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Streptamer technology allows to isolate leukemia antigen-specific $CD8^+$ T cells

Dissertation
Applying for the Doctoral Degree of Medicine (Dr. med.)
Faculty of Medicine, University of Rostock

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Rostock, November 10, 2009

urn:nbn:de:gbv:28-diss2009-0196-0

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To my family

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List of Abbreviations

ALL	Acute lymphoblastic leukemia
Allo-HSCT	Allogeneic hematopoetic stem cell transplantation
AML	Acute myelogenous leukemia
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-cyanine 7
APCs	Antigen-presenting cells
β_2-MG	β_2-Microglobulin
BM	Bone marrow
BSA	Bovine serum albumin
CD	Cluster of differentiation
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
CTLs	Cytotoxic T cells
DLI	Donor lymphocyte infusion
DMSO	Dimethyl sulfoxide
EBMT	European Bone Marrow Transplantation
EDTA	Ethylenediaminetetraacetic acid
ELISPOT	Enzyme-linked immunosorbent spot
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GMP	Good manufacturing practice

GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
HDs (HVs)	Healthy donors (Healthy volunteers)
HLA	Human leukocyte antigen
IL-2	Interleukin-2
IL-7	Interleukin-7
KLH	Keyhole limpet hemocyanin
MACS	Magnetic-activated cell sorting
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MLPC	Mixed lymphocyte peptide culture
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
RT	Room temperature
TCR	T cell receptor
WT1	Wilms' tumor gene 1

1. Introduction

1.1 Donor lymphocyte infusion (DLI)

DLI is a method of adoptive immunotherapy used after hematopoietic stem cell transplantation (HSCT). In order to augment an anti-tumor immune response or to ensure that donor stem cells remain to engraft after HSCT, lymphocytes from the original stem cell donor are infused (Porter et al. 2006, Loren et al. 2006).

DLI constitutes an approach of adoptive immunotherapy and has been identified as an important and efficient therapy for patients with chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML) relapsing after allogeneic hematopoetic stem cell transplantation (allo-HSCT) (Kolb et al. 1990). Several studies have confirmed that DLI induces complete remissions in 70-80% of patients with relapsed CML in chronic phase (Drobyski et al.1993, Porter et al. 1994, Kolb et al. 1995, Collins et al. 1997), the majority of which has proven to restore full donor chimerism and produce long-term complete molecular genetic remissions which other method, such as imatinib, does not provide a definite cure for relapsed after allo-HSCT (Kolb et al. 1995, Guglielmi et al. 2002, Raiola et al. 2003, Weisser et al. 2006). At present, DLI has been recommended by the European Bone Marrow Transplantation (EBMT) Society as a standard therapy for patients with CML relapsing after HSCT.

Several retrospective studies have proven that the main function of DLI against leukemia is based on the graft-versus-leukemia (GVL) (Loren et al. 2008). Additionally, T cell depletion of the allograft increases the risk of relapse (most markedly in patients with CML) of leukemia, suggesting that donor T cells are important effectors of the GVL response (Horowitz et al. 1990). The acted target for a GVL reaction may be leukemia-specific (Bocchia et al. 1996, Clark et al. 2001). This hypothesis is strongly supported by the fact that GVL can occur even in the absence of clinical graft-versus-host disease.

Complications of DLI include acute and chronic GVHD as well as bone marrow aplasia, resulting in immunosuppression and susceptibility to opportunistic infections (Loren et al. 2008).

1.2 Graft-versus-host disease (GVHD)

The most significant and common complication after DLI is acute and chronic

GVHD, which constitutes a result of the recognition of recipient allogenic antigens by T cells from the donor and may develop in 40-60% of the patients (Kolb et al. 1995, Collins et al.1997, Raiola et al. 2003). GVHD contributes significantly to morbidity and mortality even after treatment with fully matched donor lymphocytes. In some cases, DLI-induced GVHD may be very severe: 20-35% of DLI recipients can be anticipated to develop grade III-IV acute GVHD (Loren et al. 2008). Furthermore, acute GVHD may lead to death in up to 10% of the patients (Porter et al. 1997).

In most studies on DLI, GVHD correlated with the GVL response (Collins et al. 1997, Loren et al. 2008). Over 90% of complete responders developed an acute or chronic GVHD. Of 23 patients who did not experience GVHD, only three achieved a complete remission (Collins et al. 1997). As DLI is a highly effective salvage strategy for relapsed CML after allo-HSCT, there is a fervent need for a strategy to reduce GVHD when DLI are put into practice.

1.3 Wilms' tumor gene 1 (WT1)

WT1 is a gene of dual function, which was first considered as a tumor suppressor gene of Wilms' tumor and was demonstrated to act as an oncogene later (Haber et al. 1990, Yamagami et al. 1996, Nishida et al. 2006, Ariyaratana et al. 2007). It is located on chromosome 11p13 and encodes Wilms' tumor 1 protein, a zinc-finger transcription factor that is very important for normal cellular development and cell survival (Call et al. 1990, Mundlos et al. 1993). WT1 has been found to be a potent transcriptional regulator. The targets genes of WT1 are important for cellular growth and metabolism, including extracellular matrix components, growth factors and other transcription factors.

Although it is limited to low levels in normal adult tissues, the expression of WT1 protein is up-regulated in a high proportion of patients with acute lymphoblastic leukemia (ALL), AML, myelodysplastic syndrome (MDS), and CML and in several solid tumors, including lung, breast, prostate, and ovarian cancer (Miwa et al. 1992, Brieger et al. 1995, Oji et al. 1999). Therefore, more and more studies have focused on WT1. It has become increasingly apparent that WT1 in peripheral blood (PB) or bone marrow (BM) plays a dual role, not only as target for immunotherapy, but also as biomarker of state, stage, response to treatment,

prognosis, and relapse of the disease (Inoue et al. 1994, Sugiyama et al. 2001, Miyoshi et al. 2002, Ogawa et al. 2003, Barragan et al. 2004, Chiusa et al. 2006, Paschka et al. 2008, Rezvani et al. 2009, Weber et al. 2009)

As for immunotherapies targeting WT1, both WT1 peptide vaccination and adoptive transfer of WT1 specific $CD8^+$ T cells as a novel immunotherapy have made great progress. *Osada et al.* reported on a WT1-encoding Ad vector which is capable of inducing effective immunity against WT1-expressing malignancies (Osada et al. 2009). A clinical vaccination trial with GM-CSF, WT1.126-134 peptide and keyhole limpet hemocyanin (KLH) could provide immunological, molecular and preliminary clinical evidence of potential clinical efficacy in AML patients (Keilholz et al. 2009). A combined PR1 and WT1 vaccine showed a vaccine-driven anti-leukemia effect. These results support further studies of combination immunization strategies in leukemia patients (Rezvani et al. 2008).

Investigation on adoptive immunotherapy with WT1-specific $CD8^+$ T cells also indicated that WT1 has become an increasingly attractive target molecule for the development of efficient immunotherapy whose possibility has been strongly indicated by the feasibility and potential efficacy of cytotoxic T lymphocytes (CTLs) against WT1-specific peptides (Oka et al. 2000, Ohminami et al. 2000). For clinical applications, T cells of desired antigen specificity were isolated or engineered to express receptors that target infected or transformed cells and were then expanded in culture (Brentjens et al. 2003, Blattman et al. 2004, Morgan et al. 2006, Gattinoni et al. 2006). The generation of WT1-specific responses of healthy donors (HDs) and the establishment of T cell clones specific for WT1 indicated the potential clinical impact of *ex vivo* expanded donor-derived WT1-specific $CD8^+$ T cells for adoptive immunotherapy (Weber et al. 2009).

However, it is difficult to isolate WT1-specific $CD8^+$ T cells from HDs as for the expression of WT1 in healthy volunteers (HVs) is restricted to hematopoietic progenitor cells where WT1 is expressed at a low level. The new technology for the separation of WT1-specific $CD8^+$ T cells may open new avenues for the WT1-mediated adoptive immunotherapy.

1.4 Multimer technologies

Identification and purification of antigen-specific T cells without altering their functional status is a highly desirable goal for adoptive immunotherapy. Several

methods based on antigen specificity, such as secretion assay (affinity matrix) (Manz et al. 1995), Enzyme-linked immunosorbent spot (ELISPOT) assay (Miyahira et al. 1995), intracellular cytokine staining (Murali-Krishna et al.1998) and major histocompatibility complex (MHC) multimer procedures (Altman et al. 1996, McMichael et al. 1998, Busch et al. 1998), have been developed to directly identify antigen-specific T cells over the past years. Only multimer based selection/ separation might constitute the most straight forward methods for both the isolation and purification of antigen-specific T cells (Knabel et al. 2002).

The basic principle of multimer technologies is to use the natural T cell receptor (TCR) ligand, the MHC-peptide complex, as a staining probe (Altman et al. 1996). Multimerization causes higher binding avidity of the reagent when compared to monomeric MHC-epitope complexes to TCRs on the surface of T cells, thus allowing epitope-specific binding to T cells. MHC multimer reagents could be used for identification of T cells with high specificity and sensitivity.

The traditional MHC multimers such as tetra- and pentamer have been developed to identify antigen-specific $CD8^+$ T cells (Yao et al. 2008). Tetrameric complexes have been shown to bind stably and specifically to appropriate MHC/peptide-specific T cells (Altman et al. 1996) and to permit both the detection and isolation of antigen-specific T cells present at low numbers in the PBMCs (Busch et al. 1998). However the functional activity of MHC multimer-labeled T cells is hampered by the persistence of TCR-MHC interactions and subsequently induced signaling events (Whelan et al. 1999, Daniels et al. 2000, Maile et al. 2001, O'Herrin et al. 2001). The negative impact of surface-bound TCR-ligands on T cells makes the application of traditional MHC multimer technologies in adoptive immunotherapy difficult.

However, a new novel MHC multimer technology-designated streptamer technique-has been developed to ‘reversibly’ identify and purify the antigen-specific T cells (Knabel et al. 2002). This approach displays the specificity and sensitivity of conventional MHC multimer staining and do not affect the functional status of the T cells (Knabel et al. 2002). Therefore the streptamer technique seems to be more advantageous for adoptive immunotherapies described above (Figure 1) (Neudorfer et al. 2007).

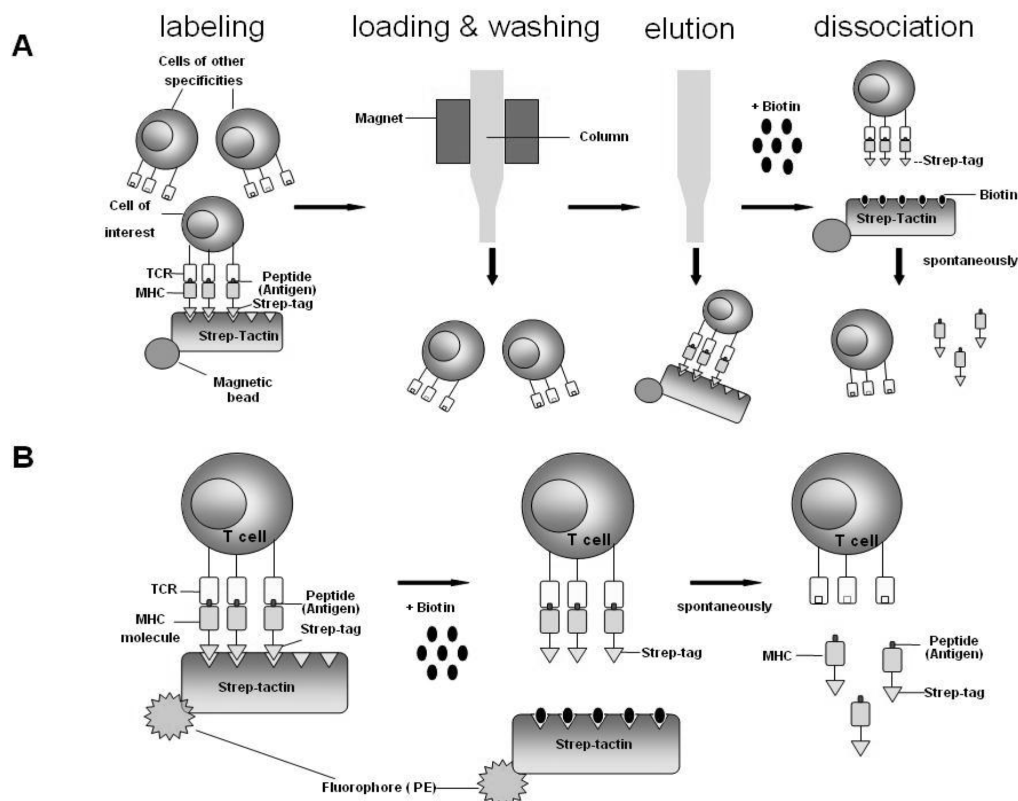

Figure 1. The streptamer techmology. A) Functional, non-induced antigen-specific T cells are purified through a magnetic field and released by D-biotin from the streptamer complex. B) A reversible antigen specific T cell is acquired by fluorescent labeled streptamer and D-biotin induced removal of the complex.

1.5 Antigen-specific cytotoxic T cells

A cytotoxic T cell (also known as TC, CTL, T killer cell, cytolytic T cell, $CD8^+$ T cell or killer T cell) is a sub-group of T lymphocytes that are capable of recognizing and inducing the death of infected somatic or tumor cells; they kill cells that are infected with pathogens, such as viruses, bacterial and fungi, or are otherwise damaged or dysfunctional.

Cytotoxic T cells could recognize antigen presented by MHC class I-derived molecules which preserve peptides from protein degradation inside the cell and present these peptides on the cell surface to CTL. This process enables CTL to scan other cells for alterations (Andersen et al. 2006). The affinity between CD8 and the MHC molecule keeps the CTL and the target cell bound closely together during antigen-specific activation. Cytotoxicity is exerted directly through the Fas or perforin pathway and/or indirectly by the release of cytokines.

The main function of the CTL is to monitor all the cells of the body, ready to destroy any threat to the integrity of the host. Therefore, it is reasonable to suggest that CTL could provide some degree of protection against spontaneous malignant tumors. When the quantitative and qualitative antigenic differences in transformed cells were detected, CTL has the ability to recognize and kill them.

WT1-specific cytotoxic T cells could specifically recognize and lyse the tumor cells that express WT1 antigen. Therefore they can be used for the treatment of patients with WT1 expressing tumors.

1.6 Rationale of the study

In order to maximize the anti-tumor effect and to minimize anti-host reactivity, several approaches to DLI have been investigated, including: (1) *Ex vivo* activation and expansion of donor T cells through co-stimulation; (2) generation and infusion of tumor-specific T cells; (3) generation and infusion of minor histocompatibility antigen-specific T cells; (4) low-dose DLI followed by dose escalation; (5) infusion of selected T cell subsets (that is, after $CD8^+$ cell depletion or $CD4^+$ cell selection); (6) inactivation of alloreactive T cells (that is, through transduction of suicide genes into donor T cells, photochemical inactivation, inactivation by chemotherapy, irradiation); (7) infusion of T regulatory cells; (8) generation and infusion of Th2-type T cells; (9) manipulation of antigen presenting cells to maximize GVL or minimize GVHD (Loren et al. 2008). Based on the data and considering the cumulative international experience, the use of tumor antigen-specific DLI is recommended to be a most attractive method of adoptive immunotherapy (Or et al. 2006) and suggested that such safer strategies above should focus on more selective targeting of specifically immune T cells and recombinant interleukin-2-activated natural killer cells (Falkenburg et al. 1993, Morecki et al. 2001, Slavin et al. 2001, Slavin et al. 2003, Slavin et al. 2004) rather than using non-selective and hazardous non-specific T cell therapy or antibody-guided recombinant interleukin-2-activated lymphocytes (Morecki et al. 2006). So, the application of specific effector cells that exert potent anti-tumor immunity with a lower risk of inducing clinical GVHD might offer an ideal approach.

Wilms' tumor protein (WT1), as an endogenous host protein, is over-expressed in the chronic phase and particularly in the blast crisis of CML as well as in a more than 70% of the patients with AML and MDS (Tamaki et al. 1999, Rosenfeld et al.

2003). WT1 could serve as a potential myeloid leukemia tumor antigen. Therefore, it was logical to start testing WT1-specific T cells generated and expanded *in vitro* as tumor-specific DLI. Some studies have identified that the selection of WT1-specific $CD8^+$ T cells may be a promising method for the adoptive T cell immunotherapy (Weber et al. 2009).

However, the problem is how to get qualified WT1-specific $CD8^+$ T cells for successful DLI. The advantage of streptamer technology above indicate that it may be the most promising technology to choose for the development of adoptive T cells transfer regimens for the treatment of patients with malignancy or infectious diseases.

Here, we investigated whether WT1-specific $CD8^+$ T cells separated by the streptamer technique for DLI will increase the GVL. Studies investigating the streptamer technology and the function of WT1-specific $CD8^+$ T cell separated by streptamer technology will indicate possible implications for adoptive immunotherapy after allogeneic stem cell transplantation.

1.7 The aim of the study

T cell depletion without selection may effectively prevent severe GVHD but profoundly impair donor-derived immune reconstitution and increase infection and disease relapse. Generation and infusion of WT1-specific $CD8^+$ T cells might be an ideal option to enhance an anti-leukemic effect and to reduce GVHD after hematopoeitic stem cell transplantation. In this study we evaluated the following aspects of WT1-specific $CD8^+$ T cells / of streptamer technology:

- Frequency of WT1-specific $CD8^+$ T cells in healthy donors (HDs)
- Frequency of WT1-specific $CD8^+$ T cells in AML patients
- Augmentation of the frequency of WT1-specific $CD8^+$ T cells through MLPC
- Purification of WT1-specific $CD8^+$ T cells by streptamer technology
- Immunophenotype of WT1-specific $CD8^+$ T cells before and after column separation

To answer these questions, the frequencies of WT1-specific $CD8^+$ T cells in HDs and patients were identified. Mixed lymphocyte peptide cultures of HLA-A*0201 positive/WT1 streptamer positive HDs and patients were performed. Antigen specific T cells were isolated by using WT1 streptamer magnetic beads from PBMCs of HDs. In order to investigate the immunophenotype of WT1-specific

$CD8^+$ T cells purified by streptamer, the samples of HDs before and after isolating were identified by the staining of phenotypic markers.

2. Material and Methods

2.1 Material

2.1.1 Samples

Blood samples were obtained from healthy donors at the Red Cross Blood Center, Ulm, Germany after informed consent was obtained.

2.1.2 Reagents

Bovine Serum Albumin (BSA)	Serva Electrophoresis GmbH, Heidelberg
D-Biotin Stock Solution for Streptamer Technology	IBA GmbH, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany
Ficoll-Biocoll Separation Solution	Biochrom AG, Berlin, Germany
Human AB Albumin	DRK Blutspendedienst, Mannheim, Germany
Human AB Plasma	DRK Blutspendedienst, Ulm, Germany
Interleukin-7 (IL-7)	Strathmann, Biotec GmbH
Interleukin-2 (IL-2)	Sigma, Steinheim, Germany
β_2-MG	Sigma, Steinheim, Germany
L-Glutamine	Biochrom AG
MHC	IBA GmbH, Göttingen,Germany
Penicillin	Invitrogen Gibco, Grand Island, USA
RPMI 1640	Biochrom AG, Berlin, Germany
Streptomycin	Invitrogen Corporation, USA
Tween 20	Sigma
WT1-derived peptide (pos: 126-134	GL Biochem (shanghai) Ltd.

RMF PNA PYL).

2.1.3 Antibodies

Alexa Fluor 647 Rat Anti-Human CD197 (CCR7)	BD Biosciences, USA
APC Mouse Anti-Human CD69	BD Biosciences, USA
APC Mouse Anti-Human CD137	BD Biosciences, USA
PE Mouse Anti-Human CD19	BD Biosciences, USA
FITC Mouse Anti-Human CD4	BD Biosciences, USA
FITC Mouse Anti-Human CD8	BD Biosciences, USA
FITC Mouse Anti-Human CD28	BD Biosciences, USA
FITC Mouse Anti-Human CD45RA	BD Biosciences, USA
FITC Mouse Anti-Human CD107a	BD Biosciences, USA
FITC Mouse Anti-Human HLA-A2	BD Biosciences Pharmigen
PerCP Mouse Anti-Human CD3	BD Biosciences, USA
PerCP Mouse Anti-Human CD8	BD Biosciences, USA
APC Rat IgG2a,κ Isotyp control	BD Biosciences, USA
PE Mouse IgG1,κ Isotype Control	BD Biosciences Pharmigen
FITC Mouse IgG1,κ Isotype Control	BD Biosciences Pharmigen
PerCP Mouse IgG1 Isotype Control	BD Biosciences Pharmigen
CD8 magnetic-activated cell sorting (MACS) beads, (CD8 microbeads, human)	Miltenyi Biotec, Bergisch Gladbach, Germany
Magnetic Beads for Streptamer Technology	IBA GmbH

Strep-Tactin PE for Streptamer Technology	IBA GmbH
BD Calibrite^{TM} Beads	BD Biosciences, USA
HLA-A*0201 Tetramer WT-1 126-134 (RMFPNAPYL) PE	Ludwig Institute for Cancer Research, Lausanne, Switzerland
HLA-A*0201 Tetramer Mutated D227k/T228A WT-1 126-134 (RMFPNAPYL) PE	Ludwig Institute for Cancer Research, Lausanne, Switzerland

2.1.4 Solutions and buffers

DPBS (10×)	Invitrogen Coporation, USA
Destilled water	Fresenius Kabi Deutschland GmbH, Homburg, Germany
FACS Buffer	PBS containing 1% BSA
BD FACS Flow	BD Biosciences, Heidelberg, Germany
BD FACS Clean	BD Biosciences
BD FACS Rinse	BD Biosciences
Separation buffer (PBS/EDTA buffer)	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
IS buffer	IBA GmbH

2.1.5 Equipment

Autoclave	WEBECO GmbH, Germany
24-well tissue culture plate (flat bottom)	BD Biosciences, New Jersey, USA
-80 °C ultra low temperature freezer	SANYO Electric Biomedical Co. Ltd, Japan
96-well tissue culture plate (flat bottom)	BD Biosciences, New Jersey, USA

96-well tissue culture plate (u-bottom)	BD Biosciences, New Jersey, USA
BD FACScan^{TM} Flow Cytometer	BD Biosciences, New Jersey, USA
Combitips plus (1.25 ml, 2.5 ml, 5 ml, 10 ml, 12.5 ml, 25 ml)	Eppendorf Ag, Hamburg, Germany
CS-15R Centrifuge	Beckman Biotechnology, Germany
CS-6R Centrifuge	Beckman Biotechnology, Germany
Ep T.I.P.S Reloads 0.1-20 µl, 2-200 µl, 50-1000 µl,	Eppendorf AG, Hamburg, Germany
Gloves	VWR International GmbH, Darmstadt, Germany
Hemocytometer chamber	Optik Labor, Berlin, Germany
Inverse microscope	Carl Zeiss, TELAVAC31, Germany
Light microscope	Carl-Zeiss, Germany
Liquid nitrogen tank	Model 8038 S/N 14830.59 Forma Scientific Inc, Germany
Magnetic stirrer	Ikamag TRC, Renner GmbH, Ludwigshafen, Germany
MS columns	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS Pre-Separation Filters	Miltenyi Biotec, Bergisch Gladbach, Germany
Non pyrogenic serologic pipettes (2 ml, 5 ml, 10 ml, 25 ml, 50 ml)	Corning Incorporation, New York, USA
Pipette Boy (IBS Pipetboy accu)	Integra Biosciences AG, Chur, Switzerland
Polypropylene Conical Centrifuge Tubes (15 ml, 50 ml)	Becton and Dickinson Labware, NJ, U.S.A
Power Pac TM HC Power Supply	Bio-Rad, USA
Refrigerator	Liebherr, Ochsenhausen, Gemany

Sterile bench (Laminar flow cabinet)	TYP. HS 18/2, Heraeus instruments, Germany
OctoMACS™ separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Syringe (1 ml, 5 ml, 10 ml, 20 ml, 50 ml)	BD Biosciences, New Jersey, USA
Thermomixer Compact	Thermon Eppendorf, Germany
Incubator Holder	Heraeus, Hanau, Germany
Vortex-2™ genie	Model G-560E, Scientific Industries, Inc. Bohemia, NY, U.S.A
Waterbath	GFL, Burgwedel, Germany

2.2 Methods

2.2.1 Ficoll density separation

All samples were taken from 40 HLA-A2^{+} healthy donors and 10 HLA-A2^{+} patients after their informed consent was obtained in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Biocoll Separation Solution density gradient centrifugation from Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood buffy coat preparations from healthy donors or from EDTA anticoagulated blood samples from patients. The viability of PBMCs obtained was always >95%, as determined by trypan blue staining (Trypan Blue Solution 0.4%). The viable cells were quantified in a Neubauer chamber. For cellular assays, Ficoll separated PBMCs were tested freshly or cryopreserved in freezing medium (RPMI 1640 containing 20% human AB serum,100 units/ml penicillin, 100 units/ml streptomycin, 2 mM L-glutamine and 10% DMSO) and stored in liquid nitrogen until further use. For recovery, cryopreserved cells were thawed quickly and washed with plain medium (RPMI 1640 containing 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM L-glutamine) for further use.

2.2.2 Flow cytometry (FACS)

To evaluate the percentage of the WT1-specific $CD8^+$ T cells, the samples were analyzed by flow cytometry. $CD8^+$ T cells were detected using a CD8-fluorescein isothiocyanate (FITC) antibody and a CD3-peridinin chlorophyll (PerCP) antibody. WT1-specific $CD8^+$ T cells were measured by using the WT1 major histo-compatibility complex with Strep-Tactin-Phycoerythrin (PE) conjugate. HLA-A*0201/ WT1 peptide streptamer*PE was specifically synthesized at IBA GmbH. Prior to staining, the WT1 major histocompatibility complex (MHC) was incubated with PE-labeled Strep-Tactin for 45 min at 4°C in the dark. WT1/MHC was used at a concentration of 0.1 µg, 0.2 µg, 0.4 µg, 0.6 µg and 0.8 µg to stain 1×10^6 cells in 100 µl per test. By this dilution chain, the appropriate amount of WT1/MHC-streptamers to stain a pellet of 1×10^6 cells was defined as 20 µl (0.4 µg) of the WT1/MHC-streptamers (0.75 µg Strep-Tactin-PE with 2 µg MHC in a final volume of 100 µl IS Buffer) and used for subsequent experiments.

For three-color staining, 10 µl anti-CD8*FITC and 10 µl anti-CD3*PerCP were added at 4 °C for 20 minutes in the dark. After washing twice with 1×PBS, stained cells were analyzed by flow cytometry (FACScan, CellQuest™). In all cases at least 100,000 events were collected for analysis. Each sample was run with an appropriate isotype control and this was used to define the stained cells. Analysis was performed on tightly gated lymphocytes to exclude dead cells and debris.

2.2.3 Mixed lymphocyte peptide culture (MLPC)

$CD8^+$ T lymphocytes were isolated from PBMCs by using CD8 magnetic-activated cell sorting (MACS) beads. Briefly, cells were centrifuged at 1,200 rpm for 10 minutes with separation buffer (1 L PBS + 0.5% human Serum Albumin +1.9 mM EDTA). Supernatants were removed carefully and cell pellets were resuspended in 80 µl of separation buffer and 20 µl of CD8 MicroBeads per 10^7 total cells. Cells were mixed well and incubated for 15 minutes at 4°C. After incubation, 5 ml of separation buffer were added to the tube and cells were centrifuged at 1,200 rpm for 10 minutes. After centrifugation, cell pellets were resuspended in 500 µl of separation buffer. MS column was placed in the magnetic field of OctoMACS™ Separator and column was rinsed with 500 µl separation buffer once. Cell suspensions above were applied onto the column. $CD8^-$ cells passed through the column, while $CD8^+$ cells were retained in the column. The column was washed

with 500 µl separation buffer three times. Then column was removed from the separator and placed on a 15 ml tube. 1 ml separation buffer was added to the column and a plunger supplied with the column was firmly applied. $CD8^+$ T lymphocytes were collected in the tube.
After the PBMCs were isolated into $CD8^+$ and $CD8^-$ fraction with a MACS column according to the MACS protocol (Miltenyi, Bergisch-Gladbach, Germany), more than 95% purity was reached in the $CD8^+$ fraction as confirmed by flow cytometry. Then, the $CD8^-$ antigen-presenting cells (APCs) were irradiated with 30 Gy and pulsed with 20 µg/ml of the WT1-peptide (pos:126-134 RMF PNA PYL) and 2.5 µg/ml of the β_2-Microglobulin (β_2-MG) for 2 hours at 37°C, followed by a washing step. After co-incubation of $CD8^-$ T lymphocytes with $CD8^+$ T lymphocytes (2×10^6 $CD8^-$ cells : 5×10^5 $CD8^+$ cells/ ml) over night at 37°C, 5% CO_2 and 99% humidity, the MLPC was supplemented with 10 U/ml recombinant human interleukin-2 (IL-2) and 20 ng/ml IL-7 on day +1. On day +8 and day +14, the steps of incubation were repeated. After 8, 14 and 21 days of culture, respectively, cells were harvested and evaluated for the proliferation of WT1-specific $CD8^+$ T cells by streptamer staining and FACS analysis as described above.
The WT1 peptide used in our study was synthesized by Thermo Electron Corporation to a minimum of 95% purity as measured by high performance liquid chromatography. The peptide was dissolved in DMSO mixed with phosphate buffered saline (PBS) at a concentration of 1 µg/µl for individual experiments.

2.2.4 Isolation of antigen-specific T cells using streptamer magnetic beads

First, the magnetic WT1/MHC-streptamer complex was used to label WT1-specific $CD8^+$ T cells according to their antigen specificity. Thereafter beads-labeled cells were separated from non-specific cells by a magnetic field. Later, D-biotin was added to the purified T cells. Briefly, this process was divided into three steps.

2.2.4.1 Preparation of WT1/MHC streptamer magnetic beads

After incubation of 50 µl streptamer magnetic beads, 8 µl WT1 MHC, and 90 µl IS buffer over night at 4°C in the dark, 1 ml IS buffer was added to WT1/MHC-streptamer magnetic bead solution and loaded on a MS column in the magnetic field to wash away unbound MHC and streptamer magnetic beads. Then the MS column was taken outside of the magnetic field and the retained beads were firmly

flushed out by 250 µl IS Buffer using the supplied plunger supplied with the column.

2.2.4.2 Preparation of peripheral blood mononuclear cells (PBMC)

The procedure was optimized to isolate antigen-specific T cells from 2×10^7 peripheral blood mononuclear cells. Higher cell numbers require larger amounts of MHC-streptamer magnetic beads. Cryopreserved PBMCs were thawed quickly and washed with 20 ml plain medium (RPMI 1640 containing 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM L-glutamine). After washed with IS buffer and resuspended in 10 ml IS buffer, these cells passed through enclosed 100 µm nylon mesh to remove cell clumps which may clog the columns. 2×10^7 cells were taken and placed on ice for further use.

2.2.4.3 Magnetic cell separation (MACS™) by columns

2×10^7 cells were mixed with 250 µl WT1/MHC-streptamer magnetic bead solution prepared above and incubated 45 minutes at 4°C in the dark. Then, these cells were washed twice with IS buffer to eliminate unbound magnetic beads which may trap cells on the column unspecifically and resuspended in 2 ml IS buffer for magnetic separation.

The MS columns were placed into the magnetic field and prepared by rinsing with 3 ml IS buffer. Then the resuspended cells were applied onto the columns allowing the cells to pass through and collect effluent. After the columns were washed three times with 2 ml IS buffer, the columns were taken outside of the magnetic field and were eluted with three times 2 ml IS buffer into a fresh vial. Thus, the positive cell fraction was collected for further study.

2.2.4.4 Dissociation of streptamers by D-biotin

The fraction of positive cells was centrifuged and resuspended in 2 ml IS buffer containing 2 mM D-biotin and incubated on ice for 20 minutes twice. Thereafter the cells were washed with 5 ml IS buffer 4 times. Then these cells were stained with PE-labeled streptamers and FACS analysis was performed as described above.

2.2.5 Cell surface immunophenotyping by flow cytometry

Samples were analyzed by flow cytometry before and after separation with the WT1 streptamer technology. The WT1/MHC-streptamer fluorochrome complex

was prepared as described above. Before staining, the cells were washed and resuspended in IS buffer, and subsequently incubated with the conjugate of WT1/MHC-streptamer*PE for 45 minutes at 4°C in the dark. Then, the cells were incubated with anti-CD8*PerCP, anti-CCR7*APC, anti-CD45RA*FITC, anti-CD107a*FITC, anti-CD69*APC, anti-CD137*APC, anti-CD28*FITC for 45 minutes at 4°C in the dark. After washing twice with 1×PBS, the cells were subjected to four-color flow cytometry (BD FACS Calibur System) and analyzed by using the FACS Weasel Software^{TM}.

3. Results

3.1 Optimization of the streptamer technology

In order to assure the isolation of fully functional, non-induced antigen-specific T cells, all the reagents and cells had reached 4°C before starting the protocol, and all steps were performed at 4°C. Background levels were determined by comparison with HLA/A2-seronegative healthy donor samples. The lowest background signals were below 0.05%. A titration of the WT1/MHC was performed for the selection of a standard concentration of streptamer WT1/MHC. WT1/MHC at a concentration of 0.1 µg, 0.2 µg, 0.4 µg, 0.6 µg and 0.8 µg, respectively, per 1×10^6 cells in 100 µl per tube was tested in triplet. By comparison, appropriate amounts of streptamer were used to stain a pellet of 1×10^6 cells, 20 µl (0.4 µg) of the MHC (0.75 µg Strep-Tactin-PE with 2 µg MHC in a final volume of 100 µl IS Buffer) was used as a standard amount for subsequent experiments (Figure 2). Higher concentrations of streptamer resulted in the detection of similar or even lower T cell frequencies, indicating saturation.

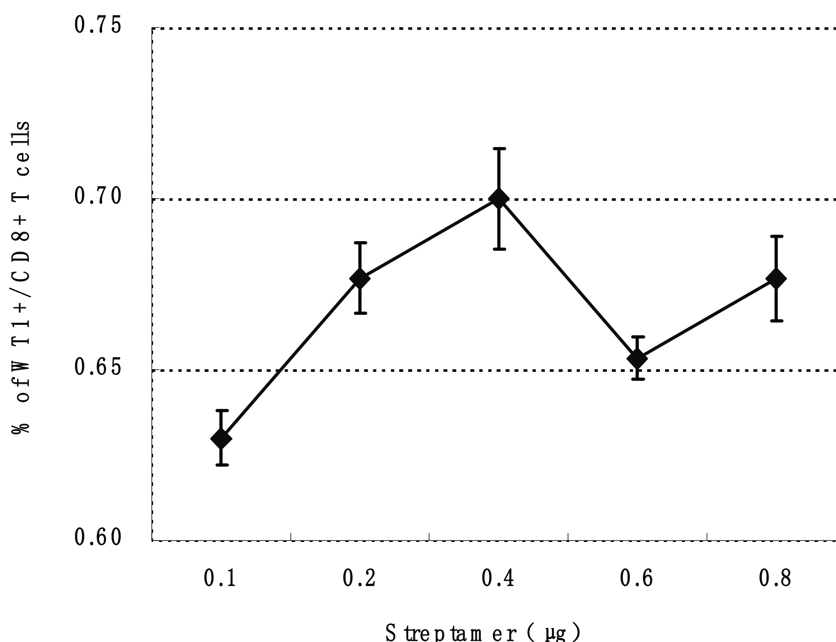

Figure 2. Different amounts of streptamer were added to obtain curves for optimal staining procedures. The data show that 20 µl (0.4 µg) per 100µl of the MHC molecules was an ideal concentration for streptamer staining. Higher concentrations of streptamer resulted in the detection of similar or even lower T cell frequencies, indicating saturation.

3.2 Identification of WT1-specific CD8⁺ T cells in healthy volunteers and in patients

In order to investigate whether WT1-specific $CD8^+$ T cells exist in healthy donors and patients with AML, forty samples from HLA/A2-seropositive healthy donors and ten samples from HLA/A2-seropositive patients were analyzed using fluorescence-activated cell sorting (FACS) staining with WT1/HLA-A*0201 Streptamers. Identification of ten HLA/A2-seronegative healthy donors, as controls, was also done and no WT1-specific $CD8^+$ T cells were founded. All 40 HLA/A2-seropositive healthy donors and ten patients with AML, we were able to detect WT1-specific $CD8^+$ T cells. The frequency of WT1-specific $CD8^+$ T cells specifically recognizing the HLA-A2 restricted WT1 epitope ranged from 0.08% to 1.61% (Figure 3). In 21 (52.5%) of 40 HLA/A2-seropositive healthy donors, the frequencies of over 0.5% of naive WT1 specific $CD8^+$ T cell when compared to all $CD8^+$ T cells were detected, among which the frequencies of 8 healthy donors over 1.0% (Table 1). In five AML patients in complete remission, 0.68 to 3.65% of WT1-specific T cells could be detected, while another five patients at time of diagnose/relapse, the frequencies ranged from 0.13 to 0.90% (Table 2).

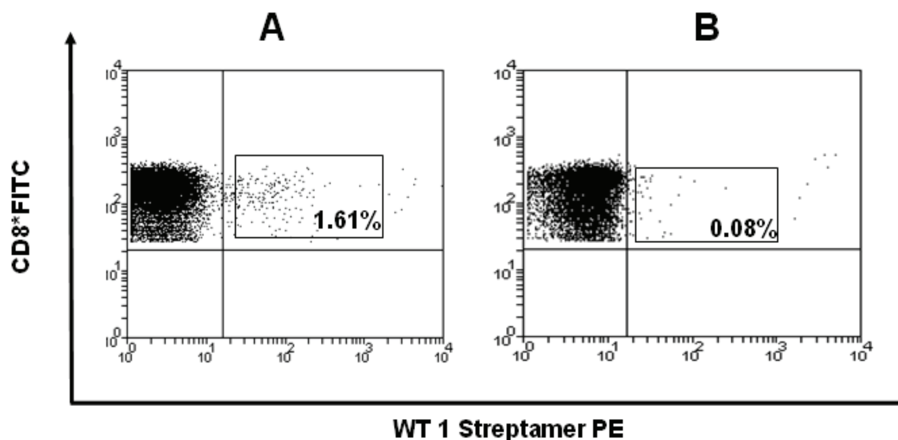

Figure 3. Frequency of WT1-specific $CD8^+$ T cells in the peripheral blood of healthy donors. Mononuclear cells of the peripheral blood were stained with monoclonal antibodies against $CD8^+$ and WT1 peptide specific streptamers as described in the Material and Method section. Panel A shows a high, Panel B a low frequency of double-positive WT1-specific $CD8^+$ T cells observed in two different healthy donors.

Table 1. The percentage of $CD8^+$ T cells specific for WT1 when compared to all $CD8^+$ T cells in HLA-$A2^+$ healthy volunteers

Volunteer	WT-1 specific $CD8^+$ T cells / $CD8^+$ T cells (%)
1	0.49
2	1.61
3	0.72
4	1.01
5	0.51
6	0.11
7	1.24
8	0.92
9	1.12
10	0.72
11	0.15
12	0.63
13	0.48
14	0.60
15	0.30
16	0.82
17	0.31
18	0.11
19	0.31
20	0.96
21	0.72
22	0.23
23	1.53
24	0.41
25	0.99
26	1.16
27	0.96
28	0.41
29	0.78
30	0.47
31	0.46
32	0.83
33	0.27
34	1.28
35	0.35
36	0.08
37	0.40
38	1.27
39	0.45
40	0.27

Table 2. The percentage of $CD8^+$ T cells specific for WT1 when compared to all $CD8^+$ T cells in HLA-$A2^+$ patients

Disease status at the time of analysis	Number	WT-1 specific $CD8^+$ T cells / $CD8^+$ T cells (%)
At time of diagnose/relapse	1	0.13
	2	0.58
	3	0.43
	4	0.90
	5	0.22
In CR	6	1.89
	7	1.68
	8	3.65
	9	1.78
	10	0.68

3.3 Mixed lymphocyte peptide culture of HLA-A*0201 seropositive/ WT1 streptamer positive healthy donors and patients

To document the induction of specific T cell responses in healthy donors and patients with AML, we tested the WT1-derived peptide (pos: 126-134 RMF PNA PYL). Flow cytometry for WT1-specific $CD8^+$ T cells from the peripheral blood was performed after MLPC.

We evaluated the proliferation of WT1-specific $CD8^+$ T cells on days 1, 8, and 15 of MLPC by streptamer staining and FACS analysis. When $CD8^+$ T lymphocytes of HLA-A*0201 seropositive and WT1 streptamer positive healthy donors and patients were subjected to two rounds of stimulation with irradiated autologous $CD8^-$ APCs pulsed with WT1 peptide, WT1-specific $CD8^+$ T cells were effectively expanded (Figure 4). The data from healthy donors as well as patients showed a ten-fold but not higher increase in the percentage of HLA-A2/WT1 streptamer (PE)-positive $CD8^+$ T lymphocytes.

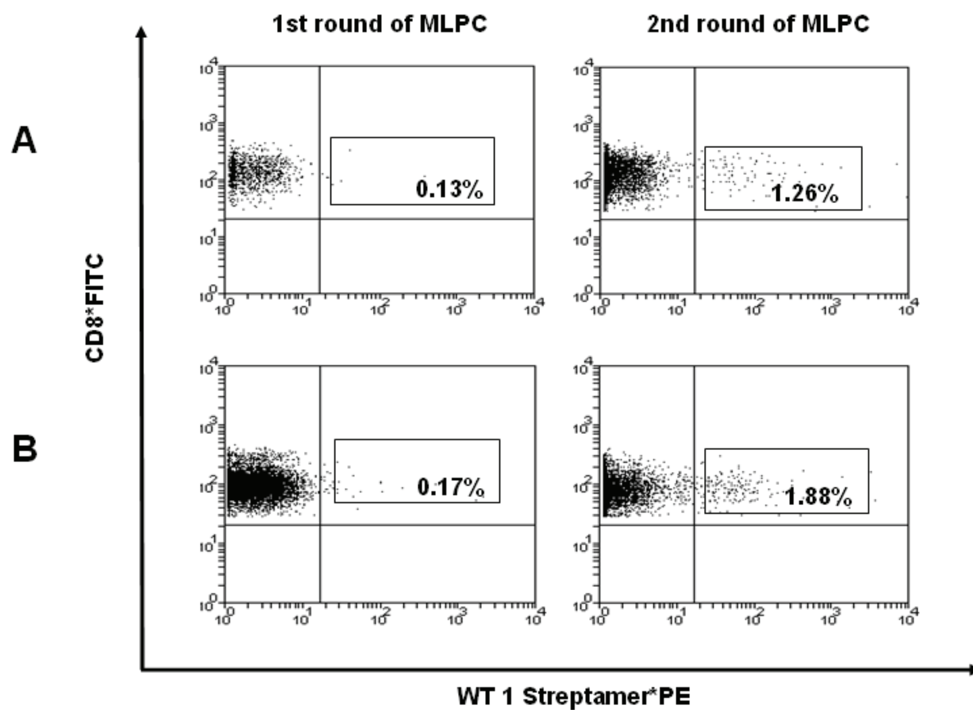

Figure 4. Augmentation in frequency of WT1-specific CD8$^+$ T cells through mixed lymphocyte peptide cultures (MLPCs) of mononuclear cells of the peripheral blood (PBMCs) from healthy donors. CD8$^+$ T lymphocytes were subjected to one or two rounds of stimulation with irradiated autologous CD8$^-$ antigen-presenting cells plused with WT1 peptide as described in the Material and Methods section. The dot plots show the percentage of HLA-A2/WT1 streptamer (PE)-positive CD8$^+$ T lymphocytes. A. The percentage of HLA-A2/WT1 streptamer (PE)-positive CD8$^+$ T lymphocytes in healthy donor after the 1st and 2nd round of stimulation. B. The percentage of HLA-A2/WT1 streptamer (PE)-positive CD8$^+$ T lymphocytes in a patient after one or two rounds of stimulation as described above. Both sets of data demonstrate a ten-fold increase in the percentage of HLA-A2/WT1 streptamer (PE)-positive CD8$^+$ T lymphocytes.

3.4 Isolation of antigen-specific T cells by WT1 streptamer magnetic beads

To evaluate the purity of the enriched antigen-specific T cells, positive fractions were stained and analyzed by flow cytometry. Before separating of PBMCs, the WT1-specific $CD8^+$ T cells ranged from 0.08% to 1.61% of the total $CD8^+$ T cells population. After separation, a purity of 30-50 fold increase could be observed for WT1-specific $CD8^+$ T cells of the total $CD8^+$ T cell population. The maximum purity we could achieve by this method was 91% (Figure 5).

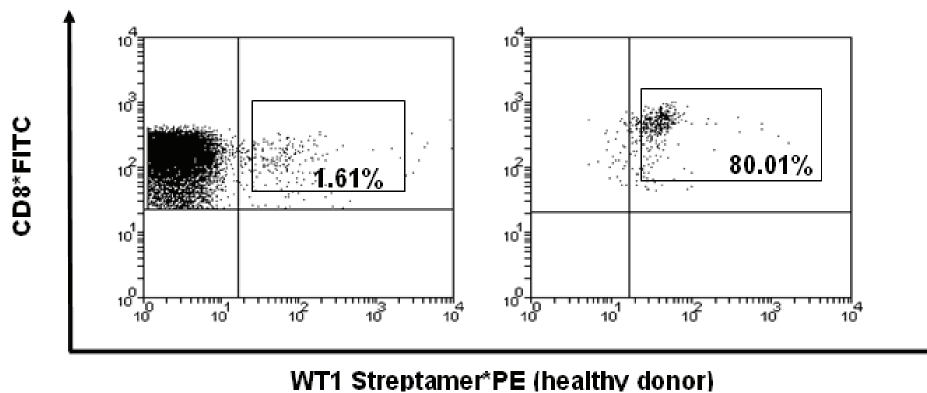

Figure 5. Isolation of WT1-specific $CD8^+$ T cells via magnetic cell separation (MACS) of PBMCs from a HLA-A2$^+$ healthy donor. The dot plots show the percentage of HLA-A2/WT1 streptamer (PE)-positive $CD8^+$ T lymphocytes before and after separation. For subpopulations of $CD8^+$ T cells, a 30-50 fold increase in the percentage of HLA-A2/WT1 streptamer (PE)-positive $CD8^+$ T lymphocytes could be observed after MACS separation.

3.5 Immunophenotyping of WT1-streptamer-specific $CD8^+$ T cells

In order to investigate the immunophenotype of WT1-specific $CD8^+$ T cells purified by streptamer, the samples of HDs before and after separation were identified by the staining of phenotypic markers. WT1-specific $CD8^+$ T cells before and after separation were consistently $CD8^+WT1Streptamer^+CD28^-CD45RA^+CD69^-CD107a^-CCR7^-CD137^-$ effector T cells, but of varying intensity for CCR7, the WT1 streptamer specific $CD8^+$ T cells after isolation expressed a high percentage of CCR7 cells. These results indicated that most purified WT1 streptamer specific $CD8^+$ T cells have an effector T cell immunophenotype (Figure 6).

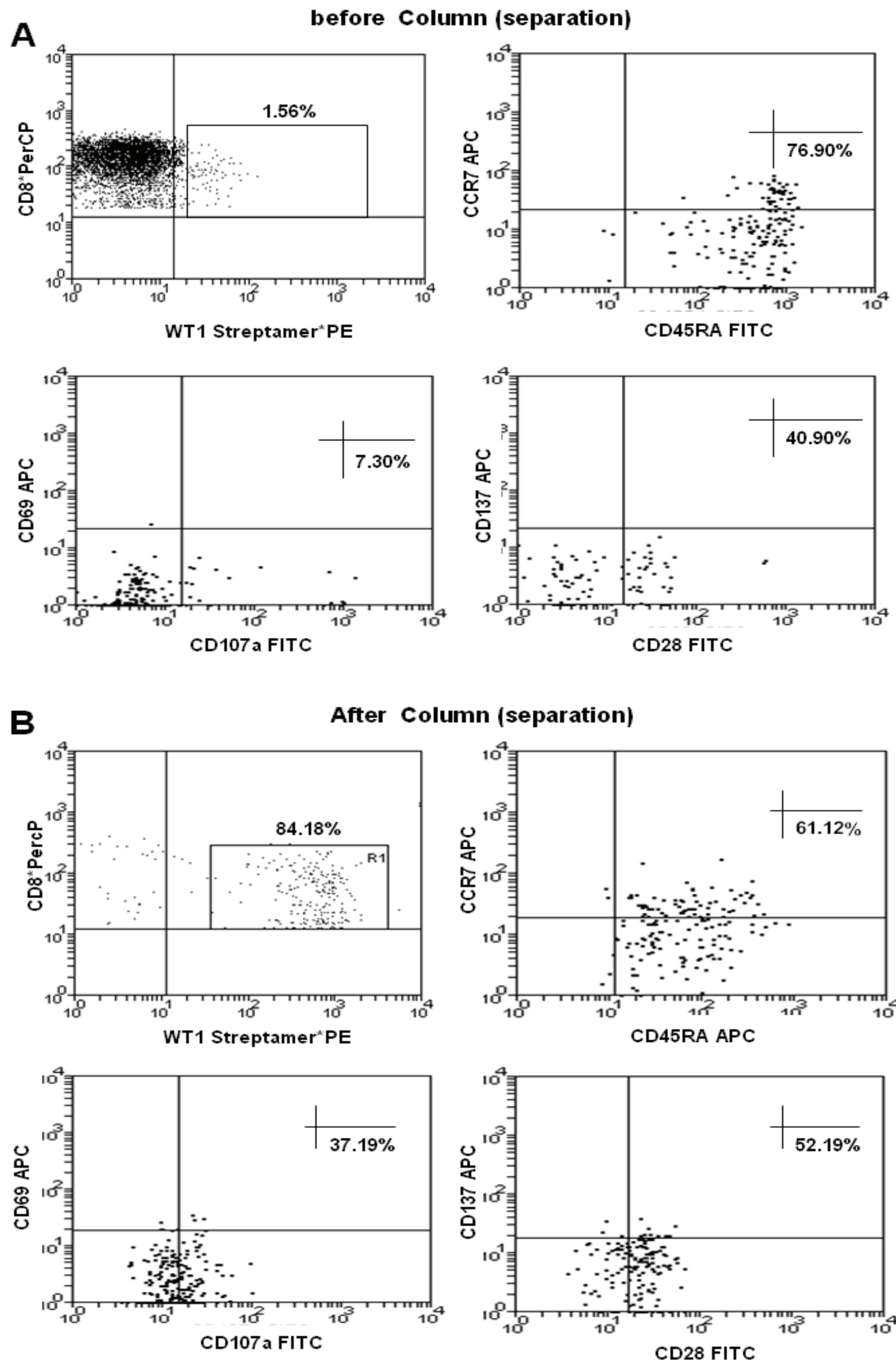

Figure 6. Immunophenotyping of WT1 streptamer specific CD8$^+$ T cells. WT1 streptamer specific CD8$^+$ T cells were further characterized by FACS analysis, demonstrating the existence of CD8$^+$WT1Streptamer$^+$CD28$^-$CD69$^-$CD45RA$^+$CCR7$^-$CD107a$^-$CD137$^-$ T cells in the peripheral blood of healthy donors, thus indicating the effector T cell status of these cells. Panel A (before separation) and Panel B (after separation) expressed a similar immunophenotype, suggesting functional, non-induced WT1-specific CD8$^+$ T cells were purified by streptamer technology.

4. Discussion

4.1 Streptamer technology

A novel multimer technology designated streptamers has been developed to identify and purify antigen-specific $CD8^+$ T cells (Neudorfer et al. 2007, Yao et al. 2008). The function of antigen-specific $CD8^+$ T cells could be preserved after separation from PBMCs by streptamer technique (Neudorfer et al. 2007). Therefore the streptamer technique may open new avenues toward an innovative immunotherapeutic approach: the selection of antigen-specific $CD8^+$ T cells at the good manufacturing practice (GMP) level for adoptive T cell transfer.

The main principle of the streptamer technology is based on strep-tags and strep-tactin. Strep-tags, which are short peptides, have a high binding selectivity for strep-tactin, an engineered streptavidin (Voss et al. 1997). The binding affinity (Kd: $1×10^{-6}$ M) of strep-tag II to strep-tactin is up to 100 times higher than to streptavidin (Voss et al. 1997). Strep-tags can be fused to recombinant protein, which allows efficient one-step purification of such fusion proteins on immobilized strep-tactin under physiological conditions, thus preserving their bioactivity. If MHC multimers based on the interaction of strep-tag II with strep-tactin could be generated for T cell staining, it should thus be possible to competitively disrupt multimers in the presence of relatively low concentrations of D-biotin, as for the molecule D-biotin, which binds with higher affinity to strep-tactin (Kd$<1×10^{-13}$ M), effectively competes with strep-tag II for the binding site (Knabel et al. 2002).

Streptamers complexes consist of peptide loaded HLA-strep-tag III molecules and strep-tactin polymers. Human leukocyte antigen (HLA)-strep-tag III is a fusion protein between one HLA monomer and two strep-tag II sequences sequentially arranged by a short linker (Schmidt et al. 1996, Junttila et al. 2005). Strep-tag II is a comprising eight amino acid residues peptide (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) showing a strong binding affinity for an engineered streptavidin derivative called strep-tactin (Voss et al. 1997). The binding affinity of strep-tag III to strep-tactin is higher than strep-tag II to strep-tactin (Kd: $1×10^{-7}$ M) (Neudorfer et al. 2007). Figure 1 showed the rationale about how a functional, non-induced antigen specific T cell preparation was yielded by streptamer technology, indicating an advance with regard to the isolation of antigen specific T cells when compared to

conventional multimers technology (Knabel et al. 2002, Neudorfer et al. 2007).
The significant characteristic of the streptamer technology is the fact that it allows the isolation of antigen-specific $CD8^+$ T cells with preserved function and that is available at GMP level (Neudorfer et al. 2007), whereas lytic function and proliferation of the T cells is impaired in the presence of conventional MHC multimers such as tetramer. Other important advantages of streptamer technology have been identified, including the absence of toxicity to T cells, the lack of adverse side effects for clinical *in vivo* application, the absence of immune responses directed against the multimer reagents and the maintenance of the capacity of the transferred T cells to migrate *in vivo* to localizations, where they are required for immune reactions (Knabel et al. 2002, Neudorfer et al. 2007). Therefore, the streptamer technique will be of great diagnostic and clinical value and might facilitate the development of adoptive T cells transfer regimens for the treatment of patients with cancer or infectious diseases (Neudorfer et al. 2007).
HLA-A2/CMV streptamers and HLA-A2/melan-A streptamers were used for the identification and purification of antigen-specific $CD8^+$ T cells. The results showed that the quality of separation in terms of the number and the purity of isolated $CD8^+$ T cells was equally high for streptamer and tetramer. The reversibility of streptamer resulted in preserved cytolytic activity of antigen-specific T cells after identification. In contrast, tetramer-treated T cells displayed a reduced cytolytic potential (Neudorfer et al. 2007). According to the manufacturer, the maximum purity of CMV antigen-specific T cells they could achieve by streptamer technology was 82%.
HLA-A2/WT1 streptamer has been developed to identify WT1-specific $CD8^+$ T cells, while little is known about the difference between the functional status of WT1-specific $CD8^+$ T cells mediated by streptamers and tetramers. We wondered whether HLA-A2/WT1 streptamer had an advantage over HLA-A2 /WT1 tetramer on the purification of $CD8^+$ T cells which may facilitate the adoptive immunotherapy of patients with malignancy tumor.

4.2 Detection of WT1 specific $CD8^+$ T cells present at low numbers in the PBMCs of healthy donors and patients

Both CMV_{pp65}-specific/tetramers and the CMV_{pp65}-specific /streptamer have been used for the detection and isolation of CMV-specific $CD8^+$ T cells present at low

number in the PBMCs (Busch et al. 1998, Keenan et al. 2001, Neudorfer et al. 2007, Yao et al. 2008), while WT1-specific/tetramers only have been used for the measurement of WT1 specific $CD8^+$ T cells immune response to the vaccine or immunotherapy (Morita et al. 2006, Rezvani et al. 2007), as the percentage of WT1-specific $CD8^+$ T cells in the PBMCs of healthy donors is too low to be detected by WT1/tetramer. Therefore we hoped to find a new technology to detect WT1-specific $CD8^+$ T cells present at low numbers in the PBMCs of healthy donors and patients.

Only when the streptamer technology was used at 4°C in the dark, T cells could be identified and purified without altering their original phenotype and functional status (Knabel et al. 2002). Based on these data, we performed all the steps, including the staining of the cells and the following dissociation, at 4°C in the dark.

In order to optimize the streptamer technology, a titration of the MHC/WT1 was performed for the selection of a standard concentration of streptamer MHC/WT1 according to the amounts recommended by manufacturer. 20 µl (0.4 µg) of the WT1/MHC (0.75 µg Strep-Tactin-PE with 2 µg MHC in a final volume of 100 µl IS Buffer) was selected as a standard amounts to stain a pellet of 1×10^6 cells for subsequent experiments (Figure 2). The results show that higher concentrations of streptamer reagent resulted in the detection of similar or even lower T cell frequency, an explanation for this observation might be a saturation phenomenon.

We evaluated the frequency of the WT1-specific $CD8^+$ T cells in healthy donors by the streptamer technology. All samples were analyzed using FACS staining with WT1/HLA-A*0201 streptamers. Ten HLA/A2 negative healthy donors were used as negative controls: no WT1-specific $CD8^+$ T cells were found. In all 40 HLA/A2 positive healthy donors, we were able to detect WT1-specific $CD8^+$ T cells. The frequency of $CD8^+$ T cells specifically recognizing the HLA-A2 restricted WT1 epitope ranged from 0.08% to 1.61% (Figure 3). In 21 (52.5%) of 40 HLA/A2 positive healthy donors, naive WT1-specific $CD8^+$ T cell frequencies of more than 0.5% out of all $CD8^+$ T cells were detected. In 8 healthy donors we detected frequencies over 1.0% (Table 1). The frequency of the WT1-specific $CD8^+$ T cells in 10 patients with AML was also investigated, in five AML patients in complete remission, also 0.68 to 3.65% of WT1-specific $CD8^+$ T cells could be detected. In contrast in another five patients at the time of diagnose/relapse, the frequencies ranged only from 0.13 to 0.90% (Table 2). These results indicated that we were

able to employ the streptamer technology to identify the WT1-specific $CD8^+$ T cells present at low numbers in the PBMCs of healthy donors and patients.
By streptamer technology, WT1-specific $CD8^+$ T cells could be detected in all HLA-A2 positive healthy donors, whereas WT1-specific $CD8^+$ T cells were not detected by tetramer staining in healthy donors (Rezvani et al. 2007). An explanation for the difference in the amount of WT1-specific $CD8^+$ T cells measured with different multimers might be same as the CMV-specific $CD8^+$ T cells: the incubation of CMV-specific $CD8^+$ T cells with CMV streptamers demonstrated high intensity T cell staining that was comparable to conventional CMV tetramers (Neudorfer et al. 2007). The other reason for higher frequency of WT1-specific $CD8^+$ T cells measured by streptamer technology is the difference of the gate: $CD8^+$ T cells, not the lymphocyte cells, were used for the gate to analyze specific cells in our study. The results showed that the streptamer technology could be used for the identification of the WT1-specific $CD8^+$ T cells present at low numbers in the PBMCs of healthy donors.
Additionally, we found that among five AML patients in complete remission, also 0.68 to 3.65% of WT1-specific $CD8^+$ T cells were detectable, while another five patients at the time of diagnose/relapse, the frequencies ranged from 0.13 to 0.90%, suggesting a WT1-driven GVL effect to the former in CR, for the emergence of WT1-specific $CD8^+$ T cells was associated with a decrease in WT1 expression (Rezvani et al. 2007).

4.3 Purification of WT1-specific $CD8^+$ T cells by streptamers

In our study, we found that streptamer technology showed a higher separation quality in term of the purity of isolated WT1-specific $CD8^+$ T cells. The data showed a ten-fold but not higher increase in the percentage of HLA-A2/WT1 streptamer (PE)-positive $CD8^+$ T lymphocytes in PBMCs of HLA-A*0201 seropositive healthy donors were acquired by MLPC, whereas a purity of 30- to 50-fold increase could be achieved through isolation by streptamer technology for WT1-specific $CD8^+$ T cells of the total $CD8^+$ T cell population. The results indicated that the streptamer technology is a promising method for the purification/positive selection of WT1-specific $CD8^+$ T cells.
Neudorfer et al. reported that the streptamer technology is not only as sensitive as conventional tetramer technology regarding the detection and purification of

minute cell amounts, but also allow the isolation of antigen-specific T cells with preserved function (Neudorfer et al. 2007). To further investigate the functional status of isolated T cells, the samples of healthy donors before and after separation were identified by the staining of phenotypic markers.

$CD8^+$ T cells can be dissected into four groups according to the state of activation/differentiation. On the basis of CD45RA and CCR7 expression, the four different subsets of $CD8^+$ T lymphocytes were previously defined: naive (N: RA^+CCR7^+), effector (E: RA^+CCR7^-), central-memory (CM: RA^-CCR7^+), and effector-memory (EM: RA^-CCR7^-) T cells (Sallusto et al. 1999). Different states of $CD8^+$ T cells activation are associated with different functional and immunophenotypic characteristics. WT1-specific $CD8^+$ T cells identified by streptamer technology were analyzed for expression of CD28, CD45RA, CD69, CD107a, CCR7, and CD137 to characterize a naïve, memory, or effector phenotype respectively. In our study, we found that WT1-specific $CD8^+$ T cells before and after isolation were consistently $CD8^+WT1streptamer^+CD28^-$ $CD45RA^+CD69^-$ $CD107a^-CCR7^-CD137^-$ effector T cells, but of little varying intensity for CCR7, the WT1-streptamer-specific $CD8^+$ T cells after isolation expressed a higher percentage of $CCR7^+$ cells. A frequency of almost 80% for $CCR7^-/CD45RA^+$ cells was acquired. These results indicated that the naïve functional status of $CD8^+$ T cells purified by streptamer technology was preserved and most isolated WT1 streptamer specific $CD8^+$ T cells had an effector T cell immunophenotype. Therefore WT1-specific $CD8^+$ T cells isolated by streptamer could confer immediate immune protection into peripheral tissues.

4.4 Streptamer technology as a promising way for DLIs

CML patients might experience a relapse after allogeneic peripheral blood stem cell transplantation (allo-PBSCT). In general, there are two treatment options: DLI or therapy with a tyrosine kinase inhibitor, such as imatinib, nilotinib or dasatinib. DLI has been identified as an efficacious therapy for patients with CML and AML at relapse after allo-HSCT. It could restore full donor chimerism and produce long-term complete molecular genetic remissions and thus eventually providing cure for the disease (Kolb et al. 1995, Guglielmi et al. 2002, Raiola et al. 2003, Weisser et al. 2006). The effect of DLI against leukemia is mainly based on the GVL reaction.

However, a severe complication of DLI might be the development of acute and

chronic GVHD, which results from the attack of the recipient's organs by T cells from the donor. GVHD may occur in 20%-60% of patients after allo-HSCT (Kolb et al. 1995, Collins et al. 1997, Raiola et al. 2003), leading to significant morbidity and mortality even after treatment with fully matched donor lymphocytes. Therefore, a development of “safer” DLI with less aggressive and more selective anti-cancer effects is urgently required. Selective targeting of specific immune T cells, recombinant interleukin-2-activated natural killer cells and antibody-guided recombinant interleukin-2-activated lymphocytes had been reported (Ruggeri et al. 1999, Slavin et al. 2001, Morecki et al. 2006). These reports above showed that a selective anti-tumor reactivity of donor lymphocytes, which could be able to induce a strong GVL effect with low grade GVHD or even no GVHD, might be a more promising way for DLI.

In this study, we focused on the immunogenic leukemia-antigen (WT1). WT1 has emerged as an important factor in normal and malignant hematopoiesis. The WT1 protein expression is up-regulated in a high proportion of patients with ALL, AML, MDS, and CML and in several solid tumors, including lung, breast, prostate, and ovarian cancer (Miwa et al. 1992, Brieger et al. 1995, Oji et al. 1999). Therefore, it has become an increasingly attractive target molecule for the development of efficient immunotherapy, the feasibility and potential efficacy of CTL against WT1-specific peptides has been identified (Oka et al. 2000, Ohminami et al. 2000, Weber et al. 2009).

Streptamer technology was selected to identify and purify WT1-specific $CD8^+$ T cells, as the streptamer reagent could be detached from the isolated WT1-specific $CD8^+$ T cells prior to transfer. Several side effects caused by reagents could be avoided, including toxicity to T cells, harm for clinical *in vivo* application, immune responses directed against the reagents and loss of the capacity of the transferred T cells to migrate *in vivo* to localizations. Therefore, a functional, non-induced, streptamer-selected antigen-specific $CD8^+$ T cell, which could be available at GMP, will facilitate the development of DLI (Neudorfer et al. 2007, Yao et al. 2008). In our study, WT1-specific $CD8^+$ T cells purified by streptamer technology showed an effector T cell immunophenotype and cytotoxic potential, indicating a promising method for adoptive immunotherapy.

In summary, streptamer technology which could be available at GMP level not only

keeps promise to improve the specificity and sensitivity when compared to tetramers, it also allows to purify antigen-specific T cells while preserving their function. Therefore, streptamer technology constitutes a novel method for the development of DLI.

5. Summary

1) Background: Donor lymphocyte infusion (DLI) may generate a desirable graft-versus-leukemia (GVL) effect, but also elicit a noxious graft-versus-host disease (GVHD). A positive selection of leukemia (antigen)-specific T cells would be highly desirable. In this study, we focused on the immunogenic leukemia-antigen Wilms' Tumor gene 1 (WT1).

2) Aim: Here we investigated whether streptamer technology allows the identification and isolation of WT1-specific $CD8^+$ T cells without altering functional status, thus facilitating the further development of DLI.

3) Material and methods: Peripheral blood samples from forty HLA/A2-seropositive healthy donors (HDs) were characterized fluorescence-associated cell separation by flow cytometry using WT1/HLA-A*0201 streptamers. We also detected the frequency of WT1-specific $CD8^+$ T cells in ten patients with AML. The purification effect of WT1-specific $CD8^+$ T cells from the healthy donors was compared between streptamer technology and mixed lymphocyte peptide culture. Furthermore, the samples of HDs before and after separation were identified by the staining of phenotypic markers to investigate the immunophenotype of WT1-specific $CD8^+$ T cells isolated by streptamer technology.

4) Results: The frequency of $CD8^+$ T cells specifically recognizing the HLA-A2 restricted WT1 epitope ranged from 0.08% to 1.61%. In 21 (52.5%) of 40 HLA/A2-seropositive HDs, naive WT1-specific $CD8^+$ T cell frequencies of more than 0.5% of all $CD8^+$ T cells were detected, in 8 HDs even frequencies higher than 1.0%. As for the frequency of WT1-specific $CD8^+$ T cells in patients with AML, 0.68 to 3.65% of WT1-specific $CD8^+$ T cells could be detected in five AML patients in complete remission, while in five patients at the time of diagnose/relapse, the frequencies ranged only from 0.13 to 0.90%. A ten-fold but not higher increase in the percentage of HLA-A2/WT1 streptamer(PE)-positive $CD8^+$ T lymphocytes in PBMCs of HLA-A*0201 positive HDs was achieved by MLPC, whereas a purity of 30- to 50-fold increase could be obtained through purification by streptamer technology for WT1-specific $CD8^+$ T cells out of the total $CD8^+$ T cell population. WT1-specific $CD8^+$ T cells before and after the separation were consistently $CD8^+WT1streptamer^+CD28^-CD45RA^+CD69^-CD107a^-CCR7^-CD137^-$ effector T cells, a frequencies of almost 80% for $CCR7^-/CD45RA^+$ cells was acquired.

5) Conclusion: Streptamer technology permits the detection of WT1-specific $CD8^+$ T cells present at low numbers in the PBMCs from both HDs and patients. The streptamer technology is a promising method for the selection of WT1-specific $CD8^+$ T cells. The naïve functional status of $CD8^+$ T cells separated by streptamer technology was preserved and most isolated WT1-specific $CD8^+$ T cells demonstrated an effector T cell immunophenotype. Taken together, our study indicated that streptamer technology available at GMP level opens new avenues for the further development of DLIs.

6. Zusammenfassung

1) Hintergrund der Studie: Infusionen von Spender-Lymphozyten generieren beim Empfänger einen erwünschten „Transplantat-gegen-Leukämie-Effekt“ (graft versus leukemia; GVL), verursachen aber gleichzeitig eine schädliche „Transplantat-gegen-Wirt-Krankheit“ (graft versus host disease; GVHD). Eine positive Selektion von T-Lymphozyten, die spezifisch gegen ein Leukämie-Antigen gerichtet sind, ist hierbei sehr wünschenswert. In dieser Arbeit haben wir uns auf das Leukämie-Antigen Wilms-Tumor-Gen 1 (WT1) fokusiert.

2) Ziel der Studie: Wir untersuchten hierbei, ob die Streptamer-Technologie die Isolation von WT1-spezifischen $CD8^+$ T-Zellen erlaubt, ohne dabei den funktionellen Status und die Entwicklung der Spender-Lymphozyten zu verändern.

3) Materialen und Methoden: 40 Proben von HLA/A2-seropositiven gesunden Spendern wurden mit WT1/HLA-A*0201-Streptamer Fluoreszenz-markiert und durchflusszytometrisch untersucht. Es wurden außerdem die Frequenzen von WT1-spezifischen $CD8^+$ T-Zellen in 10 Patienten mit AML untersucht. Wir verglichen die Reinheit von WT1-spezifischen $CD8^+$ T-Zellen von gesunden Spendern nach der Isolation mit der Streptamer-Technik und der Lymphozyten/Peptid-Kultur. Des Weiteren wurden die Proben von gesunden Spendern vor und nach Separation durch Färbung mit phänotypischen Markern untersucht, um den Immunphänotyp von WT1-spezifischen $CD8^+$ T-Zellen darzustellen.

4) Ergebnisse: Die Häufigkeit von $CD8^+$ T-Zellen, die speziell das HLA-A2 WT1-Epitop erkennen, schwankte zwischen 0,08% und 1,61%. In 21 (52,5%) von 40 HLA-A2-seropositiven gesunden Spendern wurden WT1-spezifische T-Zellen mit einer Frequenz von 0,5% detektiert. Bei acht gesunden Spendern lag die Frequenz bei über 1,0%.

Bei fünf Patienten mit einer AML in Kompletter Remission fanden wir 0,68% bis 3,65% WT1-spezifische $CD8^+$ T-Zellen, während die Häufigkeit bei weiteren 5 Patienten, die ein Rezidiv der AML aufwiesen, die Häufigkeit nur bei 0,13% bis 0,90% lag. Eine Steigerung der Ausbeute um das Zehnfache an HLA-A2/WT1-Streptamer (PE)-positiven $CD8^+$ T-Lymphozyten des peripheren Blutes von HLA-A*0201-positiven gesunden Spendern wurde mittels Lymphozyten/Peptid-Kultur erreicht. Eine Verbesserung der Reinheit von WT1-spezifischen $CD8^+$ T-Zellen um

das 30 bis 50fache konnte erreicht werden, indem man die Streptamer Technik auf die $CD8^+$ T-Zell-Population anwendete. WT1-spezifische $CD8^+$ T-Zellen waren vor sowie nach der Separation kongruent $CD8^+WT1streptamer^+CD28^-CD45RA^+CD69^-CD107a^-CCR7^-CD137^-$ T-Effektor-Zellen. Beinahe 80% $CCR7^-/CD45RA^+$ T-Zellen wurden separiert.

5）Schlussfolgerung: Die Streptamer-Technologie erlaubt die Erkennung auch einer geringen Anzahl von WT1-spezifischen $CD8^+$ T-Zellen im peripheren Blut von gesunden Spendern und Patienten. Sie ist eine zuverlässige Methode für die Isolierung von $CD8^+$ T-Zellen mit hoher Reinheit. Die Funktionalität von $CD8^+$ T Zellen, die mittels Streptamer-Technik isoliert werden, bleibt erhalten. Die meisten der Streptamer-spezifischen $CD8^+$ T-Zellen sind T-Effektorzellen. Die untersuchte Streptamer-Technik, die auf GMP-Level verfügbar ist, könnte für die weitere Entwicklung von Spenderlymphozyten verwendet werden.

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8. Acknowledgement

First of all, I would like to express my gratitude to Prof. Dr. med. Mathias Freund for accepting me as a M.D. student in the Department of Internal Medicine III at the University of Rostock.

I owe special thank to my supervisor Prof. Dr. med. Michael Schmitt, an incomparable advisor in my view, who has lent a guiding hand and been a patient teacher since I began work on this thesis. Thanks to him for inviting me to join his group and spending countless time on my research. His unique and novelty ideas and project, invaluable supervision, guidance in every progress of project, such as: data analysis, review of my thesis manuscript of its various sections and commenting on them, all those gently moved me towards my destination. I am impressed deeply by his abundant knowledge and precisely scientific attitude. His kindness and patience, carefulness and precision had a great impact on me, from which I will benefit a lot in my future. I sincerely hope more scientific cooperation and communication under his guidance will continue in the future.

I can not give enough thanks to Prof. Dr. Bao-an Chen for his encouragement and attention for me to study in this department. Without his help, I could not have opportunity to study and learn so much advanced technology and research in Germany.

My sincere thanks also go to Dr. med. Anita Schmitt, Ms. Marlies Goetz and Ms. Cornelia Herbst for the expert technical assistance and the generous help they offered me in many different ways during my study.

Special thanks to Xun XU, a very diligent and clever young fellow, and Ms. Yvonne Gross who I was never lucky enough to work with until now, for their sanguine help and others whose name have not been mentioned here, who gave me lots of support and courage in my works and life here in Germany.

I would like to take this opportunity to express my special thanks to my daughter, who had reached the age during my work on this research where her encouragement and criticism were both helpful and loving at the same time.

I am deeply grateful to my parents and parents-in-low for their continued support and endless encouragement.

I want to express my love and constant gratitude to Yuan KANG, my wife, at whom I wonder every day. She is the person to whom I look for support, wisdom and

understanding I find nowhere else; that has been especially true in every aspect of my life. She brings unusual insight and perception to all the subjects we discuss, and bring those qualities -- and her unabashed enthusiasm -- to the whole of our lives together.

9. Eidesstattliche Versicherung

Ich versichere, dass mir darüber hinaus keine weitere Hilfe zuteil geworden ist und dass ich bei der Anfertigung der Dissertation keine anderen als die in der Arbeit genannten Hilfsmittel benutzt habe.

Rostock, __. __. ____ ___________

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