuming and unpractical compared to alternative approaches. Another option is to harvest virus-specific T cells from the seropositive patient before the alloSCT procedure (autologous virus-specific T cells) and infuse these cells soon after transplantation to prevent viral reactivations and disease. However, besides a small risk of the introduction of graft rejection by infused virus-specific T cells, the major concern is the fast eradication of infused virus-specific T cells by alloreactive donor T cells that survived *in vitro* TCD. Therefore, this strategy might only boost viral immunity temporarily and is therefore not an attractive option for wide application.

Furthermore, it is also possible to isolate high-avidity virus-specific T-cell clones from seropositive donors or the naïve T-cell repertoire of seronegative donors and to utilize viral transduction to introduce a functional virus-specific T-cell receptor (TCR) into polyclonal donor-derived T cells, thereby redirecting their specificity.[46] One concern of TCR gene therapy that is currently receiving significant attention is mispairing of the introduced and endogenous TCR chains, leading to

the production of TCR of undefined specificity, which gives a potential risk of GVHD induction. Recently, the CRISPR/Cas9 gene-editing technology has been successfully used for the knockout of endogenous TCR alongside retroviral insertion of the new TCR in primary T-cell populations.[47] However, a restriction remains that gene transfer of a single high-avidity TCR results in the creation of a T-cell product containing T cells targeting a single viral antigen presented in a single HLA-allele. The feasibility and safety of CMV-pp65 TCR-transduced T cells for treatment of CMV infections in CMV-seropositive patients transplanted with a CMV-seronegative donor is currently being assessed in a phase I/II clinical trial in the United Kingdom.

Another strategy is the manufacturing of T-cell products derived from third-party virus-seropositive healthy subjects with common HLA-types. The advantage of virus-specific T cells from third-party donors is that a bank can be created of stored T-cell lines, which are immediately available ‘off the shelf’ for patients encountering viral complications, overcoming the hurdles of stem cell donor leukapheresis obtainment, T-cell isolation, processing and quality control between detection of the viral complication and the actual infusion of the T-cell product.[48] However, the safety and toxicity of the application of third-party T-cell products is likely to be associated with the level of HLA-matching between patient, alloSCT donor and third-party donor, making this approach an immunological challenge. Potential toxicity may include the risk of graft rejection by alloreactive T-cell responses of third-party donor T cells to donor stem cells. Furthermore, GVHD can be induced when third-party donor T cells recognize tissue cells of the patient. The other way around, alloreactive T cells of the stem cell donor may reject third-party donor T cells, hampering the persistence and efficacy of adoptively transferred virus-specific T cells. Multiple (pre)clinical studies evaluating the feasibility, safety and efficacy of using third-party virus specific T cells to treat (refractory) viral reactivations after alloSCT show promising results, but clinical efficacy has not yet been fully confirmed in placebo-controlled trials.[26,48-52]

TUMOR-ASSOCIATED ANTIGENS AS TARGETS FOR IMMUNOTHERAPY TO TREAT HEMATOLOGIC MALIGNANCIES

The efficacy of cancer immunotherapy relies on the use of an immunogenic target-antigen that can be recognized by the immune system and elicit protective antitumor responses resulting in clinical benefit.[53] TAA are often proposed as powerful targets to initiate antitumor responses in patients suffering from hematologic malignancies. Although several (pre)clinical studies investigated the antitumor potential of TAA, a clear causal relation between *in vivo* expansion of functional TAA-specific T cells and clinical effect has not been proven so far.[54] In **chapter 3** and **4**, we included the isolation of TAA-specific T cells along the isolation of virus-specific T cells using the major histocompatibility complex class I (MHC I)-*Strep*tamer technology and gained knowledge about their frequencies in donor PBMC. Furthermore, TAA-specific T cells have been isolated, cloned and tested for their functionality in **chapter 5**. This knowledge can be used to evaluate the applicability

6

of TAA for immunotherapeutic approaches.

Ideal tumor-antigens

The suitability of a given tumor-antigen for therapeutic applications to treat malignancies is complex to assess. The Translational Research Working Group of the National Cancer Institute pilot project of 2009 prioritized cancer antigens based on predefined and pre-weighted objective criteria with the aim to assist investigators in the immunotherapy field to select the most promising antigens for further development and testing in clinical trials, especially for vaccination approaches to treat hematologic as well as solid cancers.[55,56] The criteria handled in this project included (with an increasing weighting) number of patients with antigen-positive cancers; expression of the antigen in malignant cells, but not or only limited in non-malignant cells; oncogenicity of the gene encoding the antigen; the ability of the antigen to elicit immune responses; and antitumor reactivity observed in clinical settings. Using these criteria for 75 representative antigens, including several TAA, a ranking of antigens was generated. Although none of the representative antigens exhibited all aforementioned criteria and the weighting of the individual criteria may be argued, several of these TAA scored very well overall in this ranking, with the TAA WT1 rated as most ‘ideal’ tumor-antigen. In this thesis, we evaluated the suitability of 5 HLA-A*02:01-restricted peptides of 5 different TAA, including aberrantly expressed WT1, RHAMM and proteinase-3, as well as the cancer-germline antigens PRAME and NY-eso-1, for immunotherapeutic approaches to treat hematologic malignancies.

Immunogenicity of tumor-associated antigens

The immunogenicity of an antigen is defined as its ability to induce an immune response. The detection of high-avidity antigen-specific T cells in the peripheral T-cell repertoire of an individual can be a reflection of high immunogenicity of an antigen. The immunogenicity of TAA can therefore be investigated by addressing the functional reactivity of TAA-specific T cells in the repertoire of healthy individuals.

Before T cells enter the peripheral T-cell compartment, thymocytes need to pass thymic selection. T cells that are able to recognize peptides in the context of self-HLA are positively selected, however, high-avidity T cells directed against self-antigens presented in self-HLA are deleted in the thymus to prevent auto-immunity. Theoretically, high-avidity TAA-specific T cells are not expected to be present in the peripheral T-cell repertoire of healthy individuals because TAA are self-antigens. Low-avidity or nonfunctional T cells directed against self-antigens presented in self-HLA do pass thymic selection, but their clinical relevance is expected to be negligible. Regarding TAA-specific T cells, the most interesting are the TAA-specific T cells that pass thymic selection and perform in functional assays better than low-avidity TAA-specific T cells. This group of T cells, which we classified as intermediate avidity, is not expected to elicit auto-immune responses in a

healthy individual, but might become clinically relevant under specific circumstances.

In **chapter 3**, we confirmed that HLA-A*02:01-restricted TAA-specific T cells are actually present in the T-cell repertoire of HLA-A*02:01^{pos} healthy individuals.[57-60] Although these cells could not be visualized directly in the unmanipulated peripheral blood due to their low precursor frequencies, after several enrichment rounds using MHC I-*Strep*tamers followed by *in vitro* expansion, T cells directed against at least one of five TAA-specificities were detectable in every donor using direct tetramer staining. In **chapter 5**, T-cell clones directed against the mentioned TAA-peptides have been isolated from the self-HLA-restricted T-cell repertoire of healthy individuals. Their antigen-specific reactivity was measured by cytokine release after antigen stimulation. We showed that none of the isolated TAA-specific T-cell clones demonstrated high functional avidity in stimulation assay with targets exogenously loaded with a titration of the relevant peptide. The majority of T-cell clones appeared to be nonfunctional or of low avidity. Only a minority showed an intermediate functional avidity, which matched our hypothesis. Based on these results, we would classify TAA not ideal as target-antigens for antitumor immunotherapy. However, as addressed by the NCI project, other factors contribute to the overall suitability of antigens to elicit profound antitumor reactivity.

<u>Overexpression of tumor-associated antigens in malignant cells</u>

One important criterion applied in the NCI pilot project was the expression level of the antigen on malignant cells versus healthy cells. One of the distinguishing features of non-mutated monomorphic self-antigens like TAA is that this group of antigens is expected to be significantly overexpressed in malignant cells compared to their healthy counterparts.[61-63] This difference in antigen-expression level might influence the activation threshold for T cells resulting in activation of intermediate-avidity T cells, while preserving tolerance against healthy cells. In **chapter 5** we have shown that of the 13 TAA-specific T-cell clones of intermediate avidity, only 4 T-cell clones recognized malignant cell lines with an unrealistic high overexpression of the corresponding TAA compared to non-malignant cell lines, while none of the T-cell clones recognized (a limited set of) primary malignant samples. The TAA expression level on the RNA level of primary malignant samples appeared to be much lower than the artificial high TAA expression reached after transduction of cell lines or seen in malignant cell lines. In our study, we did not find primary malignant samples with a TAA overexpression that elicited profound cytokine production or cytolysis by intermediate-avidity TAA-specific T-cell clones. Therefore, the question is whether primary malignant cells exist with this unrealistic high TAA expression level. This discrepancy compared to literature might be due to the limited number of tested primary malignant samples in our study.[62-65] Furthermore, additional factors (eg, processing and peptide presentation) might also influence proper recognition of primary malignant cells.

Clinical application of tumor-associated antigen-specific immunotherapy

In **chapter 4**, we prophylactically administered multi-antigen specific T-cell products to patients with a variety of hematologic malignancies early after TCD alloSCT. Based on the in depth analysis of T-cell products for clinical application in **chapter 3**, we were convinced that all products contained TAA-specific T cells besides the main component of virus-specific T cells. However, we were not able to confirm expansion of TAA-specific T cells in the 5 patients with disease progression during follow-up, suggesting that TAA-specific T cells did not persist until disease progression, that the relapsing malignancies did not show an unrealistically high overexpression of the TAA or, which is most likely in light of **chapter 5**, that TAA-specific T cells were not of sufficient avidity to fight malignant cells. Based on these results, we concluded that the induction of a profound clinically relevant TAA-specific T-cell response from the unmanipulated autologous or HLA-matched T-cell repertoire might be possible, but is very unlikely due to the scarce frequency of TAA-specific T cell clones that have the potential to recognize overexpressed TAA in hematopoietic malignant cells. Therefore, we do not recommend the infusion of *ex vivo* isolated, non-expanded, transplant donor-derived TAA-specific T cells as adoptive T-cell therapy after alloSCT.

However, multiple efforts have been made to increase the chance of a functionally significant and clinically relevant TAA-specific T-cell response. Vaccination studies most often use multiple peptides or long-peptides to simultaneously initiate T-cell responses against several peptides presented in multiple HLA-alleles. Although we expect that thymic selection will work for all self-antigens in a comparable way, the induction of a very profound and diverse immune response might enhance the chance of inducing a functionally significant and clinically relevant TAA-specific T-cell response. Besides, the addition of adjuvants to vaccines targeting TAA might increase the efficiency of T-cell priming.[66,67] Another approach investigated to enhance antitumor responses is adoptive T-cell transfer after *in vitro* isolation and expansion of donor-derived TAA-specific T cells. The previously mentioned approaches have shown to be feasible in several phase I/II studies, but powerful evidence on the induction of profound T-cell responses after TAA-specific peptide vaccination or adoptive transfer of TAA-specific T cells in patients suffering from hematologic malignancies has not been described so far.[54,68-72] Therefore, recently initiated trials focusing on TAA-based immunotherapy are expected to provide additional insight in the potential of autologous TAA-specific T cells for the induction of clinically relevant antitumor responses in patients with hematologic malignancies.[73-77]

Recently, several groups investigated the possibility to equip donor T cells with a high-avidity WT1-specific TCR using TCR gene transfer. Although the first results regarding feasibility and safety sound promising in the prophylactic setting, efforts to apply this strategy in the therapeutic setting have not been successful so far.[78] Since the Nobel Prize of Medicine in 2018 was awarded for the discovery of immune checkpoint inhibitors, also this therapy has gained a lot of attention. Cancer

therapy combined with immune checkpoint inhibitors has been proposed to release the break of peripheral tolerance mechanisms by lowering the threshold for T-cell activations.[79,80] In solid cancers, an association has been suggested between the occurrence of immune-related adverse events and antitumor reactivity in patients treated with immune checkpoint inhibitors.[81,82] To investigate whether self-antigens restricted to self-HLA play a role in this antitumor reactivity, it would be interesting to analyze patients with solid tumors that received immune checkpoint inhibitors and developed auto-immune disease as well as clinical antitumor responses. If T cells directed against self-antigens restricted to self-HLA (like TAA) can be found in the tumor before disappearance and/or in the peripheral blood of the patients, this would strongly suggest that autologous T cells that passed thymic selection can provoke antitumor responses when the threshold for T-cell activation is decreased by immune checkpoint inhibitors. Confirmation of this hypothesis would strengthen the belief that intermediate-avidity T cells directed against self-antigens can become immunogenic when environmental circumstances are forced to the limit.

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APPENDICES

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

NEDERLANDSE SAMENVATTING

ALLOGENE STAMCELTRANSPLANTATIE

De ideale behandeling voor patiënten met een hematologische maligniteit zorgt voor vernietiging van maligne cellen, terwijl gezonde cellen en weefsels van de patiënt onaangetast blijven en hun normale functie kunnen blijven uitvoeren. Helaas zijn dergelijke behandelingen tot dusver niet voorhanden. De best beschikbare behandeling voor patiënten met hematologische maligniteiten bestaat uit een combinatie van chemotherapie, immunotherapie en/of radiotherapie, met als doel zoveel mogelijk maligne cellen te vernietigen (remissie-inductie therapie) en dit resultaat zo lang mogelijk te behouden (consolidatie therapie). Patiënten met een hoog-risico hematologische maligniteit kunnen als onderdeel van de consolidatie therapie behandeld worden met een stamceltransplantatie. Voordat stamcellen kunnen worden toegediend, wordt eerste chemotherapie en/of radiotherapie gegeven (conditionering) om de ziekte onder controle te krijgen en om innesteling van de toegediende stamcellen in het beenmerg mogelijk te maken. Na stamceltransplantatie, migreren de geïnfundeerde stamcellen naar het beenmerg om van daaruit te prolifereren en differentiëren in een gezond hematopoëtisch systeem. De infusie van stamcellen van de patiënt zelf die geoogst zijn vóór de consolidatie therapie, wordt autologe stamceltransplantatie (autoSCT) genoemd. Als stamcellen van een gezonde donor worden gebruikt, dan heet dit allogene stamceltransplantatie (alloSCT). Voorheen werden hematopoëtische stamcellen direct uit het beenmerg geoogst, maar tegenwoordig worden deze voornamelijk via leukaferese procedures verkregen uit het perifere bloed, na mobilisatie van de stamcellen vanuit het beenmerg door de toediening van granulocyt-kolonie-stimulerende factor (G-CSF). Bij alloSCT kan gebruik gemaakt worden van een verwante (familie) donor of een onverwante donor.

GRAFT-VERSUS-LEUKEMIE EFFECT

Van oudsher werden voorafgaand aan de SCT intensieve myeloablatieve conditioneringsschema's gebruikt om zoveel mogelijk maligne cellen te elimineren.-Vanwege aanzienlijke toxiciteit, is de toepassing van deze therapie voorbehouden aan fitte, jonge patiënten. Het is echter gebleken dat de conditionering alléén niet voldoende is om een recidief van de hematologische maligniteit na SCT te voorkomen. Dit is geïllustreerd door een hoger aantal recidieven in patiënten met een hoog-risico leukemie na autoSCT of na SCT met een transplantaat van een genetisch identieke donor (in het geval van een identieke tweeling) in vergelijking met na alloSCT. Het langdurige curatieve effect van alloSCT wordt veroorzaakt door T cellen afkomst van de donor die in staat zijn om resterende maligne cellen in de patiënt te herkennen en te vernietigen. Dit gunstige fenomeen staat bekend als het graft-versus-leukemie (GVL) effect. Sinds bekend is dat het GVL effect verantwoordelijk is voor het curatieve effect van alloSCT, zijn er minder toxische non-myeloablatieve conditioneringsschema's ontwikkeld zodat ook patiënten met een hogere leeftijd en met co-morbiditeiten in aanmerking komen voor alloSCT. Deze conditioneringsschema's hebben tot doel om hematopoëtische stamcellen van de donor zich te laten nestelen in het beenmerg zonder dat het hematopoëtische systeem van de patiënt geheel vernietigd hoeft te zijn.

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Echter, het nadeel hiervan is dat het recidief risico hoger ligt in vergelijking met myeloablatieve conditioneringsschema's.

Alhoewel donor T cellen in staat zijn om immuunresponsen te initiëren met het GVL effect tot gevolg, kunnen deze immuunresponsen ook gericht zijn tegen gezonde, niet-hematopoëtische cellen en weefsels van de patiënt, wat kan resulteren in levensbedreigende graft-versus-host ziekte (GVHD). In het onderzoek beschreven in dit proefschrift, is onderzocht hoe T cellen op verschillende momenten gedurende de alloSCT procedure gemanipuleerd kunnen worden zodat het GVL effect en immuunreacties gericht tegen pathogenen behouden, danwel gestimuleerd worden, terwijl GVHD wordt vermeden.

DIT PROEFSCHRIFT

Om het risico op GVHD na alloSCT zo klein mogelijk te maken, heeft het de voorkeur om donor en patiënt te matchen voor humane leukocyten antigenen (HLA). Echter, zelfs na HLA-gematchte alloSCT resulteert de toediening van donor T cellen vroeg na transplantatie in een hoog risico op GVHD. Daarom zijn verschillende methoden onderzocht om T cellen uit het transplantaat te verwijderen. Dit wordt T-cel depletie (TCD) genoemd. Verschillende manieren van TCD zijn beschreven, zoals de fysieke zuivering van gunstige transplantaat componenten of de fysieke depletie van potentieel schadelijke T cellen. Daarnaast worden antilichamen zoals alemtuzumab (ALT) en anti-thymocyt globuline (ATG) gebruikt voor *in vivo* en/of *in vitro* TCD. In het Leids Universitair Medisch Centrum is jaren ervaring opgedaan met het gebruik van ALT (Campath-1H), een gehumaniseerd monoclonaal antilichaam gericht tegen het antigeen CD52 dat voorkomt op het celoppervlak van met name lymfocyten, voor zowel *in vivo* als *in vitro* TCD. Echter, het exacte effect van ALT-toevoeging aan het transplantaat voor infusie in de patiënt is niet eerder onderzocht. In **hoofdstuk 2** is het effect beschreven van ALT toevoeging aan het transplantaat op de samenstelling van 60 allogene stamceltransplantaten, voordat deze aan patiënten werden toegediend. Hoewel het proces van TCD *in vivo* doorgaat na toediening van het transplantaat aan de patiënt, werd verwacht dat de *in vitro* depletie van verschillende lymfocyten en meer specifiek, van verschillende soorten T cellen door ALT, een goede voorspeller is voor het totale TCD effect van ALT. Beschreven is dat *in vitro* incubatie van transplantaten met 20 mg ALT resulteerde in een significante verandering in compositie van transplantaten voordat ze toegediend werden aan de patiënten. Van de verschillende soorten lymfocyten werden α/β T cellen het meest efficiënt gedepleteerd, in vergelijking met γ/δ T cellen, B cellen en NK cellen. Binnen de groep van α/β T cellen werden $CD4^{pos}$ T cellen efficiënter gedepleteerd dan $CD8^{pos}$ T cellen. Daarnaast werden naïeve en regulatoire T cellen efficiënter gedepleteerd dan effector en memory T cellen. Het verschil in efficiëntie van depletie van de verschillende T-cel subsets kwam overeen met de mate van expressie van het CD52-antigen op het celoppervlak. De samenstelling van het transplantaat op het moment van infusie bleek niet voorspellend voor het herstel van T cellen in het perifere

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bloed van de patiënten gemeten 3 of 6 weken na transplantatie. Dit bevestigt dat het proces van TCD na transplantatie gecontinueerd wordt *in vivo*. Er werd een lage incidentie van acute GVHD binnen 12 weken na transplantatie gerapporteerd, hetgeen bevestigt dat zowel myeloablatieve als non-myeloablatieve conditioneringsschema's waarvan TCD middels *in vitro* ALT onderdeel uitmaakt, resulteren in een efficiënte preventie van GVHD. In dit hoofdstuk is aangetoond dat op ALT gebaseerde *in vitro* TCD niet simpelweg alle T cellen uit het transplantaat verwijdert, maar dat het een gebalanceerde depletie van naïeve en regulatoire T cellen veroorzaakt, waarmee het risico op GVHD wordt gereduceerd, terwijl memory T cellen die zorgen voor antivirale bescherming grotendeels gespaard blijven. Geconcludeerd werd dat ALT toevoeging aan allogene stamceltransplantaten een gemakkelijke, veilige en breed toepasbare methode is voor efficiënte en gebalanceerde TCD.

Efficiënte TCD verlaagt het risico op GHVD. Echter, door het verlagen van het aantal of door het totaal elimineren van T cellen in het stamceltransplantaat, wordt het curatieve GVL effect en de bescherming tegen virussen ook aangetast. Om GVL te (her)introduceren en reconstitutie van virale immuniteit na TCD alloSCT te promoten, is het concept van donor lymfocyten infusies (DLI) ontwikkeld. Hierbij worden ongeselecteerde T cellen van de stamceldonor na alloSCT gegeven aan de patiënt met als doel om langdurige remissie van persisterende of recidiverende hematologische maligniteit te bewerkstelligen, naast het stimuleren van de virale immuniteit. De planning, klinische setting en dosering van DLI bepalen of het GVL effect en virale immuniteit gestimuleerd kunnen worden terwijl het risico op GVHD acceptabel blijft. Een langer interval tussen alloSCT en DLI is geassocieerd met een lagere intensiteit en een lager risico op GVHD, waarbij een hogere dosering van DLI verdragen wordt. In het algemeen wordt DLI 3 tot 6 maanden na de transplantatie toegepast.

Het voordeel van TCD alloSCT is dat het bij verreweg de meeste patiënten niet nodig is om profylactische immunosuppressieve medicatie te geven ter voorkoming van GVHD. Daarom geeft dit behandelschema de mogelijkheid om op een vroeg tijdstip na alloSCT aanvullende adoptieve T-cel therapie te geven. Adoptieve T-cel therapie is een vorm van immuuntherapie waarbij geselecteerde of gemodificeerde T cellen toegediend worden aan de patiënt met als doel specifieke T-cel responsen te promoten. De grootste uitdaging bij het genereren van T-cel producten voor toediening vroeg na transplantatie is het risico op GVHD zo laag mogelijk te houden. In het ideale geval bevatten T-cel producten alleen T cellen met een gedefinieerde specificiteit en geen T cellen waarvan de specificiteit onbekend is en die mogelijk GVHD kunnen veroorzaken. Om virus-specifieke T-cel immuniteit te verbeteren kunnen T cellen gericht tegen verschillende virale antigenen geïsoleerd worden uit het bloed van donoren die het virus eerder hebben doorgemaakt (seropositieve donoren) en toegediend worden aan patiënten. Om een GVL effect te induceren moeten T-cel responsen gegenereerd worden gericht tegen antigenen

die specifiek tot expressie komen op alleen maligne of hematopoëtische cellen van de patiënt. Genetische verschillen tussen patiënt en donor kunnen resulteren in polymorfe peptiden die worden gepresenteerd in gemeenschappelijke HLA moleculen. Wanneer deze polymorfe peptiden bij HLA-identieke donoren een immuunrespons kunnen opwekken worden deze minor histocompatibility antigenen (MiHA) genoemd. Krachtige alloreactieve T-cel responsen gericht tegen MiHA zijn gezien na HLA-gematchte alloSCT. Donor T cellen specifiek voor MiHA die uitsluitend tot expressie komen op (maligne) hematopoëtische cellen van de patiënt kunnen zorgen voor een GVL effect, terwijl donor T cellen die MiHA op gezonde, niet-hematopoëtische cellen herkennen ook GVHD kunnen veroorzaken. Daarom zijn hematopoëse-specifieke MiHA belangrijke doelantigenen voor immuuntherapie. Daarnaast zijn tumor-geassocieerde antigenen (TAA) gesuggereerd als doelantigenen voor het induceren van anti-tumor reacties. TAA zijn niet-gemuteerde antigenen met een (beperkte) expressie in niet-maligne cellen, maar waarvan wordt beschreven dat ze tot overexpressie komen in maligne cellen. In de literatuur wordt een relatie gesuggereerd tussen de expansie van TAA-specifieke T cellen in het perifere bloed van patiënten met hematologische maligniteiten en een betere ziektevrije overleving. Verschillende vaccinatie studies hebben gepoogd immuunresponsen tegen TAA te induceren in patiënten met hematologische maligniteiten. Alhoewel in een deel van de patiënten een klinische respons gepaard ging met een verhoogde frequentie van TAA-specifieke T cellen in het perifere bloed, is een causale relatie tussen expansie van potente (hoog avide) TAA-specifieke T cellen en klinische respons tot dusver niet aangetoond. Daarom is het waardevol om verder te onderzoeken of T-cel responsen tegen TAA een anti-tumor effect kunnen induceren.

De MHC I-*Strep*tameer isolatie technologie is eerder gebruikt voor de detectie en isolatie van T-cel populaties met een verwachte hoge frequentie in perifeer bloed, waarbij per isolatie één specifieke T-cel populatie werd geïsoleerd. Aangezien deze techniek heeft bewezen haalbaar en veilig te zijn voor het genereren van T-cel producten gericht tegen één enkele T-cel specificiteit, is geprobeerd het gebruik te verbreden naar de isolatie van multi-antigeen specifieke T-cel producten. In **hoofdstuk 3** wordt de haalbaarheid beschreven van de simultane isolatie van multipele antigeen-specifieke T-cel populaties uit donor perifeer bloed mononucleaire cellen (PBMC) in één procedure met de MHC I-*Strep*tameer techniek. Door de hoeveelheid isolatiecomplexen per antigeen aan te passen in de procedure, konden tot 24 verschillende T-cel populaties simultaan geïsoleerd worden uit donor PBMC voor de vervaardiging van één T-cel product. Hierbij bleef de co-isolatie van potentieel gevaarlijk contaminerende T cellen onder de vooraf gestelde veiligheidslimiet, waardoor een laag risico op GVHD gewaarborgd bleef. Daarnaast werd aangetoond dat niet alleen T-cel populaties met een hoge frequentie in donor leukaferese producten, zoals virus-specifieke T cellen van seropositieve donoren, maar ook T-cel populaties met een extreem lage frequentie zoals virus-specifieke T cellen van seronegatieve donoren, of MiHA- en TAA-specifieke T cellen simultaan geïsoleerd konden worden. Inclusie van deze laag frequente T-cel populaties

in de isolatieprocedure had geen negatieve invloed op de isolatie van andere T-cel populaties of andersom, en had ook geen invloed op de ongewenste co-isolatie van contaminerende T cellen met een onbekende specificiteit. Deze bevindingen illustreren optimale technische condities voor de isolatie van gepersonaliseerde multi-antigeen specifieke T-cel producten voor directe klinische toepassing.

In **hoofdstuk 4** worden de resultaten beschreven van de klinische toepassing van multi-antigeen specifieke T-cel producten voor de preventie van virale reactivaties en ziekte recidieven vroeg na TCD alloSCT in een fase I/II klinische studie. Aangetoond werd dat het genereren van donor-afkomstige T-cel producten gericht tegen zowel antigenen van het cytomegalovirus (CMV), Epstein-Barr virus (EBV) en het adenovirus (AdV), als tegen 5 verschillende TAA en een hematopoëse-specifieke MiHA (HA-1H) mogelijk was. In deze klinische studie werd voor 26 van de 27 geïncludeerde patiënten met succes een T-cel product gemaakt met een hoge zuiverheid van doelantigeen-specifieke T cellen en lage aantallen van contaminerende T cellen met een onbekende specificiteit. Geen van de patiënten die een studie product toegediend gekregen, hebben infusie-gerelateerde complicaties opgelopen, of acute of chronische GVHD ontwikkeld in de eerste 12 weken na transplantatie. Dit suggereert dat de infusie van deze T-cel producten veilig is. Gedurende de follow-up periode werden in het perifere bloed van patiënten aanwijzingen gevonden voor effectiviteit van het profylactisch toedienen van het T-cel product, aangezien er toename (expansie) van donor-afkomstige antigeen-specifieke T cellen werd gezien in patiënten die toch virale reactivaties ontwikkelden. Daarnaast ontwikkelde het grootste deel van de patiënten geen virale complicaties in de eerste 12 weken na infusie van het T-cel product. In de 5 patiënten die progressie van ziekte ontwikkelden gedurende follow-up werd gekeken of er expansie van TAA- of HA-1H-specifieke T cellen aangetoond kon worden in het perifere bloed: dit was niet het geval. Deze studie suggereert dat de toediening van virus-specifieke T cellen van seropositieve donoren virale complicaties kan voorkomen wanneer deze cellen kort na TCD alloSCT worden toegediend aan patiënten.

TAA zijn zelf-antigenen met een beperkte weefselexpressie in niet-maligne cellen, maar ze zijn niet afwezig in het individu. Daarom wordt verondersteld dat ook voor deze antigenen negatieve thymus selectie optreedt om auto-immuniteit door TAA-specifieke T cellen te voorkomen. Immers, T cellen met een hoge aviditeit voor zelf-antigenen die gepresenteerd worden in zelf-HLA worden geëlimineerd uit het T-cel repertoire. In **hoofdstuk 5** wordt beschreven dat thymus selectie grote invloed heeft op het T-cel repertoire gericht tegen zelf-antigenen gepresenteerd in zelf-HLA. Door gebruik te maken van een *in vitro* model van T-cel klonen gericht tegen het hematopoëse-specifieke MiHA HA-1H, geïsoleerd uit HA-1H^{neg}/HLA-A*02:01^{pos} versus HA-1H^{pos}/HLA-A*02:01^{pos} donoren, werd aangetoond dat hoog avide HA-1H-specifieke T cellen alleen aanwezig zijn in het T-cel repertoire van HA-1H^{neg}/HLA-A*02:01^{pos} donoren. Dit suggereert dat thymusselectie

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gezorgd heeft voor de eliminatie van hoog avide HA-1H-specifieke T cellen uit het repertoire van HA-1H^{pos}/HLA-A*02:01^{pos} donoren. Daarnaast werden >600 TAA-specifieke T-cel klonen gericht tegen peptiden van de TAA NY-eso-1-SLL, WT1-RMF, RHAMM-ILS, proteinase-3-VLQ en PRAME-VLD, gepresenteerd in HLA-A*02:01, geïsoleerd uit het perifere bloed van 18 HLA-A*02:01^{pos} gezonde donoren en *in vitro* getest. Alleen een zeer kleine minderheid van de geïsoleerde TAA-specifieke T-cel klonen was in staat om maligne cellijnen die het TAA endogeen tot expressie brengen *in vitro* te herkennen. Primaire maligne cellen werden echter niet door deze TAA-specifieke T-cel klonen herkend. Deze resultaten suggereren dat de inductie van een aanzienlijke anti-tumor respons door T cellen die TAA herkennen in de context van zelf-HLA mogelijk, maar zeer uitzonderlijk is.

In dit proefschrift werd de manipulatie van donor-afkomstige T cellen gedurende verschillende stadia van HLA-gematchte alloSCT voor hematologische maligniteiten bestudeerd, met als doel het aantal complicaties vroeg na alloSCT te reduceren. Geconcludeerd werd dat ALT succesvol gebruikt kan worden voor de *in vitro* differentiële T-cel depletie van transplantaten, resulterend in een zeer lage incidentie van acute GVHD in de eerste 12 weken na transplantatie. De MHC I-*Strep*tameer technologie kan gebruikt worden voor het isoleren van hoog en laag frequente, gunstige T-cel populaties uit donor-leukaferese producten, om een gepersonaliseerd T-cel product te creëren voor de preventie of behandeling van met name virale complicaties in patiënten na alloSCT. Verder werd geconcludeerd dat het induceren van een klinisch relevante anti-tumor respons door TAA-specifieke T-cel responsen uit het autologe danwel HLA-gematchte T-cel repertoire mogelijk, maar zeer zeldzaam is gezien de enorm schaarste van hoog-avide TAA-specifieke T-cellen in het perifere bloed van gezonde donoren.

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ABBREVIATIONS

AdV	human adenovirus
APC	allophycocyanin
ALL	acute lymphoblastic leukemia
alloSCT	allogeneic stem cell transplantation
AML	acute myeloid leukemia
autoSCT	autologous stem cell transplantation
ALT	alemtuzumab
ATG	anti-thymocyte globulin
ATMP	advanced therapy medicinal product
BD	Beckton Dickinson
BM	bone marrow
CLL	chronic lymphocytic leukemia
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CML	chronic myeloid leukemia
CMV	cytomegalovirus
CR	complete remission
DLI	donor lymphocyte infusion
EBV	Epstein-Barr virus
E : T	effector-to-target ratio
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FL	follicular lymphoma
Flt3	FMS-like tyrosine kinase 3
G-CSF	granulocyte-colony stimulating factor
GMP	Good Manufacturing Practice
GPI	glycophosphatidylinositol
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
HLA	human leukocyte antigen
HSA	human serum albumin
HTLV-1	human T-cell lymphotropic virus type 1
IFN-γ	interferon gamma
IMDM	Iscove's Modified Dulbecco's Medium
iv	intravenously
LPL	lymphoplasmacytic lymphoma
LUMC	Leiden University Medical Center
MA	myeloablative

MALT	mucosa-associated lymphoid tissue
MCL	mantle cell lymphoma
MDS	myelodysplastic syndrome
MHC	major histocompatibility complex
MiHA	minor histocompatibility antigen
MM	multiple myeloma
NGFR	nerve growth factor receptor
NMA	non-myeloablative
NPM1	nucleophosmin 1
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBSC	peripheral blood stem cells
PE	phycoerythrin
PeCy7	phycoerythrin-cyanine7
PerCP	peridinin-chlorophyll-protein complex
PHA	phytohemagglutinin
PR	partial remission
PTLD	post-transplant lymphoproliferative disease
(q)PCR	(quantitative) polymerase chain reaction
TAA	tumor-associated antigen
TBI	total body irradiation
TCD	T-cell depletion
TCR	T-cell receptor
TRB	T-cell receptor beta
TRBJ	T-cell receptor beta joining
TRBV	T-cell receptor beta variable
WBC	white blood cell count

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LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

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Tumor Associated Antigen Reactive Donor T Cells as Elementary Components of Streptamer Isolated Multi-Antigen Specific T Cells.

Winner Abstract Achievement Award

Dutch Society for Immunology (NNVI) Symposium 2014, Noordwijkerhout, The Netherlands (poster presentation)

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Streptamer Isolated Multi-Antigen Specific T Cells Contain Tumor Associated Antigen Reactive Donor T Cells as Elementary Component.

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Prevention of Viral Infections After T Cell Depleted Allogeneic Stem Cell Transplantation by Infusion of Multi-Antigen Specific T Cell Products.

Winner Abstract Achievement Award

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A minority of tumor associated antigen specific T cells restricted to self-HLA alleles is of sufficient avidity to recognize overexpressed endogenously processed antigen.
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A minority of tumor associated antigen specific T-cells restricted to self-HLA alleles is of sufficient avidity to recognize overexpressed endogenously processed antigen.

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*P*rophylactic Infusion of Multi-Antigen Specific T-Cell Products to Prevent Complications After T Cell Depleted Allogeneic Stem Cell Transplantation – a Phase I/II Study.
Winner Abstract Achievement Award

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Prophylactic Infusion of Multi-Antigen-Specific T-Cell Products to Prevent Complications Early after T-Cell Depleted Allogeneic Stem Cell Transplantation – a Phase I/II Study

Winner Best Young Abstract Award

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CURRICULUM VITAE

Marthe Roex werd geboren op 7 februari 1987 in Enschede. In 2005 behaalde zij haar gymnasium diploma aan het Bonhoeffer College te Enschede, waarna zij startte met de studie Geneeskunde aan het Leids Universitair Medisch Centrum (LUMC). Tijdens haar studie was zij als voorzitter van de Organizing Committee van het Leiden International Student Conference (LIMSC) verantwoordelijk voor het wetenschappelijke programma van dit grote internationale studentencongres. Voor aanvang van de coschappen in 2010 deed zij 7 maanden wetenschappelijk onderzoek aan het Kennedy Institute for Rheumatology van het Imperial College in Londen naar verschillende virale vectoren voor het bestuderen van genregulatie in primair weefsel van patiënten met reumatoïde artritis (begeleider: prof. F. Brennan). Als onderdeel van de coschappen heeft zij met veel plezier haar semi-arts stage gelopen op de afdeling Interne Geneeskunde van het toenmalige Rijnland Ziekenhuis (tegenwoordig Alrijne Ziekenhuis) te Leiderdorp. Tijdens haar laatste keuzecoschap verdiepte zij zich in de tropengeneeskunde in het St. Joseph Hospital te Kagondo in Tanzania. Na het behalen van het artsexamen in september 2012 (cum laude) heeft ze een jaar als arts-assistent op de afdeling Interne Geneeskunde gewerkt in het Groene Hart Ziekenhuis te Gouda (opleider: dr. T. Koster).

In september 2013 is zij gestart met haar promotieonderzoek op het Laboratorium voor Experimentele Hematologie van de afdeling Hematologie van het LUMC onder begeleiding van prof. dr. J.H.F. Falkenburg en dr. I. Jedema. Dit onderzoek vond plaats binnen het Europese T-Control consortium, in samenwerking met de Universitätsklinikum Würzburg in Duitsland, het Anthony Nolan Londen in het Verenigd Koninkrijk, en het toenmalige Stage Cell Therapeutics GmbH in Göttingen, Duitsland (tegenwoordig Juno Therapeutics in München). In het kader van de verschillende studies bezocht zij diverse congressen in binnen- en buitenland waar ze meerdere presentaties gaf. De resultaten van de projecten zijn beschreven in dit proefschrift.

Op 1 mei 2019 is zij gestart met de opleiding tot internist, in de regio Leiden (opleider: prof. dr. J.W. de Fijter), in het HagaZiekenhuis te Den Haag (opleider: dr. J. Lagro).

DANKWOORD

Promoveren, wat een avontuur! Al is vooraf de bestemming duidelijk, de reis daarnaartoe is dit allerminst. En wat een reis was dit! Niet alleen vanwege de inhoud, maar met name vanwege het samenwerken met zoveel inspirerende, kundige en enthousiaste collega's. En niet te vergeten de steun van vrienden en familie. Dit tezamen heeft ertoe geleid dat dit proefschrift er gekomen is.

Allereerst wil ik alle patiënten en donoren bedanken. Jullie geven ons de mogelijkheid om te leren, te onderzoeken en te experimenteren. Jullie blijven de drijfveer om onderzoek te doen en behandelingen te ontwikkelen en verbeteren: voor jullie doen we het! En er is nog zoveel winst te behalen.

Veel dank aan alle (oud-)collega's van het Laboratorium voor Experimentele Hematologie van de afdeling Hematologie. Een unieke afdeling waar jaren ervaring, hard werken en een goede organisatie samen gaan met een hele fijne sfeer. Mooi om daarvan deel uit te mogen maken. Ik kijk met heel veel plezier terug op de gezellige en nuttige koffiepauzes, de seminars op dinsdagochtend, de inspirerende werkbesprekingen, de altijd creatieve labdagen, de binnen- en buitenlandse congresbezoeken en de sportieve fietstochten die we met elkaar hebben mogen meemaken. Het was een voorrecht om met jullie te mogen samenwerken!

Graag wil ik de collega's van de interdivisionele GMP Faciliteit, de Flowcytometrie Core Faciliteit en het Laboratorium voor Speciële Hematologie van het LUMC bedanken voor de zeer gewaardeerde technische en logistieke ondersteuning bij de verschillende projecten. De Europese partners in het T-Control consortium: hartelijk dank voor de internationale samenwerking.

Met het risico iemand te vergeten wil ik toch een aantal personen in het bijzonder bedanken. Mijn promotor prof.dr. Falkenburg en co-promotor dr. Jedema, Fred en Inge: dank voor de inspirerende en motiverende begeleiding gedurende de afgelopen jaren. Ik heb enorm veel van jullie geleerd, op wetenschappelijk maar zeker ook op persoonlijk vlak. Lois, Sabrina, Conny en Esther: zowel op het translationele lab als op het GMP op J10 hebben we heel wat uren met elkaar doorgebracht. Dank voor de fijne werkplek, de hulp bij grote proeven en het samen delen van mee- en tegenvallers. Lois: ik ben jou in het bijzonder heel dankbaar voor alle hulp in het laboratorium in de afgelopen jaren. Je hebt me enorm veel werk uit handen genomen! Ellis: je hebt me in de eerste maanden wegwijs gemaakt in de wereld die laboratorium heet. Dank voor je geduld. Wesley, Aicha, Rosa, Laura, Miranda, Anna, Margot, Floris en Lorenz: het was heel fijn om met jullie de promotie-perikelen te kunnen delen. Op ons 'hok' op C5 werd hard gewerkt, maar was het ook wel eens te gezellig. Dank voor de afleiding en alle leuke momenten! Wesley en Sabrina: heel leuk dat jullie mijn paranimfen willen zijn en tijdens de verdediging naast mij staan! Peter en Stijn: met alle klinische vragen kon ik bij jullie terecht. Ik vond het heel prettig samenwerken en hoop eens een klinische collega van jullie te mogen worden. Lisette en Henk: dankzij jullie waren klinische data

in een handomdraai beschikbaar en geordend. Het was heel fijn om met jullie samen te werken. Gerrie: dank voor al je geplan, geregel en georganiseer. Charissa: het jaar dat je bij ons op de afdeling hebt gewerkt heb je ongeveer alles aan stamceltransplantaten gemeten wat mogelijk was. Wat een berg aan data. Dank voor je inzet! Seyma en José: de twee studenten die ik met plezier heb mogen begeleiden. Hopelijk hebben jullie net zoveel van mij geleerd als ik van jullie.

Ron: zonder jou was ik niet op deze afdeling aan mijn promotietraject begonnen. Je hebt me bij het zoeken van een promotieplek belangrijke vragen gesteld en voorzien van waardevolle adviezen. En zie waartoe dit heeft geleid!

Alle collega's van de interne geneeskunde van het HagaZiekenhuis: wat zijn jullie een fijne groep. Jullie collegialiteit heeft het mogelijk gemaakt dat ik tijdens de eerste tijd van mijn opleiding, mijn promotie heb kunnen afmaken. Dank daarvoor!

Een goede professionele omgeving is belangrijk om een promotie te laten slagen, maar gelukkig is er zoveel meer dan werk. Ik prijs me gelukkig met mijn vrienden en familie. Mijn oud-huisgenoten van de Hogewoerd in Leiden, meiden van de Eetclub, studiegenoten, dispuut Double Daisy, hockeyteam dames 4 van Groen Geel en alle anderen: dank voor jullie fijne vriendschap en zeer gewaardeerde afleiding in de afgelopen jaren! In het bijzonder wil ik nog bedanken: Cathalijn, Navin, Jacqueline, Nienke, Lisa, Maurits en Emily, onze vriendschappen koester ik enorm. Dank voor jullie nauwe betrokkenheid en interesse in mijn onderzoek.

En tot slot, de Roexies. Casper en Jacob: ik kan me geen betere broers wensen. Dank voor jullie altijd eerlijke, kritische en humoristische kijk op onze levens. Miyo en Anas: jullie zijn een hele fijne toevoeging aan onze familie! Lieve papa en mama: zonder jullie was ik nooit zover gekomen. Dank voor jullie steun, aanmoediging en grote betrokkenheid. Ik weet dat ik altijd bij jullie terecht kan voor hulp en oprecht advies. Daar ga ik zeker nog vaak gebruik van maken!

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