Wilms' tumor 1 (WT1) specific immune cells as a tool for cellular immunotherapy in acute myeloid leukemia

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AML	Acute myeloid leukemia
APC	Allophycocyanin
BATDA	Bis(acetoxymethyl) 2,2':6',2”-terpyridine-6,6”-dicarboxylate
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CML	Chronic myeloid leukemia
Cr	Chromium
DLI	Donor lymphocyte infusion
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme linked immunospot assay
ELN	European Leukemia Net
FAB	French-American-British Cooperative Group
FACS	Fluorescence activated cell sorting
Fas L	FAS ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMP	Good Manufacturing Practice
GrB	Granzyme B
GvHD	Graft-versus-Host Disease
GvL	Graft-versus-Leukemia
Gy	Gray
HSCT	Haematopoietic stem cell transplantation
HLA	Human leukocyte antigen
HS	Human serum
IFN-ɣ	Interferon gamma
IL	Interleukin
mA	milli Ampere
MACS	Magnetic assisted cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mRNA	messenger RNA (ribonucleic acid)
NCI	National Cancer Institute

NK	Natural killer cell
PAA	Polyacrylamide
PBMC	Peripheral blood mononuclear cell
PFA	Paraformaldehyde
PD-L1	Programmed death receptor ligand-1
PE	Phycoerythrin
PHA	Phythohemagglutinin
PI	Propidium iodide
PS	Phosphatidyl serine
PVDF	Polyvinylidene Fluoride
qRT-PCR	Quantitive real time-polymerase chain reaction
RT	Room temperature
SEM	Standard error of the mean
SFC	Spot-forming cells
SSO	Sequence-specific oligonucleotides
SSP	Sequence-specific primers
TAA	Tumor-associated antigen
TCR	T cell receptor
TDA	2,2':6',2"-Terpyridine-6,6"-Dicarboxylic Acid
TNF-α	Tumor necrosis factor alpha
Trail	Tumor necrosis factor-related apoptosis-inducing ligand
U	Unit
WHO	World Health Organization
WT1	Wilms' Tumor 1

1. Introduction

1.1. Acute myeloid leukemia

1.1.1. Epidemiology

Acute myeloid leukemia (AML) is a haematopoietic malignancy of the bone marrow involving haematopoietic progenitor cells, especially in the myeloid lineage. The pathogenesis is defined by a clonal expansion and proliferation of immature myeloid progenitor cells (so-called blasts) interfering with the normal haematopoiesis (Vardiman J, Brunning R et al. 2008, Showel and Levis 2014). While there is an abnormal production of white blood cells, AML can present with a decreased blood count for erythrocytes and platelets (Lowenberg, Downing et al. 1999, Brunning, Matutes et al. 2001). The abnormal production and the transformation of myeloid progenitor cells in the bone marrow define the state of the leukemia. With more than 20 % blasts in the bone marrow, the myeloid malignancy is considered “acute” according to the World Health Organization (WHO) classification (Brunning, Matutes et al. 2001, Vardiman 2010). AML is classified by two different systems, either by the WHO or the French-American-British Cooperative Group (FAB) classification. Both systems have in common that AML is classified according to cell morphology and immunological detection of lineage-specific markers. The WHO classification additionally includes molecular features and cytogenetic criteria and differentiates between clinical features. These factors are of great importance because the development of AML is associated with a variety of risk factors (Vardiman 2010). The systems differ in the minimum blast percentage at diagnosis which is at least 30 % for FAB classification (Cheson, Cassileth et al. 1990, Maurillo, Buccisano et al. 2013).

Although AML is a relatively rare cancer type, it is the most common acute leukemia in adults (Teague and Kline 2013). The incidence of AML reaches 2.5 cases in 100,000 persons when adjusted to the world’s population. In Europe, the incidence is slightly higher with a 3.7 cases per 100,000 persons (Deschler and Lubbert 2006). Newly diagnosed patients have a median age of 65 years and the incidence increases with age. Therefore AML is primarily a malignancy of later adulthood. In Europe the 5-year survival rate is approximately 19 % (Visser, Trama et al. 2012). Like in other cancers, different risk factors have been identified to have an impact on epidemiology. These include genetic disorders, exposure to physical and chemical agents as well as ionizing radiation and antecedent chemotherapies with alkylating

drugs (Smith, Skibola et al. 2004, Bowen 2006). Moreover, previous haematological complications such as myelodysplastic syndrome also play a role in leukemia onset (Catenacci and Schiller 2005). Molecular genetic features are of great importance when considering treatment options and prognostic outcome (Kayser, Dohner et al. 2011). Genetic abnormalities play an important role as diagnostic criteria for further sub-classification into prognostic groups with either favourable, intermediate or adverse outcome by European Leukemia Net (ELN) guidelines. The three groups are associated with complete remission but with different risk for relapse (Mrozek, Marcucci et al. 2012). Favourable outcome results from beneficial mutations, adverse prognosis from severe genetic abnormalities (Lowenberg, Downing et al. 1999, Brunning, Matutes et al. 2001, Estey 2014). However, the specific cause of AML remains to be elucidated because it is a multigenetic disease like most cancer (Groschel, Schlenk et al. 2013). Especially relapse is still a major drawback in AML treatment. Conventional therapeutic approaches aiming at long term cure are considered the standard treatment and will be explained in the following.

1.1.2. Conventional treatment

As an acute leukemia, AML progresses rapidly and is typically fatal within weeks or months if left untreated. Treatment involves two phases: induction and consolidation. Induction therapy aims at the so-called complete remission, a condition that is defined by less than 5 % percent of remaining blast cells in the bone marrow that do not have the leukemic phenotype (Chen, Newell et al. 2015). Moreover, remission is specified by haematological parameters such as the presence of more than 1,000 neutrophils per µL blood and a platelet count of more than 100,000 cells per µL (de Greef, van Putten et al. 2005). This condition is normally achieved with intensive chemotherapy that usually consists of a combination of different drugs. However, which drug or drug combination may work best highly depends on the patient and also on the genetics of his AML type (Brunning, Matutes et al. 2001, Kaur, Constance et al. 2015). Complete remission can be achieved in approximately 70 % of patients younger than 60 years of age. It is inversely correlated to age meaning that the older the patient gets, the smaller is the chance of complete remission and the shorter is the duration of remission. Even if induction therapy destroys most of the normal bone marrow cells as well as leukemic cells, a small number of residual blasts often remain. Without subsequent treatment such as consolidation therapy, leukemia is likely to return within several months (Gratwohl, Baldomero et al. 2012). After successful remission, post-remission therapy known as “consolidation” is given to the patient in order to eradicate residual leukemic cells and to

achieve a long-term cure. Consolidation therapy is usually administered as another chemotherapy that differs from induction therapy in a higher dose intensity of drugs. This chemotherapy is then followed by haematopoietic stem cell transplantation (HSCT) to reconstitute the healthy bone marrow of the patient (Dvorak, Lysak et al. 2015).

1.2. Haematopoietic stem cell transplantation

1.2.1. The importance of haematopoietic stem cell transplantation (HSCT)

If the first complete remission failed, the selection of the consecutive therapy is crucial. After a certain conditioning regimen in chemotherapy as mentioned as "conventional treatment", the patient will not have a functional immune system and requires the administration of cells that are able to reconstitute the bone marrow. Reconstitution of the hematopoietic system can be achieved by autologous HSCT with either healthy cells from the host himself or by allogeneic transplantation of cells from an human leukocyte antigen (HLA)-matched donor (Loh, Koh et al. 2007). In autologous transplantation, stem cells are removed from the patient and re-infused if needed (Gratwohl, Baldomero et al. 2012). In AML, this concept is rather difficult because even at complete remission, patients may have residual leukemic blasts in the bone marrow that have to be separated from healthy stem cells before autologous transplantation. For this reason, autologous transplants are accompanied with high relapse rates (von Grunigen, Raschle et al. 2012). The most common type of transplantation is allogeneic HSCT (Estey and Kantarjian 2005). Here, stem cells are isolated from an appropriate donor that closely matches the patient's HLA antigen pattern confirmed by standard tissue typing of the major HLA molecules. Allogeneic HSCT can derive from related or unrelated donors as long as immunological similarity is confirmed (Gratwohl, Baldomero et al. 2012). Since allogeneic transplantation aims at the reconstitution of the patient's bone marrow and likely supports the long-term cure of the disease, it is the treatment of choice for AML patients. The importance of allogeneic transplantation becomes clear when considering the rising numbers of AML patients in Germany (Beelen and Mytilineos 2014). Figure 1 shows increasing numbers of allogeneic HSCT due to AML over a period of 16 years (1998-2013). As mentioned before, AML is the most frequent form of leukemia and is conventionally treated with allogeneic HSCT (Estey and Kantarjian 2005).

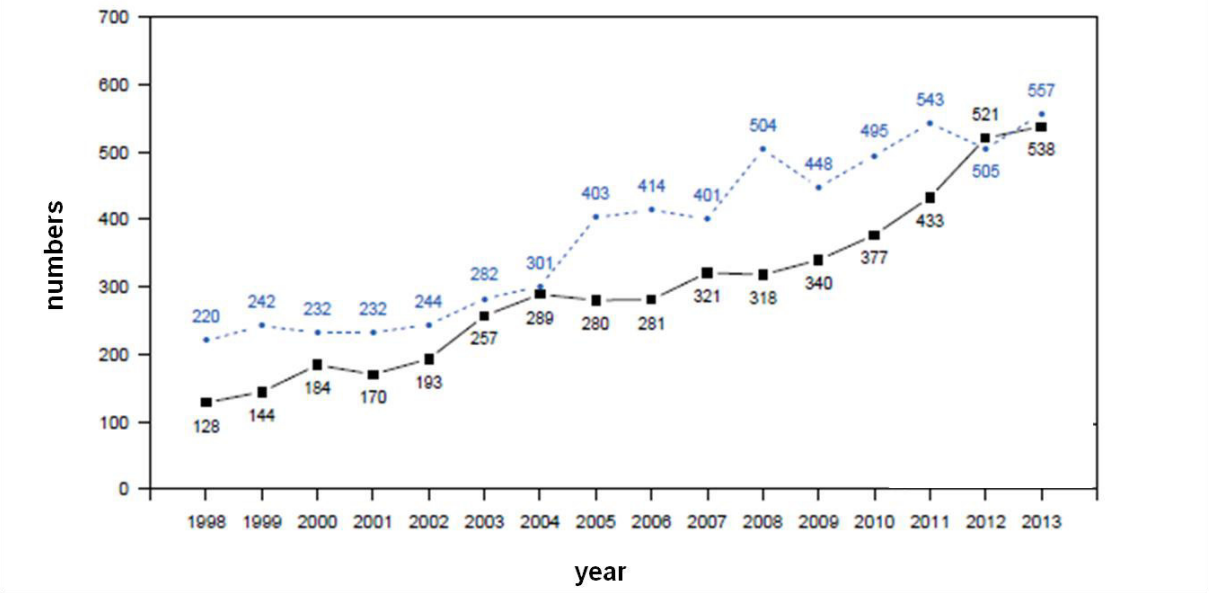


Fig. 1: Allogeneic stem cell transplantation in patients with Acute Myeloid Leukemia (AML)
Increasing numbers of allogeneic stem cell transplantations in Germany. AML patients with (black) and without (blue) complete remission, **adapted from www.drst.de**

1.2.2. The role of human leukocyte antigens (HLA)

The success of allogeneic transplantation requires optimal donor selection. Optimal conditions are dependent on matching of HLA antigens, also known as major histocompatibility (MHC) antigens, which are surface proteins on almost all nucleated cells of the donor and the patient. HLA typing methods such as serological analysis and molecular techniques of sequence-specific priming (SSP) and sequence-specific oligonucleotide (SSO) probing can discriminate unique alleles encoded by HLA class I and II genes (Sheldon and Poulton 2006). The allele combination of HLA antigens is unique for every individual and enables immune cells to discriminate 'self' from 'non-self'. Therefore, HLA antigen matching has a great clinical impact in bone marrow transplantation because they are the major molecules initiating graft rejection (Okumura, Yamaguchi et al. 2007). If there is an immunological incompatibility due to mismatches in HLA alleles, complications *post* transplantation can occur because engrafted cells can see the host's immune system as 'non-self'. Even if the donor and the patient have identical HLA antigens, transplantation can result in Graft-versus-Host Disease (GvHD). This complication may arise from disparities in minor histocompatibility antigens (Spierings 2014). Complications such as graft rejection and

GVHD-related mortality also increase with increasing numbers of HLA mismatches. In the following chapter, complications after HSCT are introduced in detail.

1.3. Complications after haematopoietic stem cell transplantation

1.3.1. Graft–versus-Host Disease (GvHD)

One of the most serious complications of allogeneic HSCT is known as GvHD. GvHD is initiated by the introduction of a new immune system into the patient's body. The newly transplanted cells can recognize the recipient's body as 'non-self' and can attack the patient's cells. This immune response is especially mediated by reactive T and NK cells present in the graft. Reactive immune cells can also react against antigens of the patient's tissue expressed by the skin, liver or gastrointestinal tract (Jacobsohn and Vogelsang 2007). Complications like acute GvHD or graft rejection are usually controlled by immunosuppressive treatment given as a prophylaxis directly after transplantation. Acute GvHD is classified into grade 0-IV by the number and extent of organ involvement. Grade III/IV acute GvHD tend to have a poor outcome. Despite optimal prophylaxis, grade II-IV acute GvHD develops in 35-50 % after transplantation (Jacobsohn and Vogelsang 2007, Okumura, Yamaguchi et al. 2007).

A more beneficial consequence of incompatibility of donor and recipient is the so-called "Graft versus Leukemia (GvL)" effect, a major therapeutic response of donor derived T cells directed against the HLA antigens on residual leukemic blasts of the patient (Horowitz, Gale et al. 1990, Baron, Maris et al. 2005). This immunological advantage has been a matter of intensive research because it allows eradication of residual tumor burden instead of total cell destruction in the patient by subsequent chemotherapy. The recognition of potent effector cells is subject of the concept of adoptive immunotherapy and will be introduced in this thesis.

1.3.2. Relapse after haematopoietic stem cell transplantation

After induction and consolidation therapy, it is generally assumed that a considerable burden of leukemia blasts remains undetected. Even when an AML patient is in complete remission, residual blasts can cause a relapse within a few weeks or months if no further post-remission therapy was administered. This in turn means that attainment of complete remission is often

neither a long-term guarantee for disease-free survival nor a cure (Larson, Lowenberg et al. 2014). If complete remission fails, a rate of 20-30 % of all AML patients will relapse. After HSCT, a relapse refers to the recurrence of residual leukemic blasts in the bone marrow and peripheral blood of a patient that could not be eradicated completely by induction therapy.

According to the National Cancer Institute (NCI) there is currently no standard therapy for relapse treatment of AML patients. Formerly, a second transplantation was considered the only treatment option. However, transplantation requires intensive treatment and can be accompanied with life-threatening complications. A number of drug combinations have been reported (Hiddemann, Kreutzmann et al. 1987, Brown, Herzig et al. 1990, Lang, Earle et al. 2005, Altman, Sassano et al. 2011, Lu, Zhang et al. 2014) active against residual leukemic blasts. Patients treated with these drug combinations may have extended disease-free survival, however complete response to drugs was only found in a maximum of 60 % of relapsed patients. Donor lymphocyte infusions (DLI) are considered more useful even if no proper research on its beneficial potential was shown in prospective trials yet. Retrospective analysis of 400 relapsed patients after HSCT done by Schmid and colleagues (Schmid, Labopin et al. 2007) only resulted in a relapse remission rate of 34 % concluding that the benefit of DLIs was quite limited. According to large retrospective analyses, the time between transplantation and relapse accounts for six to twelve months (Barrett and Battiwalla 2010). The longer the time between the first transplantation and the relapse occurrence, the longer is the leukemia-free survival (Michallet, Tanguy et al. 2000, Hemmati, Terwey et al. 2015). To maximize survival rates, several research groups began studying concepts of additional immunotherapies in order to prevent relapse after HSCT.

1.4. Current strategies of immunotherapies for AML

Conventional therapy such as allogeneic stem cell transplantation controls residual tumour burden *post* remission but residual leukemic blasts can re-occur that have been hidden from reactive cells of the donor. Therefore, supportive immunotherapies are of urgent need. Current strategies involve treatment with cytokines or antibodies, vaccine trials with antigen-loaded dendritic cells or peptides, and adoptive transfer of effector cells with either genetically engineered T cell receptor (TCR) or chimeric antigen receptor (CAR). The aim of an immunotherapy is either to confer immunity by adoptive cell transfer or to boost the patient's immune system after relapse.

Cytokine-derived therapy mainly uses interleukin-2 (IL-2) because of its known potential to activate and boost effector T cells that destroy cancer cells. Among other cancer types treated with IL-2 cytokine therapy such as colorectal cancer, ovarian carcinoma and bladder cancer, various trials (Macdonald, Jiang et al. 1991, Hamon, Prentice et al. 1993, Meloni, Trisolini et al. 2002, Stone, DeAngelo et al. 2008) have shown that the use of IL-2 has an impact of residual leukemic blasts. However, if the IL-2 receptor is expressed by leukemic cells, IL-2-induced relapse can occur. Therefore, IL-15 was under intense research because it also targets the IL-2 receptor (Szczepanski, Szajnik et al. 2010).

By contrast to IL-2, IL-15 is responsible for a prolonged maintenance of memory T cells, a preferred cellular phenotype for adoptive immunotherapy. Other cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interferon also have pivotal roles in upregulating MHC molecules in order to improve antigen presentation (Borrello, Levitsky et al. 2009) which is necessary for effector cell attack. However, Gurion and colleagues compared the safety and efficacy of GM-CSF from randomized clinical trials and concluded that GM-CSF does not affect overall survival in AML patients (Gurion, Belnik-Plitman et al. 2012). So far, usage of interferon in immunotherapies against AML has been disappointing in clinical trials but lately gained new interest (Anguille, Lion et al. 2011).

AML blasts not only express cytokine receptors but a variety of surface markers that have been explored as potential targets for monoclonal antibody therapy. CD33, as the most prominent surface molecule representing a myeloid-specific transmembrane receptor is extensively studied because it is highly expressed on more than 80% of haematopoietic malignancies including AML (Ehninger, Kramer et al. 2014). CD33 is not expressed on normal progenitor cells which makes the CD33 antibody (anti-CD33) a potential candidate for targeting residual leukemic blasts when relapse occurs. However, Kobayashi and colleagues reported that anti-CD33 therapy accompanies with liver toxicity (Kobayashi, Tobinai et al. 2009) and is nowadays rather used as a target for CD33-directed chimeric antigen receptors on modified T cells (CART-33) (Pizzitola, Anjos-Afonso et al. 2014, Wang, Wang et al. 2015). Because this is a fairly new technology, CARs will be explained in more detail in the following text.

The first idea of preventing relapse was a vaccine trial with autologous leukemic blasts that were irradiated and administered to the patient with the addition of bacille Calmette-Guérin (BCG). BCG unspecifically stimulated the autoimmunization, a process where the patient's

immune system develops antibodies against the patient's own tissue such as leukemic blasts. Researchers thought that the capacity of the patient's immune system increased by BCG and the patient would resist a subsequent trigger in form of leukemic cells. Because of the fact that autoimmunization only had a transient effect, 10^9 blast cells administered per therapy were selected for increase in reactivity (Powles, Crowther et al. 1973). In order to increase susceptibility of leukemic cells to immune attacks, research focussed on whole cell vaccines in form of dendritic-like leukemia cells. Dendritic cells are antigen presenters and were differentiated from AML blasts in order to obtain leukemic characteristics. In a study of Roddie and colleagues, these cells were administered to AML patients as a vaccine. Consequently, T cell responses were initiated and monitored in 22 patients over 12 months *post* vaccination. Because only two out of 22 patients remained in remission, this vaccination was not broadly applicable (Roddie, Klammer et al. 2006). Other whole cell vaccinations and loading strategies for dendritic cell based vaccination have been investigated extensively *in vitro* (Galea-Lauri, Darling et al. 2002, Duncan and Roddie 2008, Kremser, Dressig et al. 2010) but clinical data are often limited to individual patient cohorts (Hardwick, Chan et al. 2010). In other studies, whole cell vaccination was replaced by peptide vaccination including antigen-specific peptides that are highly expressed in AML (Greiner, Dohner et al. 2006, Keilholz, Letsch et al. 2009, Oka, Tsuboi et al. 2009). However, peptides are mostly restricted to MHC class I epitopes that do not represent the entire sequence of the antigen. Furthermore, they are restricted to a certain HLA antigen and therefore might not be applicable to a broad range of AML patients.

Novel approaches focus on the generation of specific effector cells rather than on leukemia-derived cells or peptides. Adoptive cell therapies with T or NK cells are currently under intensive investigation. One strategy, already described in the context of relapse, is the administration of DLIs from the original HLA-matched stem cell donor that was selected for the transplantation (Gozdzik, Rewucka et al. 2015). This immunotherapy has the advantage that a decent number of effector cells can be re-infused in the patient's body. Even if HLA antigens donor and patient match, there is still a chance of GvHD which is a major complication in this setting. In order to circumvent GvHD, autologous cell isolation is considered. Autologous cell isolation does not necessarily result in sufficient cell numbers and isolated effector cells are mostly short-lived and of low avidity towards leukemic antigens so that clinical efficiency cannot be achieved (Lichtenegger, Lorenz et al. 2014, Steger, Milosevic et al. 2014, Uttenthal, Martinez-Davila et al. 2014).

Immune responses rely on the specific TCR-dependent recognition of peptides presented by MHC. Naturally occurring TCRs that recognize a self-antigen are only of low affinity because of thymic selection (Purbhoo, Sutton et al. 2006, Linette, Stadtmauer et al. 2013). Since tumor antigens are also expressed by germ line cells and selected adult tissues, T cells directed against these peptides express TCRs of lower avidity for their antigenic ligands making isolation difficult (Schmid, Irving et al. 2010). Modification of T cells using genes encoding antigen receptors can be used to generate high-avidity T cells in a process termed genetic redirection of specificity (Kershaw, Westwood et al. 2014). By introduction of genetically modified TCRs in a specific effector cell culture, a T cell product without GvHD reactivity can be generated. To improve the duration of T cell control in the patient, T cells with memory function were pre-selected in many studies (Stauss, Thomas et al. 2008, Thaxton and Li 2014).

Another strategy of a specific adoptive therapy is the generation of non MHC-restricted chimeric antigen receptors (CARs) on modified T cells. CARs are artificial TCR which typically graft the specificity of a monoclonal antibody onto a T cell (Ramos and Dotti 2011). This reprogramming of the T cell antigen receptor is usually done by retroviral vector systems that are specific for malignant cells. This technology combines the antigen recognition domain of an antibody, directed against leukemia-specific antigens on AML cells, with an intracellular signalling domain in the T cells that stimulate the cell's activity (Mardiros, Brown et al. 2013). Different generations of CAR modified T cells aim at enhanced activation signals, T cell proliferation and effector function of CAR-modified T cell in pre-clinical trials (Maus, Grupp et al. 2014). This technique also has the advantage of rapid generation of modified T cells that recognize leukemic antigens without HLA restriction and is therefore broadly applicable. These two strategies, TCR editing and generation of CARs, include modification of T cells resulting in highly specific effector cells for immunotherapy and are currently state of the art (Sadelain, Brentjens et al. 2009, Lipowska-Bhalla, Gilham et al. 2012).

1.5. Wilms' tumor 1 antigen

In order to eradicate residual leukemic blasts, a number of different research groups focussed on the identification of AML peptide antigens (Greiner, Ringhoffer et al. 2000, Bae, Martinson et al. 2004, Berlin, Kowalewski et al. 2014). These so called tumor-associated antigens (TAA) are of great interest because they are overexpressed by AML cells. Among these AML peptide antigens, the Wilms' tumor 1 (WT1) antigen plays a significant role and is the target structure of interest for the generation of an adoptive immunotherapy for AML in this project.

WT1 protein, a transcription factor that "shuttles" between the nucleus and the cytoplasm of cells (Niksic, Slight et al. 2004), is encoded by the *WT1* gene which is responsible for differentiation of genitourinary tissues (Hirose 1999, Toska and Roberts 2014, Bandiera, Sacco et al. 2015). In healthy tissue, WT1 expression is limited to developing kidney, testis and ovaries. In adult haematopoiesis, WT1 is also expressed at low level in a few progenitor cells, where it directs stage-specific quiescence and differentiation of $CD34^+$ progenitors (Ellisen, Carlesso et al. 2001, Hosen, Sonoda et al. 2002). In contrast, WT1 is associated with nephropathies such as Wilms' tumor and gonadal dysgenesis in Denys-Drash syndrome. Although *WT1* was originally described as a tumour suppressor gene in Wilms' tumor and other related syndromes, WT1 positively affects carcinogenesis (Huff 2011). WT1 is found in tumor cells from a wide range of cancers and is highly associated with haematopoietic malignancies supporting WT1's oncogenic function. WT1 is especially overexpressed in AML as shown by several studies that report elevated WT1 expression for approximately 80% of all AML patients (Miwa, Beran et al. 1992, Miyagi, Ahuja et al. 1993, Menssen, Renkl et al. 1995, Ostergaard, Olesen et al. 2004, Osborne, Frost et al. 2005). WT1 expression also accompanies with poor prognosis of AML (Lyu, Xin et al. 2014). According to a project on prioritization of cancer antigens of NCI, WT1 was ranked first because it fulfils most of the predefined criteria of an 'ideal' cancer antigen. Criteria included expression level, oncogenicity, immunogenicity, therapeutic function, cellular localization and the number of antigen epitopes (Cheever, Allison et al. 2009). Its limited expression in healthy tissue makes WT1 a leukemia-associated antigen with therapeutic function because it can be used as a target structure for adoptive immunotherapy in order to treat AML (Uttenthal, Martinez-Davila et al. 2014).

Although current therapies can be promising, they are mostly restricted to small patient cohorts in clinical trials or even individual patients (Barrett and Le Blanc 2010). It seems that optimization of immunotherapies does not only depend on one therapy but rather on a combination of approaches. Also, the entire status of the immune system after transplantation has to be taken into account which means that research should not only focus on the generation of potent effector cells but also on the analysis of residual target cells. Therefore, this project investigates a novel immunotherapy for the treatment of relapsed AML and focuses on the characterisation of putative effector cells and target cells that are specific for the WT1 antigen.

1.6. Aim of the project

Although allogeneic haematopoietic stem cell transplantation is considered as a curative treatment for AML, a relapse rate of 20-30 % still remains (Leopold and Willemze 2002). The generation of new immunotherapies for relapse treatment is under intense research but still seems to be challenging. This project investigates adoptive immunotherapy as a novel approach for relapse treatment in AML. This therapy should be administered to the patient as a prophylaxis after transplantation. The overall aim of the project is the *ex vivo* generation of WT1-specific effector cells as shown in figure 2.

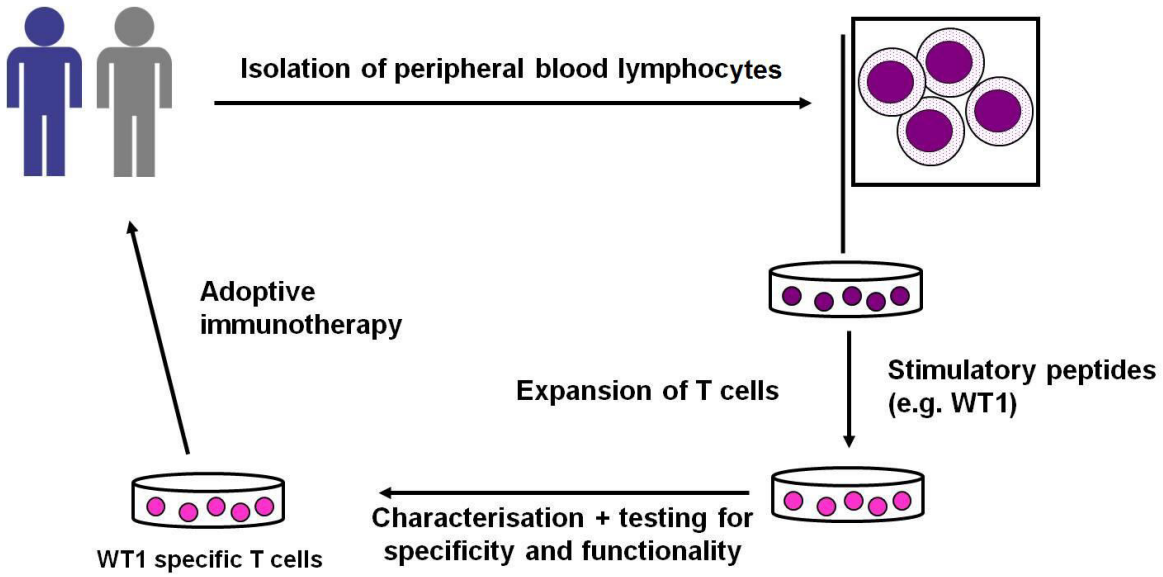


Fig. 2: WT1-specific T cell immunotherapy
Peripheral blood mononuclear cells of healthy controls and AML patients, respectively are cultured ex vivo and activated upon peptide stimulation (here WT1). T cells specific for the peptide are selected and expanded. In the next step, these cells are characterized and tested for their specificity and functionality. If cell numbers of WT1-specific cells are adequate for adoptive immunotherapy, the product is infused in the patient.

In order to achieve this aim, two different subjects were addressed. The first part of the project dealt with the analysis of WT1-specific effector cells that were characterised according to their frequency, their cytokine production and proliferation potential and were also tested for their functionality. Isolated peripheral blood mononuclear cells and their subpopulations were analysed in ELISpot assay in order to determine WT1-specific cell frequencies of healthy controls and AML patients *pre* and *post* transplantation, respectively. Therefore, cells were stimulated with WT1 peptide and tested for their cytokine secretion pattern. Frequencies of cytokine secreting cells were further confirmed by FluoroSpot assay

that measures simultaneous cytokine production of WT1-specific cells. This was of interest for selecting a suitable starting culture for WT1-specific cell expansion. WT1 specificity was further analysed with flow cytometry-based Streptamer technology. In addition, cells were analysed for functional activity in cytotoxicity assays. Cytotoxicity of WT1-specific cells was tested by their potential to lyse specific target cells in an Europium release assay. Lysis of target cells was also visualized in video experiments. WT1-specific cells were expanded to generate cell numbers sufficient for a WT1-specific immunotherapy. To achieve this aim, different expansion strategies of WT1-specific cells were tested, including various activation strategies and growth supplements such as cytokines, autologous feeder cells and artificial MACSi beads. After expansion, the phenotype of cultures was analysed by flow cytometry. Optimal expansion results in cytolytic WT1-specific cells with memory function because these cells are long-lived and target specific lysis of residual leukemic blasts. Expanded cells were tested for WT1 specificity by ELISpot and WT1-specific Streptamer which is an antibody directed against HLA-A*02 restricted WT1 peptide.

In order to test the cytolytic behaviour of WT1-specific cells, the characterisation of suitable leukemic target cells was subject in the second part of the project. Since WT1 is overexpressed in haematopoietic malignancies such as AML (Koeffler and Golde 1980, Bergmann, Maurer et al. 1997, Lowenberg, Downing et al. 1999), leukemic cells lines and AML blasts were chosen as targets. Target cells were analysed for their WT1 mRNA and protein levels. mRNA levels were examined by qRT-PCR, the WT1 protein amount by Western Blot and extra- and intracellular WT1 expression by flow cytometry. Additionally, target cells were tested in Europium release assay for their lysis potential.

In summary, WT1-specific effector cells were characterized according to their frequency, cytokine production and cytolytic behaviour. These cells need to be expanded with an expansion strategy that results in highly specific cells in an appropriate cell number. Also, analysis of suitable target cells that express WT1 was crucial in order to establish optimal condition for a WT1-specific immunotherapy.

2. Materials and Methods

2.1. Materials

2.1.1. Instruments, materials, chemicals and reagents

Tab. 1: Instruments

Instrument	Model	Company
CO_2 Incubator	Hera cell 240	Thermo Scientific (Waltham,USA)
Centrifuges	Heraeus Varifuge 3.0R	Thermo Scientific (Waltham,USA)
	Heraeus Megafuge 40	Thermo Scientific (Waltham,USA)
Cell counting device	KX-21N	Sysmex (Norderstedt)
ELISpot reader	iSpot FluoroSpot	AID Diagnostika (Straßberg)
Fluorescent lamp	FL 009751	AID Diagnostika (Straßberg)
Fluorometer	Wallac Delfia 1232	Perkin Elmer (Waltham, USA)
Scintillation counter	Wallac 1450 MicroBeta TriLux	Perkin Elmer (Waltham, USA)
Flow cytometer	FC500	Beckman Coulter (Krefeld)
	Software: Kaluza 1.1	Beckman Coulter (Krefeld)
	FACS Aria I	BD Biosciences (San Jose, USA)
	Software: Diva 6.1.2	
	FlowJo vX.0.6	
Gel documentation	FX 7	Vilber Lourmat (Eberhardzell)
Microscope	Axio Observer Z1	Zeiss (Jena)
Cell-bead separator	MACSiMAG	Miltenyi Biotec (Bergisch Gladbach)
Software	GraphPad Prism 6	GraphPad Software (La Jolla, USA)

Tab. 2: Materials

Material	Company
MAIPN 4450 Multiscreen Filter Plates	Merck Millipore (Darmstadt)
Suspension cell culture plates (6, 12, 24, 48, 96 wells)	Greiner Bio-One (Frickenhausen)
Suspension cell culture plates, V bottom	Sarstedt (Nümbrecht)
Tissue culture plates, U bottom	BD Biosciences (San Jose, USA)
Cell culture flasks (25, 75, 175 cm^2), filter caps	Greiner Bio-One (Frickenhausen)
Cell culture dishes 100x20 mm^2	Greiner Bio-One (Frickenhausen)
MACS separation columns (MS, LS)	Miltenyi Biotec (Bergisch Gladbach)
Cell culture tubes (12 mL)	Greiner Bio-One (Frickenhausen)
Cryo tubes (1 mL)	Greiner Bio-One (Frickenhausen)
Heparin monovettes (9 mL)	Sarstedt (Nümbrecht)
Polypropylene tubes, conical (15, 50 mL)	Greiner Bio-One (Frickenhausen)
Reaction tubes (0.5 - 2 mL)	Eppendorf (Hamburg)
Combi tips advanced (2.5 µL)	Eppendorf (Hamburg)
Finnpipette (from 5 - 300 µL)	Thermo Scientific (Waltham, USA)
Multipette ® plus	Eppendorf (Hamburg)
Pipettes (from 0.5 - 1000 µL)	Gilson (Middleton, USA)
Serological pipettes (5, 10, 25 mL)	Greiner Bio-One (Frickenhausen)
Mr FrostyTM Freezing Container	Nalgene (Neerijse, Belgium)

Tab. 3: Chemicals and reagents

Chemical/reagent	Company
Aqua, sterile water	Braun (Melsungen)
Chlorpromazine	Sigma-Aldrich (Taufkirchen)
DMSO (Dimethylsulfoxide)	Wak-Chemie (Steinbach)
DNase (100 µg)	Roche (Basel, Switzerland)
D-PBS (Dulbecco's phosphate buffered saline)	Gibco, Life Technologies (Darmstadt)
EDTA (Ethylendiamintetraacetate)	Sigma-Aldrich (Taufkirchen)
Ethanol	Roth (Karlsruhe)
Ficoll-Paque™ plus, separation	GE Healthcare (Upsala, Sweden)
H_3 thymidine	Hartmann Analytic (Braunschweig)
L-Glutamine	Sigma-Aldrich (Taufkirchen)
Liquemin N25000	Roche (Basel, Switzerland)
MACS rinsing solution	Miltenyi Biotec (Bergisch Gladbach)
NaCl 0.9 % (sodium chloride)	Braun (Melsungen)
Pelispot buffer (50 mL)	Sanquin (Amsterdam, Netherlands)
Penicillin-Streptomycin	Gibco, Life Technologies (Darmstadt)
Streptavidin poly HRP (horse raddish peroxidase)	Sanquin (Amsterdam, Netherlands)
TMB substrate (18 mL)	Sanquin (Amsterdam, Netherlands)
Trypan blue	Invitrogen (Karlsruhe)

2.1.2. Cell culture media and corresponding supplements

Tab. 4: Cell culture media

Product name	Company
AIM V	Gibco, Life Technologies (Darmstadt)
IMDM	Lonza (Basel, Switzerland)
RPMI 1640	Gibco, Life Technologies (Darmstadt)
TexMacs, research grade	Miltenyi Biotec (Bergisch Gladbach)

Tab. 5: Sera and supplements

Product name	Company
Fetal calf serum	Biochrom (Berlin), PAA (Pasching, Austria)
Human AB serum	Own production
L-Glutamine (200 U/mL)	Sigma-Aldrich (Taufkirchen)
Penicillin-Streptomycin (1 %)	Gibco, Life Technologies (Darmstadt)

2.1.3. Cytokines for expansion cell culture

Tab. 6: Cytokines

Product name	Company
IL- 2 (50 µg)	Miltenyi Biotec (Bergisch Gladbach)
IL- 7 (100 µg)	Miltenyi Biotec (Bergisch Gladbach)
IL-15 (100 µg)	Miltenyi Biotec (Bergisch Gladbach)
IL- 21 (100 µg)	Miltenyi Biotec (Bergisch Gladbach)

2.1.4. Kits and assays

Tab. 7: Kits and assays

Kit	Company
Dead cell removal kit	Miltenyi Biotec (Bergisch Gladbach)
EuTDA cytotoxicity reagents, Delfia	Perkin Elmer (Waltham, USA)
FITC Annexin V dead cell apoptosis kit	Invitrogen (Karlsruhe)
FluoroSpot assay, IFN-ɣ/granzyme B kit	Mabtech (Nacka Strand, Sweden)
Pan T/ CD137/ CD4/ CD8 cell isolation kit	Miltenyi Biotec (Bergisch Gladbach)
Bradford assay	BioRad (Hercules, USA)

Tab. 8: Western Blot equipment and buffers

Additional material	Forceps, conical tubes for gel preparation
Blocking solution	5 % skim milk powder (Sigma-Aldrich, Taufkirchen) in D-PBS (Gibco, Life Technologies, Darmstadt) + Tween 20 0.05 % (Thermo Scientific, Waltham, USA)
Blotting buffer	D-PBS (Gibco, Life Technologies, Darmstadt) + Tween 20 0.05 % (Thermo Scientific, Waltham, USA)
Femto substrate	Thermo Scientific (Waltham, USA)
Filter paper	Whatman, 11µm pore size (BioRad, Hercules, USA)
Gel running buffer	2.5 mM TRIS, 0.1 % SDS, glycine (Sigma-Aldrich, Taufkirchen)
Power supply	Thermo Scientific (Waltham, USA)
Pre-stained molecular marker	PageRuler™ Prestained Protein Ladder (10-170 kDa), Fermentas (Thermo Scientific, Waltham, USA)
PVDF membrane	0.45 µm pore size, Millipore (Darmstadt)
Running gel	Polyacrylamide and acrylamide (PAA, BioRad, Hercules, USA), TRIS pH 8.8 (Sigma-Aldrich, Taufkirchen), 10 % APS (Thermo Scientific, Waltham, USA), TEMED (Sigma-Aldrich, Taufkirchen)
Sample buffer	6 % SDS, 0.25 M TRIS (pH 6.8), 10 % glycerol, bromophenyl blue, 20 mM DTT (all: Sigma-Aldrich, Taufkirchen)
Semi-dry transfer apparatus	Bio Rad Criterion™ Blotter (BioRad, Hercules, USA)
Stacking gel	PAA (BioRad, Hercules, USA), TRIS pH 6.8, 10 % APS (Thermo Scientific, Waltham, USA), TEMED (Sigma-Aldrich, Taufkirchen)
Transfer buffer	TRIS, 20 % methanol, pH 10.4 (Sigma-Aldrich, Taufkirchen)
Wetting solution	100% methanol (Sigma-Aldrich, Taufkirchen)

2.1.5. Antibodies

Tab. 9: Antibodies for ELISpot assay

Specificity	Conjugation	Stock solution	Company
Granzyme B	Primary mAb, unconj.	1 mg/mL	Thomas Ehret Consults (Nidderau)
Granzyme B	Secondary mAb, biotin	1 mg/mL	Thomas Ehret Consults (Nidderau)
IFN-ɣ	Primary mAb, unconj.	1 mg/mL	Mabtech (Nacka Strand, Sweden)
IFN-ɣ	Secondary mAb, biotin	1 mg/mL	Mabtech (Nacka Strand, Sweden)
IL-10	Primary mAb, unconj.	1 mg/mL	Mabtech (Nacka Strand, Sweden)
IL-10	Secondary mAb, biotin	1 mg/mL	Mabtech (Nacka Strand, Sweden)
IL-12	Primary mAb, unconj.	1 mg/mL	Mabtech (Nacka Strand, Sweden)
IL-12	Secondary mAb, biotin	1 mg/mL	Mabtech (Nacka Strand, Sweden)
IL-13	Primary mAb, unconj.	1 mg/mL	Sanquin (Amsterdam, Netherlands)
IL-13	Secondary mAb, biotin	1 mg/mL	Sanquin (Amsterdam, Netherlands)
IL-17a	Primary mAb, unconj.	0.5 mg/mL	Mabtech (Nacka Strand, Sweden)
IL-17a	Secondary mAb, biotin	0.5 mg/mL	Mabtech (Nacka Strand, Sweden)
IL1-β	Primary mAb, unconj.	1 mg/mL	Sanquin (Amsterdam, Netherlands)
IL1-β	Secondary mAb, biotin	1 mg/mL	Sanquin (Amsterdam, Netherlands)
IL-2	Primary mAb, unconj.	0.5 mg/mL	BD Biosciences (San Jose, USA)
IL-2	Secondary mAb, biotin	0.5 mg/mL	BD Biosciences (San Jose, USA)
IL-4	Primary mAb, unconj.	1 mg/mL	Mabtech (Nacka Strand, Sweden)
IL-4	Secondary mAb, biotin	1 mg/mL	BD Biosciences (San Jose, USA)
Perforin	Primary mAb, unconj.	1 mg/mL	Mabtech (Nacka Strand, Sweden)
Perforin	Secondary mAb, biotin	1 mg/mL	Mabtech (Nacka Strand, Sweden)
TGF-β1	Primary mAb, unconj.	1 mg/mL	Sanquin (Amsterdam, Netherlands)
TGF-β1	Secondary mAb, biotin	0.5 mg/mL	Sanquin (Amsterdam, Netherlands)
TNF-α	Primary mAb, unconj.	1 mg/mL	BD Biosciences (San Jose, USA)
TNF-α	Secondary mAb, biotin	0.5 mg/mL	BD Biosciences (San Jose, USA)

***Antibodies species**: monoclonal mouse IgG1anti-human have been used for analysis*

Tab. 10: Antibodies for Western Blot and staining of WT1

Antibody	Species	Dilution	Clone	Company
Fab_2	Donkey α goat	1:300	polyclonal	Santa Cruz Biotech. (Santa Cruz, USA)
Fab_2 PE	Donkey α goat	1:100	polyclonal	Santa Cruz Biotech. (Santa Cruz, USA)
Human WT1	Goat IgG	1:1000	polyclonal	R&D Systems (Minneapolis, USA)
Isotype	Goat IgG	1:800	polyclonal	R&D Systems (Minneapolis, USA)
WT1-streptamer	Human	1:10	-	IBA solutions (Göttingen)

Tab. 11: Antibodies for FluoroSpot analysis

Antibody	Conjugation	Clone	Company
IFN-ɣ	unconjugated	1-D1K, monoclonal	Mabtech (Nacka Strand, Sweden)
Granzyme B	unconjugated	GB10, monoclonal	Mabtech (Nacka Strand, Sweden)
IFN-ɣ	FITC	7-B6-1, monoclonal	Mabtech (Nacka Strand, Sweden)
Granzyme B	Cy3	GB11, monoclonal	Mabtech (Nacka Strand, Sweden)

***Antibodies species**: monoclonal mouse IgG1anti-human have been used for analysis*

Tab. 12: Antibodies for flow cytometry

Specificitcy	Conjugation	Clone	Company
CD3	FITC	SK7	BD Biosciences (San Jose, USA)
CD3	PerCP	UCHT1	BioLegend (San Diego, USA)
CD4	FITC	SK3	BD Biosciences (San Jose, USA)
CD4	APC	SK3	BD Biosciences (San Jose, USA)
CD8	PE	SK1	BD Biosciences (San Jose, USA)
CD8	APC	SK1	BD Biosciences (San Jose, USA)
CD14	APC	NP9	BD Biosciences (San Jose, USA)
CD16	PE	3G8	BD Biosciences (San Jose, USA)
CD19	APC	SJ25C1	BD Biosciences (San Jose, USA)
CD45	PerCP	HI30	BioLegend (San Diego, USA)
CD56	PE	NCAM16.2	BD Biosciences (San Jose, USA)
IgG 2a	PE	MOPC-21	BD Biosciences (San Jose, USA)

***Antibodies species**: monoclonal mouse IgG1anti-human have been used for analysis*

Tab. 13: Peptides and Peptivators®

Peptide	Stimulation		Company
WT1 peptide pool	$CD4^+$ and $CD8^+$ T cells	15 mer	Miltenyi Biotec (Bergisch Gladbach)
CEF peptide pool	$CD8^+$ T cells	23 peptides	Mabtech (Nacka Strand, Sweden)
Candida albicans	polyclonal	extract	Allergopharma (Hamburg)
Phythohemagglutinin	polyclonal	extract	Remel, Thermo Scientific (Waltham, USA)
CMV pp65 NLVPMVATV peptide	CMV-specific $CD8^+$ T cells, HLA-A*0201	single peptide	IBA solutions (Göttingen)
RMFPNAPYL peptide	WT1-specific $CD8^+$ T cells, HLA-A*0201	single peptide	IBA solutions (Göttingen)

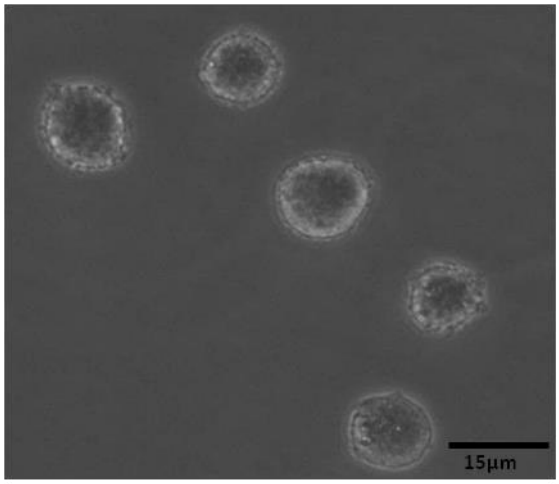
2.1.6. Cell lines

Tab. 14: Cell culture media for cell lines

Cell line	Medium	Supplement	Serum	Antibiotics	Reference
HL60 8322	IMDM	L-Glutamine	10 % FCS	Pen/Strep.	Collins *et al.,* 1978
K562	RPMI 1640	L-Glutamine	10 % FCS	Pen/Strep.	Lozzio *et al.* 1979
Kasumi-1	RPMI 1640	L-Glutamine	20 % FCS	Pen/Strep.	Tashiro *et al.* 1991
KG-1	RPMI 1640	L-Glutamine	10 % FCS	Pen/Strep.	Koeffler *et al.,* 1980
T2	RPMI 1640	L-Glutamine	10 % FCS	Pen/Strep.	Salter *et al.,* 1985
THP-1 8354	RPMI 1640	L-Glutamine	10 % FCS	Pen/Strep.	Tsuchiya *et al.,* 1982

Cell lines were thawed and cultivated at 37°C and 5 % CO_2. Before using the cells in experiments, HLA typing was done in our institute using SSP and SSO technologies.

K562

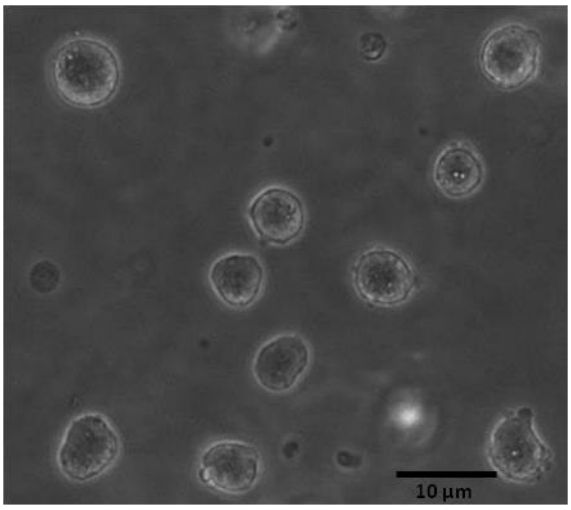


K562 is a suspension cell line with large, round, non-adherent cells. It derived from human origin and was established in 1970 from a blast crisis of a pleural effusion of a 53-year-old woman suffering from chronic myeloid leukemia (CML) (Lozzio and Lozzio 1979, Koeffler and Golde 1980). This cell line is commonly used as a target cell line for natural killer cell assays and was used for assay establishment in this project. Cells were maintained at 300.000 cells per mL and fresh medium was added every two to three days.

Kasumi-1

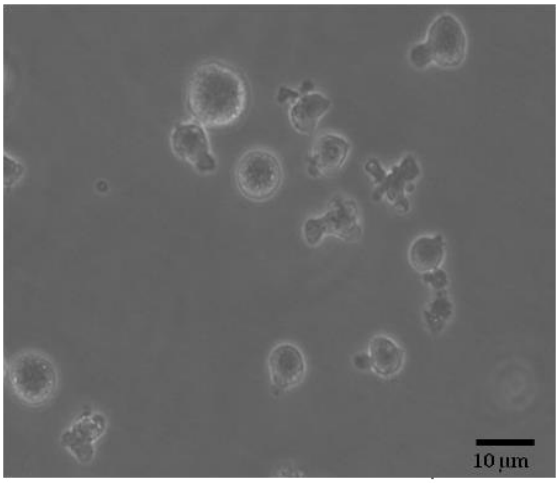
Kasumi-1 is a suspension cell line with large, round cells that have a myeloblast-like morphology. It derived from the peripheral blood of a Japanese patient who suffered from AML (Asou, Tashiro et al. 1991). Kasumi-1 is a commonly used target cell line for cytotoxicity assays which analyse the cytolytic behaviour of AML cells by effector T cells. A frozen aliquot of Kasumi-1 cells was kindly provided by the Department of Bone Marrow Transplantation in Essen and was thawed directly before use. According to HLA typing in our institute, Kasumi-1 cells express HLA-A*26, B*40 and B*48, C*03 and C*08. Cells were maintained at 300.000 cells per mL and fresh medium was added every two to three days.

HL-60



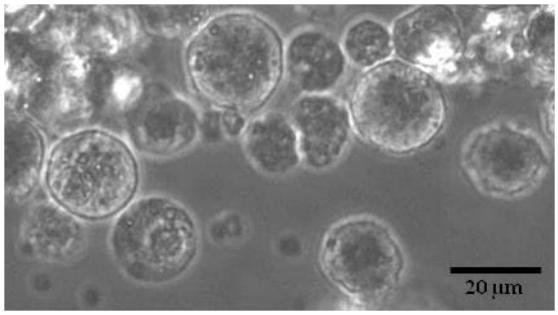
The HL-60 cell line consists of round, single cells in suspension that tend to grow in clusters. Cells were obtained by leukopheresis from a 36-year-old Caucasian female who suffered from acute promyelocytic leukemia (Collins, Ruscetti et al. 1978). HL-60 was used as a target cell line for cytotoxicity assays because of its leukemic origin. The cells express both HLA-A*01 and B*57 on their cell surface according to our HLA genotyping. HLA antigens are homozygous which can be an advantage for tumor escape strategies. Cells were maintained at 300.000 cells per mL and fresh medium was added every two to three days.

KG-1



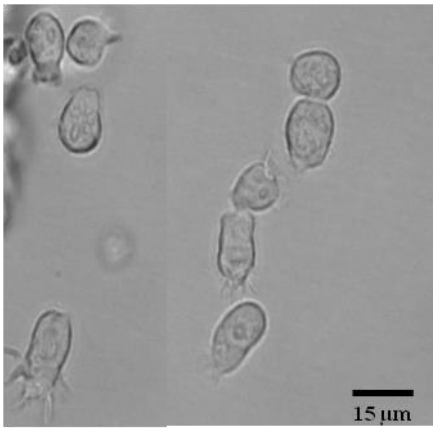
KG-1 cells grow as single cells with different morphology. They are derived from a bone marrow aspirate of a 59-year-old Caucasian male with erythroleukemia that developed into acute myeloid leukemia. The cell line was described by H.P. Koeffler and D.W. Golde (Koeffler and Golde 1980). This suspension cell line expresses HLA-A*30, B*53 and B*78 on the cell surface according to our HLA typing. Cells were maintained at 200.000 cells per mL and fresh medium was added twice a week.

THP-1



THP-1 cells derived from a patient with acute monocytic leukemia and are characterised as large, round, single cells in suspension. The cell line was first described in 1982 by Tsuchiya and colleagues (Tsuchiya, Kobayashi et al. 1982). According to HLA typing, THP-1 cells express HLA-A*02, B*15 and B*35. The THP-1 cell line was used as a target cell for cytotoxicity assays because of its leukemic origin and its common HLA-A*02 antigen. Cells were maintained at 200.000 cells per mL and kept in culture with fresh medium added twice a week.

T2



The suspension cell line T2 is a mutant human suspension cell line which is negative for class II HLA antigens. Confirmed by HLA typing, T2 cells only express HLA-A*02 on their surface making the cells suitable targets for studying antigen processing and effector T cell recognition (Salter, Howell et al. 1985). In culture, cells tend to migrate and have different morphology. The T2 cell line was maintained at 300.000 cells per mL and kept in culture with fresh medium added twice a week.

Cell lines as frozen aliquots were kindly provided by the Institute for Transfusion Medicine in Essen unless otherwise noted.

2.2. Methods

2.2.1. Collection and processing of cells

2.2.1.1. Blood sample isolation of effector cells

Blood samples of both healthy donors and AML patients *pre* and *post* transplantation were subject of this project. Peripheral blood from AML patients and healthy volunteers was collected after written informed consent was given according to the approval of the local ethics committee. For peripheral blood mononuclear cells (PBMC) isolation, heparinised blood or blood samples in ethylene-diamine-tetraacetic acid (EDTA) containing tubes were isolated using Ficoll-Paque™ Plus density gradient centrifugation at 2800 rpm for 30 min in a swinging bucket rotor without brake. After centrifugation, PBMCs of healthy donors were resuspended in fresh RPMI 1640 medium supplemented with 10 % human serum pool (HS) or in AIMV medium for AML patients, respectively. Cells were then counted with the Sysmex cell counter that does not distinguish between viable and dead cells but determines the cell size only. Counting of cells was double-checked by viable cell staining with Trypan Blue. After counting, cells were adjusted to the required cell number and kept in the corresponding medium before use.

2.2.1.2. Freezing and thawing of cells

For cryopreservation of cells and cell lines, cells were pelletized by centrifugation and resuspended in foetal calf serum (FCS) containing freezing medium. Freezing medium in 1 mL vial contained 20 U/mL Liquemin, a heparin derivate, and 10 µg/mL DNAse and was tested with optimal performance in previous experiments in our laboratory. After addition of the freezing medium, 100 µL DMSO was added to the cells. The vials were transferred to a freezing device filled with isopropanol which was then put into a -80°C freezer for two to three days and cells were afterwards transferred into liquid nitrogen cryotanks for long-time storage.

In order to process frozen cells, vials were transferred to a water bath with 37°C until the cell suspension was partly thawed. Subsequently, the cell suspension and a residual ice core were transferred to a new tube filled with the thawing medium that consisted of 9 mL of freezing medium without DMSO. The vials were centrifuged at 300g for 10 min and the supernatant was discarded. Then, two washing steps with D-PBS followed in the same procedure. After discarding the supernatant, the cell pellet was resuspended in fresh RPMI medium supplemented with 10 % HS. Then, the cells were counted with trypan blue solution and

checked for viability. If more than 80 % of viable cells were present, the sample was considered for further experiments. If less than 80 % of viable cells were present, dead cells were removed using the Dead Cell Removal kit (Miltenyi Biotech). The kit contains magnetic microbeads labelled with annexin V that recognizes phosphatidyl serine exposed by dead cells. By magnetic separation, dead cells are retained in the column and non-labelled viable cells are collected in the effluent and can be applied to the experiment. This procedure was especially necessary for AML cell lines.

2.2.1.3. Magnetic separation of cell subpopulations

Magnetic assisted cell sorting (MACS) is a technology that enables separation of cell subpopulations. MACS technology mainly consists of reagents containing magnetic microMACSi beads and separation columns composed of magnetic matrices (Miltenyi, Muller et al. 1990). For the project, T cell subpopulations such as CD3 (referred to as Pan T cells) and $CD8^+$ T cells have been isolated by negative selection to obtain untouched T cell populations. For both cell populations, isolated PBMCs were centrifuged at 300 g for 10 min and the supernatant was discarded. The pellet was then resuspended in 40 µL of MACS buffer (0,1 % BSA, 2 mM EDTA in D-PBS) per 10^7 PBMCs. Additionally, 10 µL of "$CD8^+$ T cell Biotin-Antibody Cocktail" containing antibodies against CD4, CD15, CD16, CD19, CD34, CD36, CD56, CD123, TCRγ/δ, and CD235 for CD8 isolation or "Pan T cell Biotin-Antibody Cocktail" containing antibodies against CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123 and CD235 for Pan T cell isolation, respectively was added to the cells and incubated for 5 min at 4°C. After incubation, 30 µL of MACS buffer and 20 µL of the corresponding MicroBead Cocktail were mixed with the cells. An additional incubation step of 10 min at 4°C followed before cells were processed by magnetic separation. In this project, LS columns with a cell number capacity of $2*10^9$ total cells have been used as suggested by the manufacturer's instruction. After placing the columns into the magnetic field, they were rinsed with MACS buffer. Then, the cell suspension was applied onto the column and the flow-through was collected in tubes. Due to the fact that this separation technique is a negative selection method, the flow-through contains the unlabelled and therefore untouched cells of interest. The other subpopulations of PBMCs retain in the column because cells are bound to microMACSi beads that are coated with highly specific antibodies against all other subpopulations except the one of interest. After isolation, cells were pelletized by centrifugation and resuspended in the corresponding medium that was needed for further experiments. The level of purity was checked by flow cytometry and samples were considered pure when at least 96 % of cells displayed the desired immunophenotype ($CD3^+$ or $CD8^+$).

2.2.2. ELISpot assay

The Enzyme Linked Immuno Spot (ELISpot) assay detects various cytokine secreting cells on a single cell level (Fujihashi, McGhee et al. 1993, Merville, Pouteil-Noble et al. 1993) and is a highly sensitive technique for the quantification of cells (Czerkinsky, Nilsson et al. 1984). In this project, isolated PBMCs as described above have been measured for their cytokine secretion profile. Cytokine secreting cells were counted and the median frequency of cells specific for a certain cytokine secretion pattern was determined.

Pre-incubation: For ELISpot assay, isolated PBMCs or magnetically separated PBMC subpopulations were pre-incubated overnight at 37°C in 5 % CO_2 if not stated differently. Cells were therefore plated in 200 µL medium in 96-well U bottom plates and incubated in the presence or absence of stimuli. Stimulation of cells was done with either phythohemagglutinin (PHA), *Candida albicans* extract or with the **CEF** peptide pool consisting of viral peptides from the human Cytomegalovirus (**C**MV), Epstein-Barr virus (**E**BV) and influenza (common **f**lu) virus (Mabtech) at a concentration of 2 µg stimulus/mL as positive controls. Cells were also stimulated with 1 µg WT1 peptide pool/mL, a pool of lyophilized WT1 peptides that consists of 15-mer sequences with 11 amino acids overlap, covering the complete sequence of the WT1 protein (Peptivator WT1, Miltenyi Biotech).

Coating: 96-well Multiscreen plates for ELISpot analysis containing polyvinylidene fluoride (PVDF) membranes were activated with 100 µL of 70 % ethanol for 2 min. Wells were washed with D-PBS in order to remove residual ethanol and coated overnight with 60 µL of monoclonal cytokine antibodies as listed in table 9. Antibodies were incubated overnight at 4°C. If pre-incubation of cells was not required, primary antibodies were incubated for 2-3 hours at 37°C in 5 % CO_2. In the next step, ELISpot plates were washed five times with D-PBS and then blocked with 100 µL of 1x PELISPOT blocking buffer for 1 hour at 37°C incubation. After incubation, blocking buffer was discarded without washing wells.

Incubation and detection: Cells with or without stimulus were transferred from the U plates to the ELISpot plates. After 48 hours of incubation at 37°C, wells were washed five times with D-PBS. Then, 60 µL per well of secondary detection antibodies diluted in PELISPOT buffer were added to the wells and incubated for 1 hour at room temperature (RT). After five additional washing steps with D-PBS, 100 µL of diluted streptavidine-poly-horseradish peroxidase conjugate complex in PELISPOT buffer was added to the wells and plates were incubated for 1 hour at RT. After incubation, plates were washed with D-PBS as previously described. For spot detection, 50 µL of 3,3',5,5'-tetramethylbenzidine (TMB substrate) was

added and plates were kept for spot development a few minutes in the dark. The ELISpot procedure is summarized in figure 3.

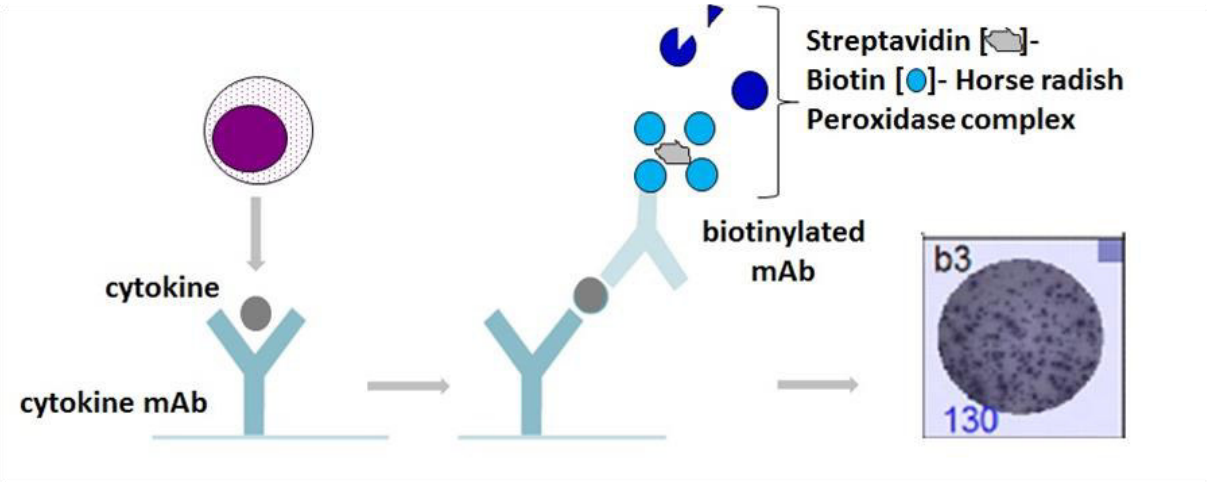


Fig. 3: Scheme of the ELISpot procedure

The primary monoclonal antibody (mAb) coated on an ethanol-treated polyvinylidene difluoride (PVDF) membrane binds to the cytokine which is secreted by the cell upon stimulation. After cell removal, a secondary, biotinylated detection antibody specifically binding the respective cytokine is added to the mixture followed by a streptavidin-biotin-horse radish peroxidase conjugate complex. This enzyme complex is changed into an insoluble precipitate when a colorimetric substrate (TMB) is added. The resulting spots on the PVDF membrane corresponding to single cells can be counted and evaluated by the ELISpot reader.

2.2.3. FluoroSpot assay

The FluoroSpot assay is a fluorescent-labelled ELISpot with the advantage that secretion of two cytokines can be measured simultaneously on a single cell level. Measuring simultaneous cytokine secretion is possible because detection antibodies are labelled with different fluorescent tags that are excited by a fluorescent lamp and two different filters. Both filters create separate images of the cytokines and the reader generates an overlay of both images. Therefore, fluorescent spots that correspond to single cells secreting either one or both cytokines can be counted.

After isolation of PBMCs, cells were magnetically separated through MACS columns in different T cell subpopulations (Pan T cells and $CD8^+$ T cells). For FluoroSpot analysis, T cell subpopulations were directly used after magnetic separation. The protocol for the assay procedure was adjusted according to the manufacturer's guidelines of the "Human IFN-ɣ/ granzyme B (GrB) FluoroSpot Kit" from Mabtech. 150,000 cells per well were plated in 200 µL RPMI medium supplemented with 10 % HS in 96-well U-bottom plates and incubated in the presence or absence of stimuli overnight at 37°C and 5 % CO_2. Stimulation of cells was done with either PHA, *Candida albicans* extract or with peptide pools of CEF and WT1 used at the same concentration as for ELISpot assays. Preparation of a 96-well plate, a so called IPFL (short for **I**mmobilon **P**late **F**luorescence **L**ow) plate, was done by activating the wells with 15 µL of 35 % ethanol for 1 min. This plate is equipped with a low-fluorescent PVDF membrane and clear for suitable fluorescent reading. Wells were washed five times with 200 µL D-PBS in order to remove residual ethanol and coated overnight with 100 µL of monoclonal antibodies against IFN-ɣ and granzyme B at 4°C. In the next step, ELISpot plates were washed five times with D-PBS and then blocked with 200 µL of the same medium containing 10 % of serum as used for the cell suspension. Incubation was done for at least 30 min at RT. After incubation, medium was removed without additional washing. For cell incubation, pre-incubated cells were transferred from the 96–well U-bottom plates to the FluoroSpot plate and placed in the incubator for 24-48 hours at 37°C, 5 % CO_2. Plates were wrapped in foil to prevent evaporation. After incubation, wells were washed five times with D-PBS to remove cells. Then, 100 µL/well of secondary detection antibodies, tagged with fluorescein isothiocyanate (FITC) for IFN-ɣ and cyanine 3 (Cy3) for granzyme B diluted in D-PBS-0.1 % BSA buffer, were added to the wells at a concentration of 2 µg/mL and incubated for 2 hours at RT. After five washing steps with D-PBS, 100 µL of diluted anti-fluorescent antibodies was added to the wells and plates were incubated for another hour at

RT without light exposure. After incubation, plates were washed with D-PBS as previously described and 50 µL of fluorescence enhancer solution was added and left on the wells for 15 min at RT. The procedure of cell labelling is summarized in figure 4.

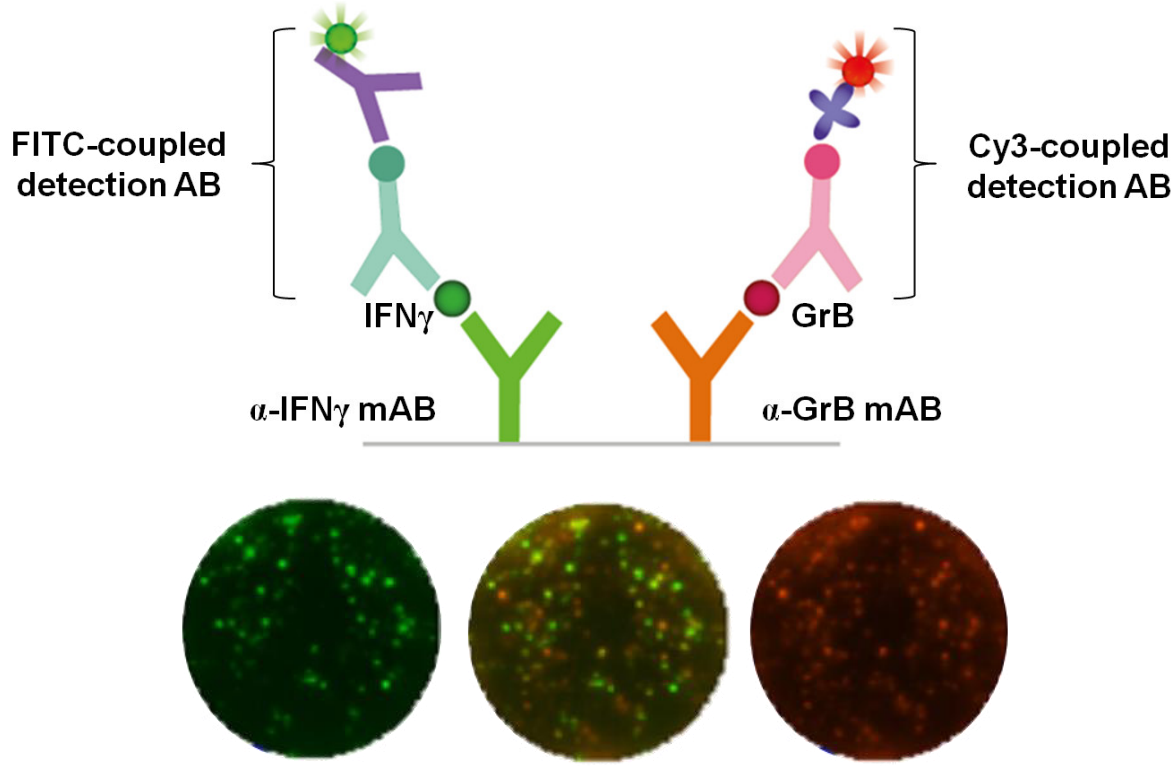


Fig. 4: Scheme of the FluoroSpot procedure
A mixture of primary monoclonal antibodies (mAB) specific for the cytokines IFN-γ and granzyme B are coated on an ethanol-treated polyvinylidene fluoride (PVDF) membrane. In the presence of stimulated cells, these two cytokines are released and bound by the antibodies. After cell removal, a mixture of secondary fluorescence-labelled detection antibodies is added. Green fluorescence (FITC) is labelled indirectly to anti-IFN-γ via a biotinylated antibody, red fluorescence (Cy3) is directly labelled to anti-granzyme B. Enhancer solution is added to the complex and resulting spots can be counted and evaluated with the ELISpot reader.

2.2.4. Fluorescence activated cell sorting (FACS) analysis

Flow cytometry is a method for analyzing expression of cell surface and intracellular molecules that can be used for cell characterisation. The technique is predominantly used to detect and count individual cells by passing in a stream through a laser beam. For analysis, cells have to be tagged by antibodies labelled with a fluorophore. Then, the cell mixture is applied to the stream flowing through a nozzle that allows for single cell counting. As each cell passes through the laser the fluorophore is excited and light will be scattered. Sensitive

photomultiplier tubes equipped with a set of filters and mirrors detect both scattered light and fluorescence emissions. Each fluorophore has a characteristic peak excitation and emission wavelength. The energy of the light photons is converted into an electronic signal which is measured by a detection device. When cells are labelled with a single fluorophore, data are usually plotted by the software in form of histograms, dot plots, density plots or contour plots. Overlapping emission spectra by two different fluorophores excited by the same laser have to be compensated to ensure that the fluorescence detected in a particular detector derives from the fluorochrome that is being measured (BD Biosciences 2000). In this project, flow cytometry was mainly used for phenotypic analysis of cells *pre* and *post* expansion and for assessing the purity of isolated subpopulations after MACS technology.

2.2.5. WT1 expression levels in AML patients

WT1 expression levels from AML patients in relapse and from patients *pre* and *post* transplantation were measured with quantitative real time PCR in the Department of Bone Marrow Transplantation in Essen. After collecting RNA samples from PBMCs of more than 200 patients and healthy controls, WT1 mRNA levels relative to GAPDH, a housekeeping control gene, were measured and values were kindly provided for this project. Relative WT1 expression levels were evaluated using descriptive statistics. The aim of this analysis was the determination of a correlation between WT1 expression levels and AML prognosis to answer the question whether WT1 could be used as a putative prognostic marker for relapse. Relative WT1 mRNA expression levels were correlated to the time between sample analysis and transplantation, in case of patients *post* transplantations, and relapse occurrence in case of relapsed AML patients. Since WT1 is a putative tumor-associated antigen and highly enriched on AML blasts (Inoue, Sugiyama et al. 1994, Menssen, Renkl et al. 1995), its expression level should be increased during relapse and decreased during reconstitution of the bone marrow after transplantation.

2.2.6. Expansion of WT1-specific cells

Expansion of WT1-specific cells depends on various factors. The quality of the primary material, which is mainly peripheral blood from AML patients, is one of the crucial factors. In general it is better to use freshly isolated cells instead of thawed cultures. Another factor is the initial cell number after isolation which can be limited in AML patients. The amount of WT1-specific cells in AML patients is dependent on the presence of leukemic blasts in the peripheral blood which is the limiting factor before starting an *ex vivo* expansion experiment. Since heavily proliferating blasts gradually replace haematopoietic cells in the bone marrow, they are likely to migrate into the periphery over time. However, this is not the case in every patient and the successful isolation of a feasible amount of AML blasts from peripheral blood strongly depends on the frequency and severity of the disease. For this project, different protocols for optimal cell expansion after isolation from peripheral blood have been tested. Expansion protocols have been established by using freshly isolated blood cells from healthy donors. These experiments were done prior to the expansion of WT1-specific cells from AML patients and are not subject of this project. In the following section, the most suitable protocol for appropriate cell expansion is described and variations are stated if applicable. Expansion of specifically stimulated cells was monitored with ELISpot assay and cells were therefore taken from expansion cultures at distinct time points.

Day 0: If frozen material was used, isolated PBMCs of AML patients were thawed as previously described and incubated overnight in fresh RPMI 1640 medium supplemented with 10 % HS.

Day 1: The next day, cells were washed in order to remove dead material, counted and adjusted to 10^7 cells/mL. A subset of cells was analysed by flow cytometry for their immunophenotype. After thawing, $CD14^+$ monocytes could hardly be detected in the cell culture. If needed, dead cells remaining after centrifugation were removed using the Dead Cell Removal Kit (Miltenyi, Muller et al. 1990). In case of freshly isolated PBMCs, cells were treated likewise except the overnight incubation from day 0 to day 1. Cells for negative and positive controls in ELISpot assay were removed as aliquots from the bulk culture and kept in 96-well U-bottom plates until analysis. Positive controls were stimulated with 2 µg/mL PHA and CEF peptide pool in a total of 200 µL culture per well. Unstimulated cells were used as negative controls. Cell numbers for ELISpot analyses varied according to the measured cytokine. In parallel, regardless of fresh or thawed cells, cultures were stimulated with 1 µg WT1 peptide/mL culture. Cultures were incubated for 20-24 h at 37°C and 5 % CO_2.

Day 2: Cultures were washed, counted and checked for viability by Trypan Blue staining. Enrichment of untouched Pan T cells or untouched CD8 positive T cells was done according to the instructions of the isolation kit from Miltenyi as described in section 2.2.1.3. Purity of resulting subpopulations was assessed by flow cytometry. Negative fractions bound to the column (i.e. all other cells excluding the untouched fraction) were eluted, centrifuged, irradiated with 30 Gy, and used as autologous feeder cells for antigen presentation in the expansion cell culture. T cell cultures and irradiated feeder cells were resuspended in RPMI 1640 + 10 % HS.

After isolation, different T cell activation strategies have been tested. One strategy to directly enrich for $CD8^+$ $CD137^+$ T cells was the subsequent use of the CD137 MicroBead Kit from Miltenyi Biotech after the isolation of $CD8^+$ T cells. CD137 is a surface marker expressed on activated $CD8^+$ T cells but does not occur on resting cells (Wolfl, Kuball et al. 2007).
A second activation strategy was deviated from the T cell activation/expansion kit from Miltenyi Biotech, which employs anti-biotin MACSiBead particles that are coupled to biotinylated antibodies against the human surface markers CD2, CD3 and CD28 for the activation of resting T cells. MACSi beads also mimic antigen presentation as another stimulus for T cell activation. Independent of T cell activation strategies, cultures were resuspended in TexMACS expansion medium supplemented with a certain cytokine cocktail. This cocktail consisted of 200 IU/mL Proleukin (IL-2), 10 U/mL of each IL-7 and IL-15 and 0.3 U/mL IL-21. Cells were sowed with a density of $2.5*10^6$ cells/cm^2 in an appropriate cell culture plate. In case of artificial bead activation, different bead-to-cell ratios were tested. For the third expansion strategy, culture conditions with autologous, irradiated feeder cells were determined and different ratios of feeder cells-to-effector cells were employed. The expansion strategy with MACSi bead activation was considered most effective and therefore summarized in figure 5.

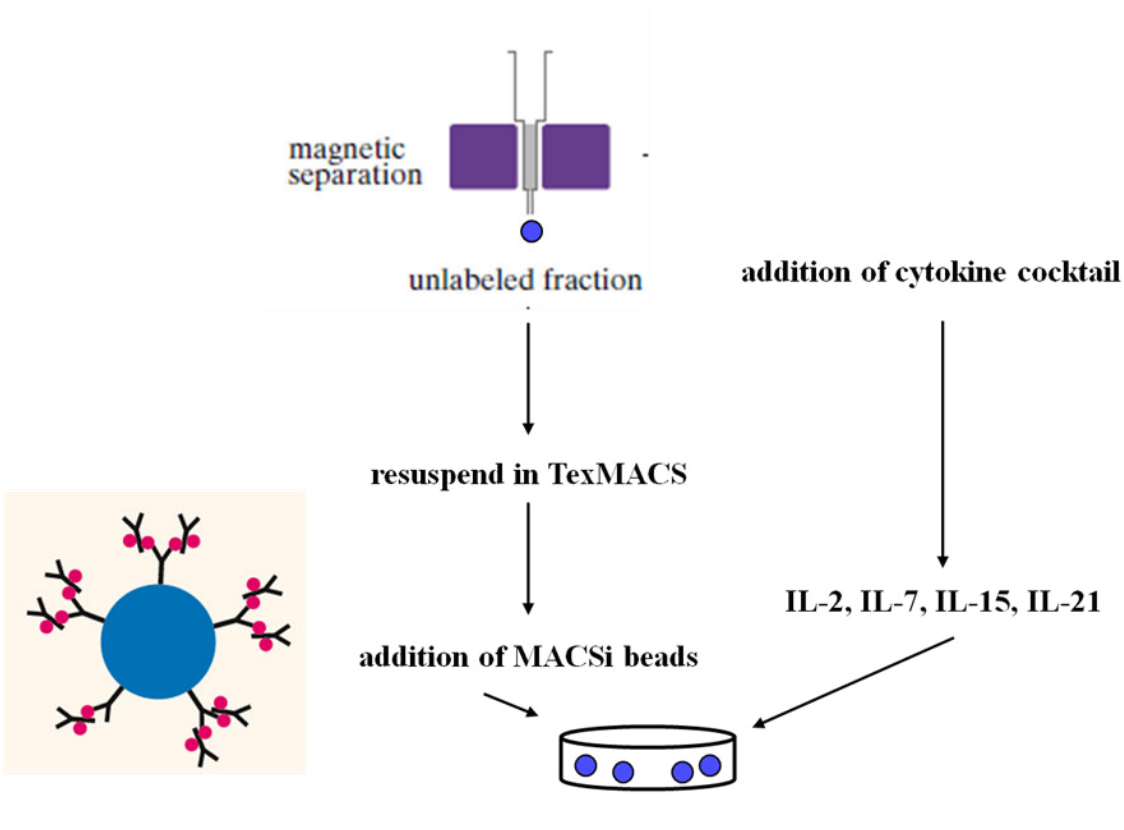


Fig. 5: WT1-specific cell activation and expansion strategy with MACSi bead particles
After magnetic separation of PBMCs into T cell subpopulations, unlabelled Pan T cells are cultured in TexMACS expansion medium with the addition of artificial MACSi beads that mimic antigen-presenting cells. Expansion of WT1-specific T cells is supplemented with a cytokine cocktail of IL-2, IL-7, IL-15 and IL-21 that support cell expansion into functional memory cells, ***adapted from Miltenyi Biotech***

Day 4 and 7: Cells were observed microscopically for viability and appearance. Images were taken for monitoring colony growth. Afterwards, half of the medium was removed and the remaining cell culture was pelletized at 500 g for 5 min. Pellets were resuspended in fresh TexMACS medium supplemented with the cytokine cocktail that was adjusted to its original concentration. Cultures were incubated at 37°C and 5 % CO_2.

Day 9/10: For cell recovery from expansion medium, cells were washed three times with D-PBS and pelletized at 500 g for 5 min. Expansion medium was replaced by RPMI 1640 with 10 % HS for cell recovery. Cultures were kept at a density of $2*10^6$ cells/mL and incubated at 37°C and 5 % CO_2 for 24 hours.

Day 10/11: Cells were observed microscopically for viability and appearance and images were taken. Cells were then counted and adjusted to 10^7 cells per 1 mL medium. For restimulation, cultures were treated with 1 µg WT1 peptide pool/mL culture. Prior

stimulation, cells for ELISpot analysis were removed from the culture and processed in 96-well plates described on day 1. Cultures were incubated at 37°C and 5 % CO_2 for 24 hours.

Day 11/12: After restimulation with WT1 peptide and cytokines, cultures were transferred to fresh expansion medium. With every medium change from culture medium to expansion medium, cytokines and artificial MACSi beads were added at the same concentrations as before. For medium change, cultures were pelletized at 500 g for 5 min, counted and resuspended at a density of $2.5*10^6$ cells/mL culture and further incubated

Day 14/15 and 16/17: Cells were processed as described for day 4 and 7. During this time, cultures were regularly observed and images were taken for cell growth monitoring. After this expansion period, another expansion round was initiated as described for day 9/10. For functional analysis, artificial MACSi beads were removed as follows: Cultures were harvested by centrifugation and resuspended in MACS buffer at a density of 10^7 cells/mL and transferred to 15 mL Falcon tubes. The tubes were placed in the magnetic field of the MACSiMAG Separator, a magnetic device from Miltenyi Biotech. After adherence of bead particles to the tube walls for 2 min at RT, the supernatant with bead-depleted cells was carefully removed and the procedure was repeated. Bead-free supernatants were collected in a new tube and collected cells were ready for further analysis. T cell activation was monitored in ELISpot assays by measuring cytokine secretion of IFN-ɣ, IL-10, granzyme B and perforin. Expanded cells were considered active when cells stimulated with the WT1 peptide pool resulted in twice as much cytokine secreting cells than controls without stimulation.

2.2.7. Europium Release assay

The Europium TDA (short for 2,2':6'2"-terpyridine-6,6"-dicarboxylic acid) (EuTDA) release assay based on PerkinElmer's DELFIA technology is a non-radioactive cytotoxicity assay that offers sensitivity comparable to the well-known ^{51}Cr release assay. In this project, the assay aimed at measuring the amount of lysed tumor cells by functional WT1-specific effector cells. Instead of using radioactive chromium, target cells are loaded with an acetoxymethyl ester of fluorescence enhancing ligand (BATDA, short for bis (acetoxymethyl) 2,2':6',2"-terpyridine-6,6"-dicarboxylate)) which can enter the cell through the cell membrane. Inside the cell, a hydrophilic ligand (TDA) is formed due to hydrolysis of ester bonds. As a consequence, TDA stays in the cytoplasm of target cells since it can no longer pass the membrane (Blomberg, Granberg et al. 1986).

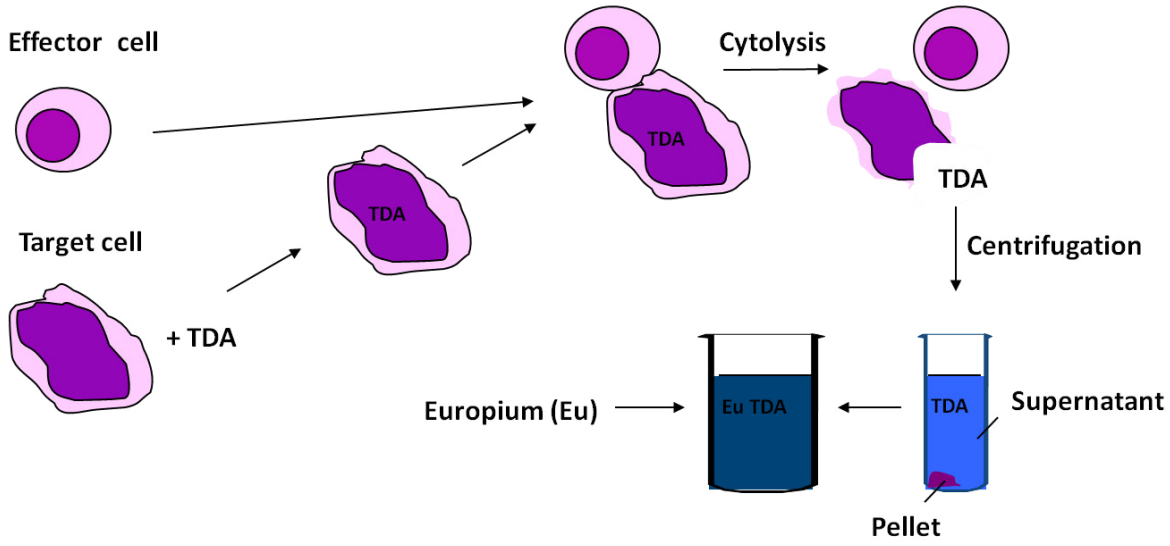


Fig. 6: Principle of Europium Release Assay
Target cells are labelled with 2,2':6'2"-terpyridine-6,6"-dicarboxylic acid (TDA) ligand, which is released upon target cell lysis by specific effector cells. After cytolysis, TDA is present in the cells' supernatant and labelled with Europium solution resulting in a stable, fluorescent complex. Fluorescence is then measured with time-resolved fluorometry.

As summarized in figure 6, TDA is released upon lysis of target cells by effector cells and coupled to Europium solution, forming a highly stable chelate complex whose fluorescence can be measured with time-resolved fluorometry.

Prior to target cell labelling, AML cell lines were checked for viability. Cell numbers were adjusted to 10,000 cells/well. Experiments were performed in 96-well V bottom plates and cells were cultured in RPMI 1640 medium supplemented with 10 % HS for analysis. An appropriate number of target cells needed for analysis was washed in D-PBS in order to remove culture medium containing 10 % of FCS. Cells were adjusted to 10^6 cells/mL for labelling. 2.5 µL/mL cell suspension of the fluorescent ligand BATDA was added and cells were incubated for 25 min at 37°C and 5 % CO_2. The cells were then washed three times with D-PBS and the cell density was adjusted to 10^5 cells/mL for analysis. PBMCs as effector cells of AML patients were stimulated with the appropriate peptide and adjusted to a target cell : effector cell ratio of 1:4 to 1:100. Before loading target cells with varying concentrations of effector cells, cells for background and spontaneous release were added. For the background control, 100 µL of cell supernatant without target cells and 100 µL medium was added in triplicates to the wells. For measuring spontaneous release, 100 µL of target cells without effector cells was incubated with 100 µL medium. For maximum release, 100 µL of target cells was incubated with 100 µL medium supplemented with 15 µL of DELFIA lysis buffer.

For cell lysis experiments, 100 µL of target cells were loaded with the respective amount of effector cells and the volume was adjusted to 200 µL with fresh medium. The plate was then incubated for 2.5 hours at 37°C. Fifteen min before incubation ended, lysis buffer was added to the corresponding control well for maximum release. The plate was centrifuged at 500 g for 5 min and 20 µL of supernatant from every well was transferred to a DELFIA microtitration plate containing 200 µL of Europium solution/well. The plate was further incubated for 15 min at RT on a plate shaker. The signal was measured with time-resolved fluorometry using the Wallac Delfia 1232 fluorometer from Perkin Elmer. For measurement, the instrument used the following parameters:

Tab. 15: Parameter settings for fluorometry

Parameters	**Setting**
Assay type	Counts
Blanks	0
Unknown Replicates	12
Sysset: Flash cycle	1.00
Delay time	0.40
Window time	0.40
Dead time	10
Emission filter	615 nm
Excitation filter	320 nm

After counting fluorescence, results were calculated. The background control was subtracted from spontaneous and maximum releases and percentage release was determined by counts with the following formulas:

$$\% \text{ spontaneous release} = \frac{spontaneous\ counts - background\ counts}{maximum\ counts - background\ counts} * 100$$

$$\% \text{ specific release} = \frac{measured\ counts - spontaneous\ counts}{maximum\ counts - spontaneous\ counts} * 100$$

2.2.8. Cell lysis of CMV positive target cells by CMV-specific effector cells

In cooperation with the company Cell Medica, a cell lysis experiment was conducted. For this purpose, a cell lysis assay was established. In order to establish experimental conditions, K562 cells were taken as target cells. For observation of target cell lysis, e.g. by apopotosis, during microscopy, the FITC Annexin V/ Dead Cell Apoptosis Kit from Invitrogen was used. The following protocol was developed for K562 cells treated with chlorpromazine, an endocytosis inhibitor, for apoptosis induction. Therefore, titration of chlorpromazine was performed prior to the actual experiments. Negative controls were prepared by incubating K562 in the absence of chlorpromazine. After incubation, target cells were washed with cold D-PBS (an ion-free formulation without calcium and magnesium as annexin will desolve) and propidium iodide (PI) and annexin working solutions were prepared according to the kit's instructions. 10^6 cells/mL were selected as the best cell density for incubation in 24-well suspension plates. 10 µL of the annexin V-FITC conjugate and 1.5 µL red-fluorescent PI working solution containing 100 µg PI/mL was added to 100 µL of K562 suspension and cells were incubated for 15 min at RT before visualization. The incubation temperature during microscopy was kept at 37°C in the built-in conditioned cell incubation chamber. For analysis, cells were separated into different subgroups according to the intensity of their fluorescent signal. Living cells show no or little fluorescence while apoptotic cells show a high degree of surface fluorescence. Viable cells have intact membranes impermeable for PI, thereby it stains only dead cells. Apoptotic cells appear with green surface fluorescence, dead cells show both membrane staining by annexin V and nuclear staining by PI. Cells were observed for 2 hours. Annexin bleached out over time and had to be added again in case of longer exposure.

After setting up experimental conditions with K562 cells, experiments were conducted with T2 cells as targets. As described in 2.1.6. T2 cells are HLA-A*02 positive and have a defect in antigen processing. Therefore, T2 cells can be loaded with exogenous peptides that are presented via HLA-A*02 (Salter, Howell et al. 1985). Freshly isolated or thawed PBMCs of a CMV-HLA-A*02-positive healthy control were selected as effector cells and resuspended in culture medium. On the same day, effector cells were stimulated with the CMV peptide pp65 NLVPMVATV to activate the cells. Cells were incubated overnight at 37°C and 5 % CO_2. To assure that NK cells do not take over effector cell function, NK cell removal was performed with MACS technology. The NK cell-negative fraction ($CD56^-$) was taken into culture and incubated for 6 days at 37°C and 5 % CO_2. During this time, daily observation of cell growth

and viability was crucial. After incubation, the culture was again magnetically separated into $CD3^+$ and $CD3^-$ fractions and $CD3^+$ cells were restimulated with the CMV peptide. After overnight incubation, cells were prepared for analysis. In the meantime, T2 target cells were counted and checked for viability. In the next step, target cells were loaded with the CMV peptide for 15 min at 37°C and 5 % CO_2. Cell cultures of effector and target cells were then mixed at a ratio of 10:1 and transferred into a 24-well suspension plate for analysis. Controls were prepared as described for K562. T2 cells stained with annexin V were carried along with the sample in a control well that did not contain effector cells. Additionally, a culture of pure effector cells was observed in order to see if incubation conditions were appropriate during microscopic analysis as shown in figure 7.

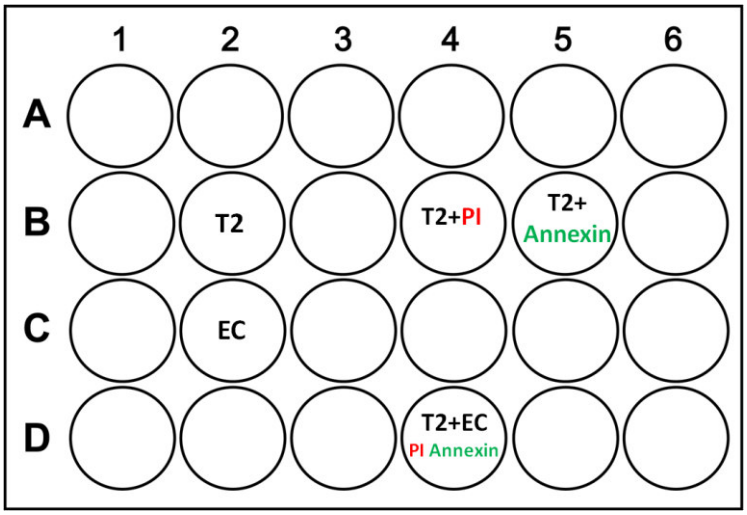


Fig. 7: Loading of a 24-well plate with effector cells, target cells and corresponding controls
Target cells (T2) are cultured in control wells without dye (T2, B2) and with either propidium iodide (T2+PI, B4) or annexin (T2+annexin, B5). As a control for effector cell viability, effector cells (EC) are cultured alone (C2) and in addition with T2 cells, propidium iodide and annexin (D4) for cell lysis. The colour code indicates the fluorescent colour of the dyes.

In the next experiment, $CD8^+$ HLA-A*02 positive T cells specific for the CMV epitope pp65 NLVPMVATV (Gratama, van Esser et al. 2001) were purified and confirmed as 98 % pure in flow cytometry. Cells were then cultured in RPMI 1640 supplemented with 10 % HS and incubated at 37°C and 5 % CO_2 until use. In the meantime, the cell line T2 was cultured and cells were counted and checked for viability by Trypan Blue staining. 50.000 T2 cells in 250 µL medium were loaded with 2.5 µL pp65 NLVPMVATV peptide and incubated for 15 min at 37°C and 5 % CO_2. In the next step, $CD8^+$ effector cells were added to T2 target cells in a 24-well suspension plate at a ratio of 10:1. Immediately after combining effector and target cells, 5 µL of FITC-coupled annexin V was added to 500 µL of the cell suspension and the culture was observed under the microscope for 2 hours. After 2 hours another 5 µL of the

dye was added because of bleaching. Control wells containing target cells with the addition of either red-fluorescent PI or FITC-labelled annexin V were treated in the same manner. Cell cultures were visualised microscopically with Zeiss Axio Observer X. Results were displayed in time-lapse images.

2.2.9. Western Blot to determine WT1 protein level in AML cell lines

The WT1 protein is 52-54 kDa in size (Morris, Madden et al. 1991). In previous experiments, WT1 expression in fractionated protein lysates (nuclear and cytoplasmic) was found to be almost not detectable by Western Blot analysis. Therefore, all other experiments were done with the whole cell lysate.

Sample preparation: Cells of AML cell lines were counted and tested for viability. For analysis, $1*10^6$- 10^7 cells/100 µL medium were removed from cell culture and centrifuged at 1000 g for 1 min. After discarding the supernatant, total cell lysates were prepared by solubilising cells in sample buffer. Cell extracts were denatured at 95°C for 3.5 min and sonicated for 5-10 sec with 5 bursts each at 50 % amplitude.

Gel preparation and electrophoresis: After appropriate preparation of the running and stacking gel containing a mixture of polyacrylamide and acrylamide (PAA) in a ratio of 37.5:1, the electrophoresis chamber was adjusted and filled with gel running buffer. 5 µL of PageRuler™ and 20 µL of sample adjusted to the same cell number were loaded onto the gel. The electrophoresis unit was then connected with a power supply and the electrophoresis was started at 20 mA for approximately 20 min until the dye front has completely migrated into the running gel. Afterwards the current was increased to 400 mA until the dye front reaches the last 2 mm of the bottom of the gel. This distance was considered as complete electrophoresis. Gels were dislocated from glass plates and prepared for membrane transfer.

Transfer of proteins: The PVDF membrane was prepared as follows: for activation, the membrane was submerged in 100 % methanol for 10 sec and then placed in de-ionized water until assembly of transfer unit done. For assembly, the following stacking with wet components was done: sponge on anode plate → 2 Whatman filter papers (wet in transfer buffer) → PVDF membrane → gel → 2 Whatman filter papers → sponge → cathode plate. The transfer unit was filled with transfer buffer and transfer was initiated at 4°C. A constant current of 400 mA was applied for 120 min. After transfer, the membrane was prepared for immunostaining.

Immunostaining: The electrophoresed proteins were transferred to a PVDF membrane and incubated in blocking solution for 1 hour at room temperature on a shaker. After one hour, the blocking buffer was poured off and the membrane was probed with 1 µg/mL of goat-α-human WT1 polyclonal antibody (1:1,000) in blocking solution and incubated overnight at 4°C on a shaker. The next day, the membrane was washed with blotting buffer 5 times for 10 min. The membrane was then incubated for one hour at RT with the secondary antibody solution containing horseradish peroxidase (HRP)-conjugated donkey-α-goat antibody (1:10,000). After incubation, the membrane was washed with buffer as described and treated with substrate solution (Thermo Fisher) for protein detection. In order to stop the colour development, the membrane was rinsed with de-ionized water.

2.2.10. Intra- and extracellular staining of WT1 protein

For immunofluorescent labelling, AML cells were counted and checked for their cells' viability. For intracellular staining in flow cytometry the cellular membranes had to be permeabilizied without losing the cells' integrity. Fixating agents such as paraformaldehyde (PFA) stably cross-link protein structures on the cell surface before membranes are permeabilizied by Triton X 100 or other detergents (Sander, Andersson et al. 1991). $4*10^6$ cells were washed with D-PBS and incubated in 4 % PFA for 20 min at 4°C in the dark for fixation. Afterwards cells were washed with isotonic solution supplemented with decreasing concentrations of Triton X 100 (from 0.1- 0.01 %) and 5 % donkey serum. Cells were incubated for 30 min at 4°C in the dark with the primary antibodies of either goat-α-human WT1 or the isotype control, respectively. Unbound antibodies were removed by washing the cells with isotonic solution and 0.01 % Triton X 100 in three centrifugation steps. For indirect staining, a second incubation with a fluorescence-coupled antibody specific for the primary antibodies (here: donkey-α-goat-PE) followed. After incubation of 20-30 min at 4°C in the dark, cells were washed twice with isotonic solution and resuspended in 100 µL FACS buffer for immediate analysis. For extracellular staining, $1*10^6$ cells were stained with the unconjugated primary WT1-specific antibody or corresponding isotype control antibodies. Therefore cells were pelletized and pellets were resuspended in buffer containing an excess amount of antibodies, which was determined by titration in previous experiments, for 15 min at 4°C in the dark. Unbound antibody was washed off with FACS buffer containing D-PBS supplemented with 0.5-1 % BSA. The second antibody specific for the primary antibody was labelled with phycoerythrin (PE) and incubated with the cells for 20 min at 4°C in the dark.

After washing steps as described above, cells were directly used for flow cytometry. Cell staining was analysed on an FC 500 (Beckman Coulter) flow cytometer. After gating on size and granularity of cells in the forward/side scatter (FSC/SSC), 10^4 events were analysed with Kaluza® Flow analysis software from Beckman Coulter. The mean fluorescence intensity (MFI) of the isotypic control staining was compared to the MFI of the WT1-specific staining and shown as histograms.

2.2.11. WT1 mRNA level in AML cell lines

WT1 expression levels were also determined on mRNA level in AML cell lines. To assess expression levels of WT1 in AML cell lines for comparison to AML patient samples, RNA was extracted from cell lysates by phenol-chloroform-precipitation and cDNA was produced by reverse transcription. Subsequently, quantitative PCRs were performed employing specific primers for WT1 and a housekeeping gene (GAPDH), respectively. To obtain a comparable value for the expression of WT1, it had to be normalized to the corresponding GAPDH expression level. These experiments were done and kindly provided by the Department of Bone Marrow Transplantation at the University Hospital in Essen.

2.2.12. Statistical analysis

For statistical analyses, GraphPad Prism 6 (GraphPad software, La Jolla, USA) was used. Data of cell frequencies were analysed with descriptive statistics and are indicated as median and interquartile ranges or as mean and standard error of the mean (SEM). The nonparametric t test has been used for comparing non-Gaussian distributed groups of data measured with the same analyte. In order to measure how well the relationship between two variables can be described, the Spearman rank correlation coefficient r was calculated and interpreted by the Dancey's and Reidy's categorisation (Dancey and Reidy 2004). The probability is given as values that were considered significant with $p < 0.05$.

3. Results

3.1. Analysis of WT1-specific effector cells

3.1.1. Cytokine secretion of WT1-specific cells

Subpopulations of immune cells have different cytokine secretion profiles (Street and Mosmann 1991, Ziegler-Heitbrock, Ströbel et al. 1992). Immune cells in the human body fulfil different tasks and secrete cytokines upon antigen stimulation to activate or stimulate other cells. For the generation of an immunotherapy, it is important to know which cell population secretes which cytokines. A pro-inflammatory response employs cellular agents in order to attack the tumor environment. Measuring an anti-inflammatory response would rather indicate regulatory immune cell function. In order to characterize WT1-specific cells, cytokine secretion profiles were measured with ELISpot assay. These experiments gave a first hint how cells behave on WT1 peptide stimulation. A broad range of pro-inflammatory (IFN-ɣ, granzyme B, perforin, TNFα, IL-2, IL-12, IL-17a) and anti-inflammatory (IL-4, IL-10, IL-13) cytokines was tested (Sanchez-Correa, Bergua et al. 2013). In the ELISpot assay, cytokine secretion results in different spot sizes and requires individual counting for each cytokine. Whereas pro-inflammatory cytokine secretion mainly appears as big spots, anti-inflammatory cytokine secretion varies from small to tiny spots. Resulting spots are converted into frequencies of WT1-specific immune cells which is shown in figure 8.

For the establishment of assay conditions, experiments were firstly set up with PBMCs of healthy controls. Freshly isolated PBMCs from a buffy coat, a concentrated leukocyte suspension, of 3 healthy individuals were divided into two parts where one part was used for negative selection of CD3 cells via magnetic separation to isolated untouched Pan T cells. The other part remained as the unfractioned PBMC fraction. Cells of both fractions were either stimulated with the mitogen PHA (1 µg/mL) or the antigen *Candida albicans* (62.25 µg/mL) as positive controls or with the peptide pool of the human WT1 protein (1 µg/mL). Unstimulated cells were used as a negative control. Both fractions were stimulated with peptides overnight and further incubated for 2 days for measuring cytokine secretion.

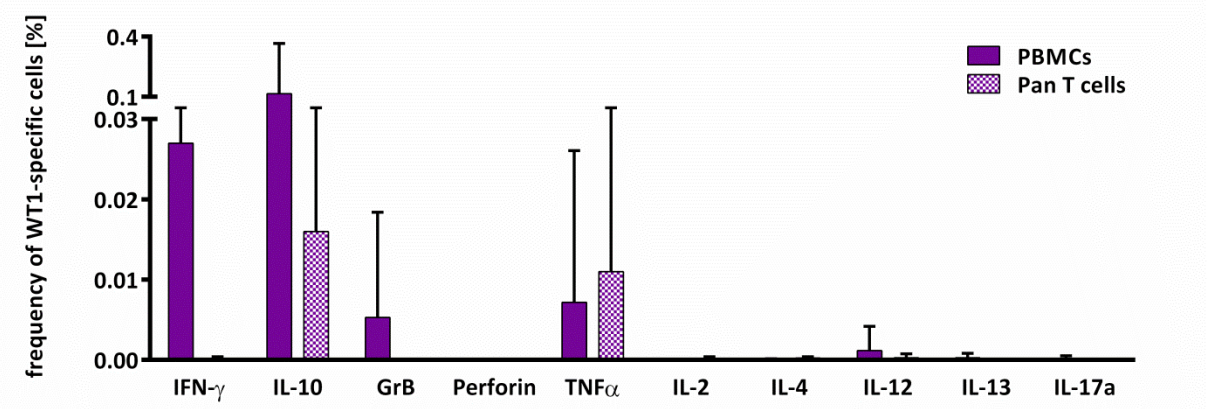


Fig. 8: Mean frequencies of WT1-specific PBMCs and Pan T cells in healthy controls
Cytokine secretion was analysed in RPMI 1640 with 10% human serum pool for healthy controls. Pan T cells were isolated via magnetic separation. Cells were used without stimulus and with WT1 peptide stimulation and measured in ELISpot assay. Mean frequencies of WT1-specific cells were calculated and shown with standard error of the mean (GrB= granzyme B, n=3)

The PBMC fraction that consists of monocytes, B cells and NK cells next to the T cell fraction, secreted both pro- and anti-inflammatory cytokines (figure 8, n=3). The production of IFN-γ, IL-10, granzyme B, IL-12, IL-13 and IL-17a was higher in the PBMC fraction than in the Pan T cell fraction. Pan T cells also secreted cytokines belonging to both immune responses but mainly IL-10 and TNFα upon WT1 stimulation. In case of IL-10, a representative cytokine in anti-inflammatory immune response, the Pan T cell fraction only secreted one fifth compared to the whole PBMC fraction. This leads to the conclusion that PBMC subpopulations such as monocytes mainly secrete IL-10 and are therefore contribute to regulatory function.

In the next experiments the PBMC and Pan T cell fraction was further separated into subpopulations and analysed for cytokine secretion. PBMCs were magnetically separated into two different T cell subpopulations ($CD4^+$ and $CD8^+$), an NK cell population ($CD56^+$) and a monocytes population ($CD14^+$). Resulting WT1-specific frequencies are shown in figure 9.

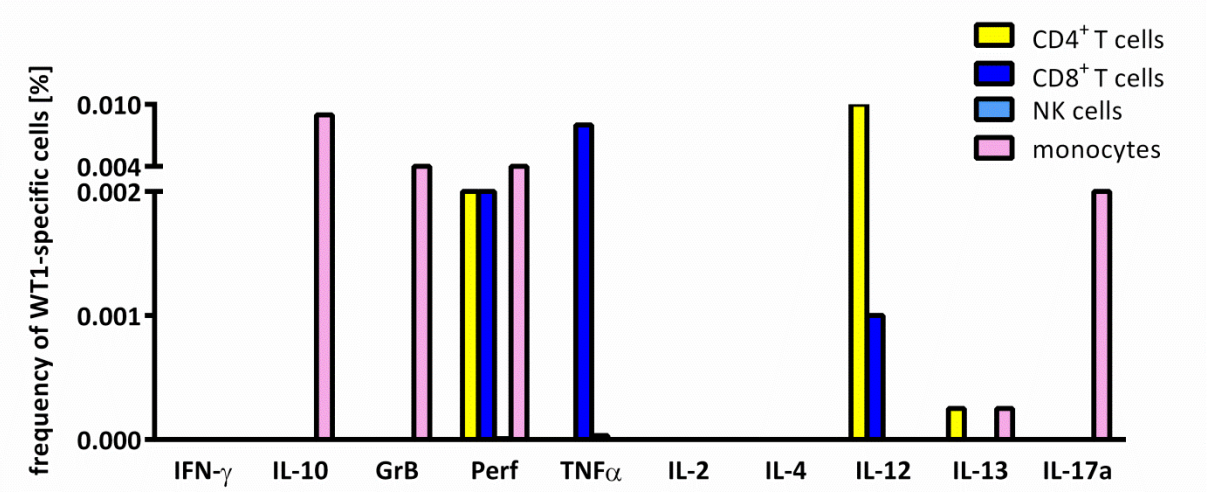


Fig. 9: Cytokine screening analysis of cell subpopulations

PBMCs of one healthy control were separated into different subpopulations by magnetic cell isolation (MACS columns). CD4 and CD8 positive T cells, NK cells and monocytes were analysed in ELISpot assay. Cell cultures were pre-incubated with WT1 peptide overnight and further incubated for 48 h before ELISpot analysis. Frequencies of WT1-specific cells are shown in percent (GrB= granzyme B).

Whereas the $CD4^+$ T helper cells secreted perforin, IL-12 and IL-13, the cytotoxic $CD8^+$ T cell fraction only secreted pro-inflammatory cytokines such as perforin, TNFα and IL-12. The NK cell fraction that is said to have a similar secretion profile as $CD8^+$ T cells did not secrete any cytokine in this experiment. Stimulated monocytes secreted both cytolytic enzymes and the cytokines IL-10, IL-13 and IL-17a. In conclusion, immune cells from the same bulk culture of a healthy individual have different cytokine secretion profiles and WT1 stimulation results in very low cell frequencies.

Because of these low frequencies in the measured subpopulations and due to limited patient material, it was decided that following experiments were conducted with the entire PBMC fraction. For analysis, IFN-ɣ as a pro-inflammatory cytokine and cytolytic enzymes granzyme B and perforin were selected. Additionally IL-10 was measured to monitor potential regulatory immune response by monocytes present in the PBMC fraction. In the following experiments, WT1-specific, cytokine secreting PBMCs were characterised and compared in healthy individuals (figure 10) and AML patients *pre* and *post* transplantation (figure 11 and 12). As WT1 is a leukemia-associated antigen, it is overexpressed in malignant cells but can be also found in normal tissue. This would explain that even in healthy controls some WT1-specific spots can be detected by ELISpot but median frequencies are equal to zero for all measured cytokines.

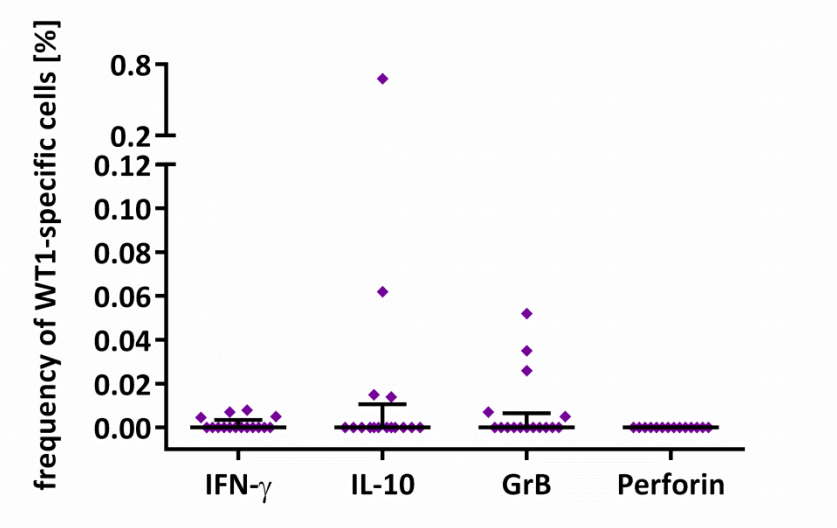


Fig. 10: Median frequencies of WT1-specific cells in healthy controls
PBMCs of healthy donors (n=16) were pre-incubated without stimulus and with WT1 peptide. Cytokine secretion of IFN-ɣ, IL-10, granzyme B (GrB) and perforin was measured in RPMI 1640 medium supplemented with 10% human serum pool in ELISpot assay. Median frequencies of WT1-specific cells and interquartile ranges are shown.

Compared to healthy controls (figure 10) WT1-specific spots were slightly higher in patients *pre* transplantation (figure 11). Even if the median frequencies for WT1-specific cells were similar to the ones of healthy controls, the range of detected spots was broader. Figure 11 shows that AML patients (n=18) have different cytokine profiles especially for IL-10. In some patients high frequencies of IL-10 producing cells could be measured. This also holds true for some cells secreting perforin. IFN-ɣ and granzyme B production was presumably low because AML patients do not have a fully functional immune system to fight leukemic blasts.

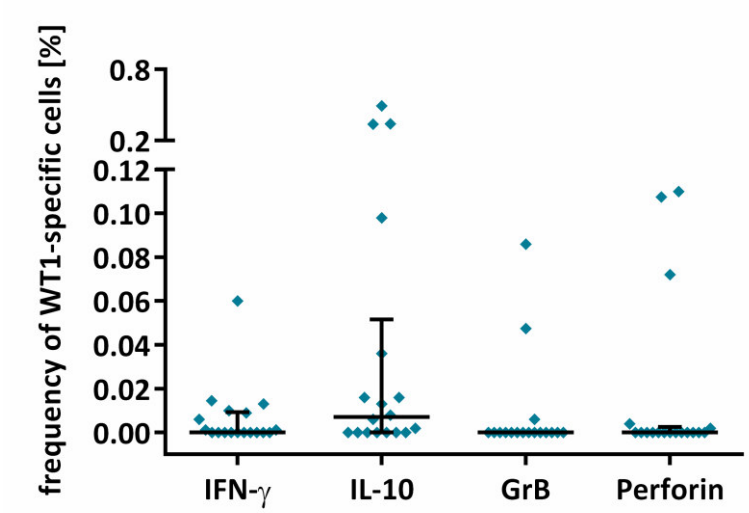


Fig. 11: Median frequencies of WT1-specific cells in AML patients *pre* transplantation
PBMCs of AML patients pre transplantation (n=18) were pre-incubated without stimulus and with WT1 peptide. Cytokine secretion of IFN-γ, IL-10, granzyme B (GrB) and perforin was measured in AIMV medium in ELISpot assay. Median frequencies of WT1-specific cells and interquartile ranges are shown.

AML patients *post* transplantation (n=33) showed comparable median frequencies of WT1-specific cells to the other two groups as represented in figure 12. However, interquartile ranges were even broader compared to ranges in healthy controls and AML patients *pre* transplantation. Single spots counts measured for all cytokines were higher in AML patients *post* transplantation than in the other cohorts. IL-10 secretion was detected highest among the four measured cytokines which could be dependent on the number of monocytes in the patient's PBMC population.

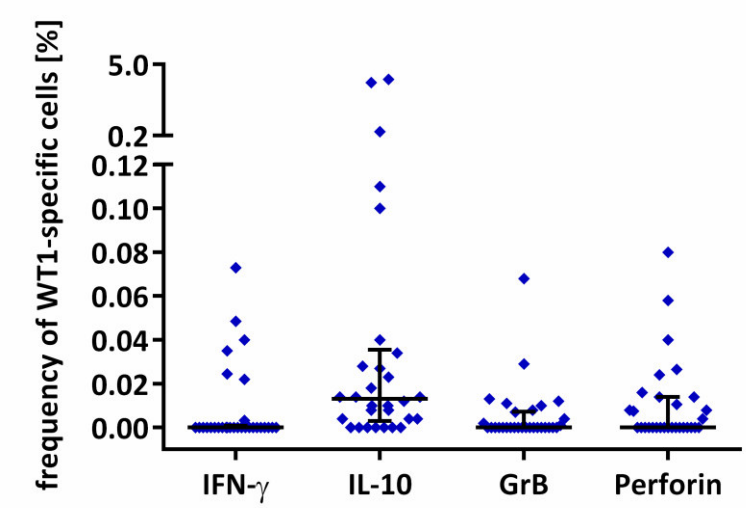


Fig. 12: Median frequencies of WT1-specific cells in AML patients *post* transplantation
PBMCs of AML patients post transplantation (n=33) were pre-incubated without stimulus and with WT1 peptide. Cytokine secretion of IFN-γ, IL-10, granzyme B (GrB) and perforin was measured in AIMV medium in ELISpot assay. Median frequencies of WT1-specific cells and interquartile ranges are shown.

Median frequencies for all three cohorts are summarized in table 16 and do not differ significantly between the groups as already mentioned. However, single frequencies of WT1-specific cells from patients *post* transplantation are already higher than in the other two groups and thus cells seem to be more active. Therefore, cells are taken as starting material for expansion and functional analysis.

Tab. 16: Comparison of median frequencies for WT1-specific cells in AML patients and healthy controls

	IFN-γ	IL-10	granzyme B	perforin
Healthy controls	0.0	0.0	0.0	0.0
pre HSCT	0.0	0.007	0.0	0.0
post HSCT	0.0	0.013	0.0	0.0

IFN-γ-, IL-10-, granzyme B-and perforin-secreting mononuclear cells in healthy controls and AML patients pre and post haematopoietic cell transplantation (HSCT). Frequencies measured in ELISpot assay are given in percentage.

In the next step, the relationship between the time from transplantation until sample analysis and the WT1-specific frequency at the measured time point was determined with Spearman's correlation coefficient r. This correlation shows how two variables, here time and frequency are related to each other. If there is no relationship, the coefficient equals zero. Values greater than zero describe the strength of the relationship (Dancey and Reidy 2004). With increasing time until sample analysis, IFN-γ secreting cells did not increase in number and showed no

correlation (r = -0.11). Cells secreting the cytolytic enzyme granzyme B slightly increased over time (r =0.17) (figure 13).

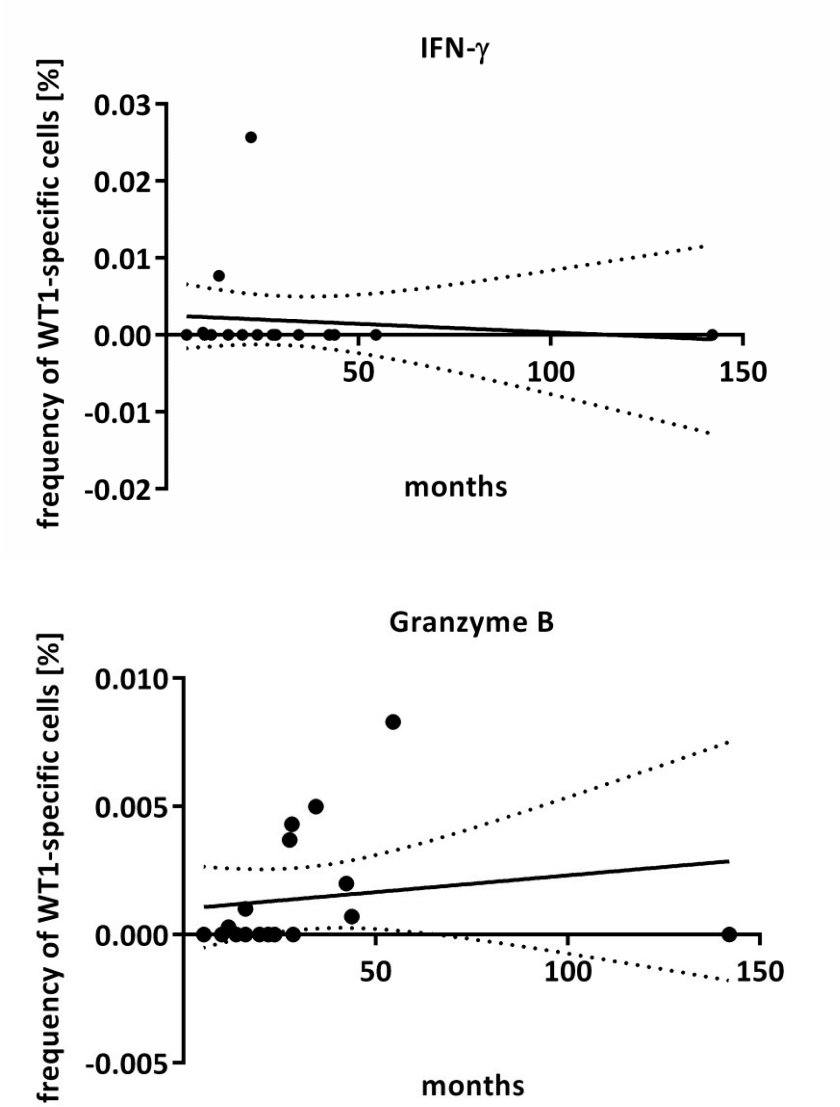


Fig. 13: Correlation between frequencies of WT1-specific cells secreting IFN-ɣ/ granzyme B and time after transplantation

WT1-specific cell frequencies of AML patients post transplantation (n=18) were analysed for correlation with time after transplantation. Correlation was investigated by Spearman's rank correlation coefficient r (IFN-ɣ-secreting cells r= -0.11, p= 0.7, granzyme B-secreting cells r= 0.17, p= 0.5).

Whereas conventional ELISpot analyses cytokine-secreting cells separately for different cytokines, a fluorescent detection assay (FluoroSpot) enabled simultaneous enumeration of WT1-specific cells secreting two cytokines in the same well. In order to compare assays and to test sensitivity, WT1-specific, spot-forming cells (SFC) from 20 patients after

transplantation were measured with both assays in parallel and results are shown in the figure 14.

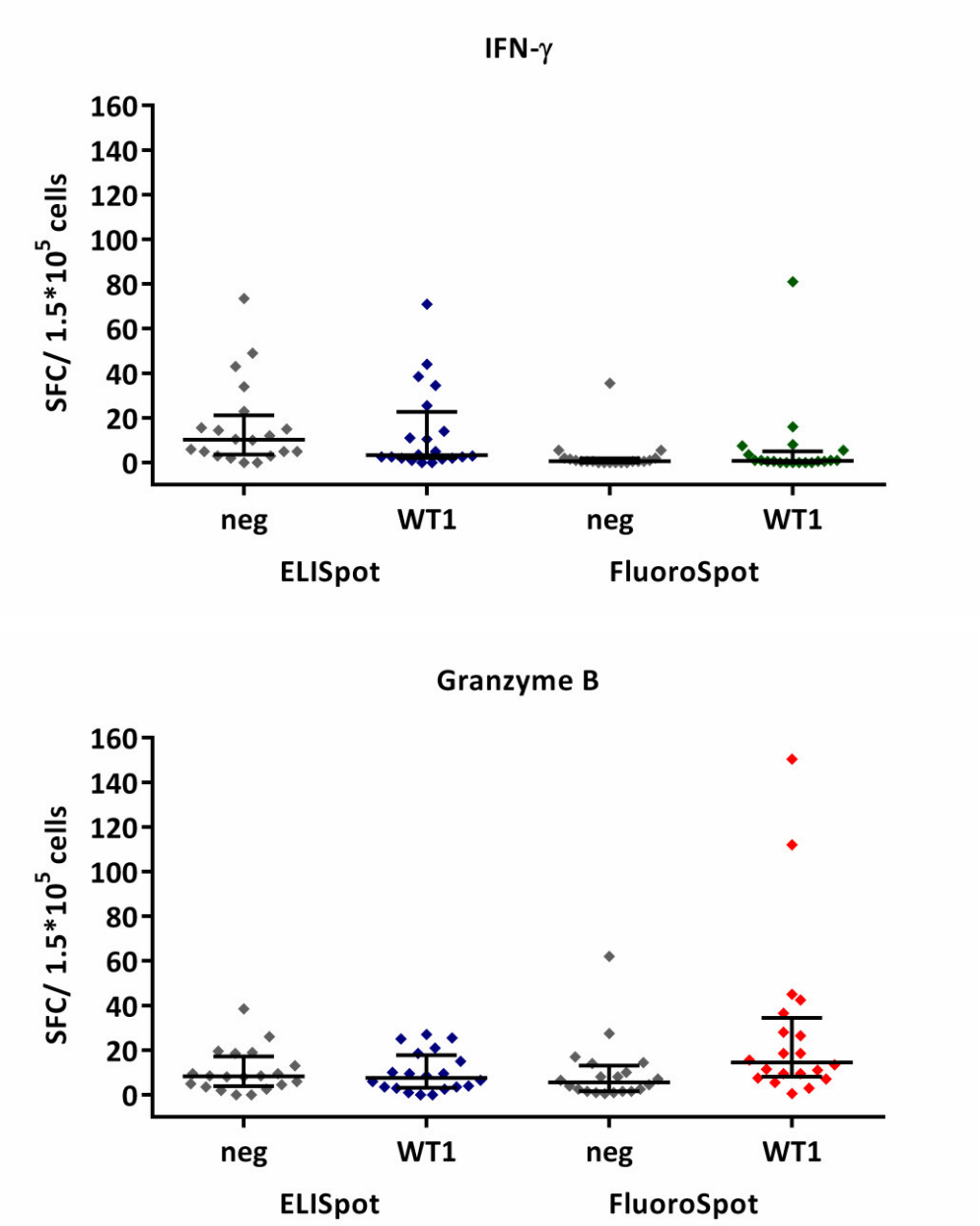


Fig. 14: ELISpot and FluoroSpot analysis of spot-forming cells secreting IFN-ɣ and granzyme B

Cytokine secretion was analysed in AML patients after transplantation (n=20) by ELISpot and FluoroSpot. Unstimulated (neg) Pan T cells were compared to WT1 stimulated Pan T cells. After overnight incubation with WT1, cells were further incubated for 2 days and WT1 spots are shown in colour corresponding to the labelling fluorochrome. Median spot-forming cells (SFC) are shown with interquartile ranges.

IFN-ɣ and granzyme B were selected as analytes for cytokine secretion in ELISpot procedure in order to further characterize the pro-inflammatory WT1-specific response. In general, in non-stimulated controls, FluoroSpot results were lower as compared to ELISpot results (figure 14 and 15). This refers to the fact that detection of fluorescent spots is more accurate due to lower background noise. In ELISpot, frequencies for WT1-specific cells secreting both cytokines could not be observed as negative controls were even higher than WT1-specific spot numbers. In FluoroSpot analysis, however, SFC in negative controls were lower than SFC in WT1-specific stimulation. As a result, WT1-specific cell frequencies can be measured by FluoroSpot. Median cell counts of both assays are summarized in table 17. According to statistical analysis, median spot-forming cells did not differ significantly.

Tab. 17: Comparison of median spot-forming cells in ELISpot and FluoroSpot

	ELISpot		FluoroSpot	
	neg	**WT1**	**neg**	**WT1**
IFN-ɣ	10.25	3.25	0.50	0.75
Granyzme B	8.25	7.50	5.50	14.50

Data are given in median spot-forming cells per 150,000 Pan T cells, isolated of AML patients post haematopoietic cell transplantation, n=20. Unstimulated cells (neg) were compared to WT1 stimulated cells.

WT1-specific responses in ELISpot and FluoroSpot were also compared by Spearman correlation. Values showed a slightly positive correlation (data not shown). The FluoroSpot assay was more sensitive than the ELISpot assay which is shown in a representative example in figure 15.

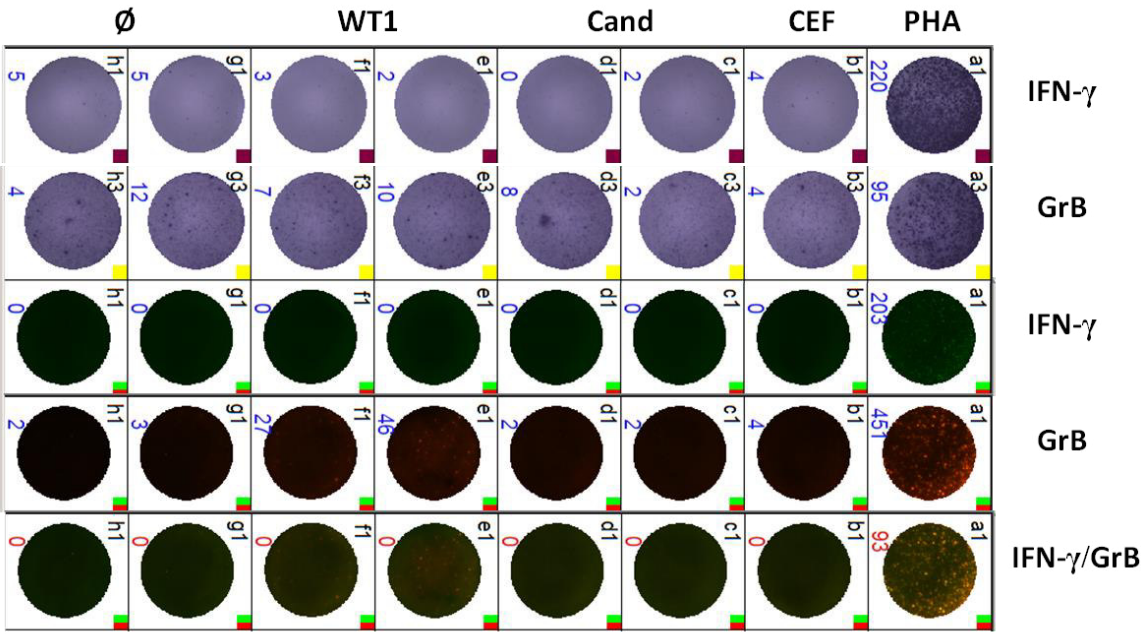


Fig. 15: Representative example of spot-forming cells secreting IFN-ɣ and granzyme B (GrB) in ELISpot and FluoroSpot analysis of the same sample

Cytokine secretion was analysed in an AML patient after haematopoietic stem cell transplantation in ELISpot (two upper rows) and FluoroSpot (three lower rows). Unstimulated (neg) Pan T cells were compared to WT1 stimulated Pan T cells measured in both assays. Cells stimulated with Candida albicans (Cand), CEF peptide pool and phythohemagglutinin (PHA) served as positive controls. After overnight incubation with stimuli, cells were further incubated for 2 days.

Therefore, FluoroSpot was also used in the next experiments where simultaneous secretion of IFN-ɣ and granzyme B in patients after transplantation was analysed. Here, FluoroSpot permits the detection of a T cell subpopulation defined by their simultaneous secretion of these cytokines. This is of great importance when culture conditions for WT1-specific cell expansion are selected. Double positive cells producing IFN-ɣ and granzyme B simultaneously would be most suitable for expansion because IFN-ɣ-granzyme B-positive cells have cytolytic activity. This double positive population covers different immune responses that are necessary for the generation of an immunotherapy. FluoroSpot results summarized in figure 16 did not show high frequencies of WT1-specific double positive cells (0.0003 %).

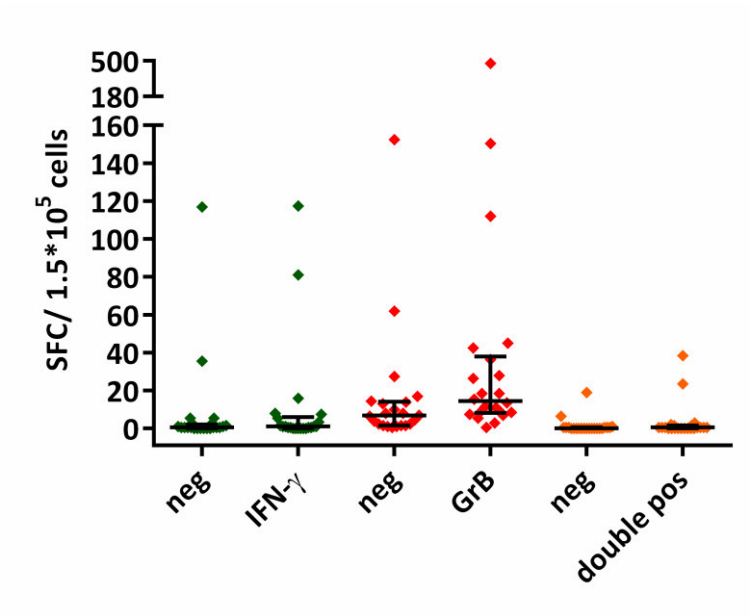


Fig. 16: FluoroSpot analysis of spot-forming cells secreting IFN-ɣ, granzyme B or both cytokines simultaneously (double pos) in AML patients *post* haematopoietic cell transplantation

Cytokine secretion was analysed in AML patients post transplantation (n=22). Unstimulated (neg) Pan T cells were compared to WT1 stimulated Pan T cells. After overnight incubation with WT1, cells were further incubated for 2 days and measured in FluoroSpot assay. Median spot forming cells (SFC) are shown with interquartile ranges for IFN-ɣ, granzyme B (GrB) and cells simultaneously secreting these cytokines (double pos).

FluoroSpot was also performed in healthy controls and AML patients *pre* transplantation (figure 17). WT1-specific spots were low in healthy controls compared to AML patients. Double positive spots could also be detected in AML patients *pre* transplantation. However, overall frequencies were lower. Granzyme B secreting WT1-specific cells had a higher frequency than IFN-ɣ secreting cells.

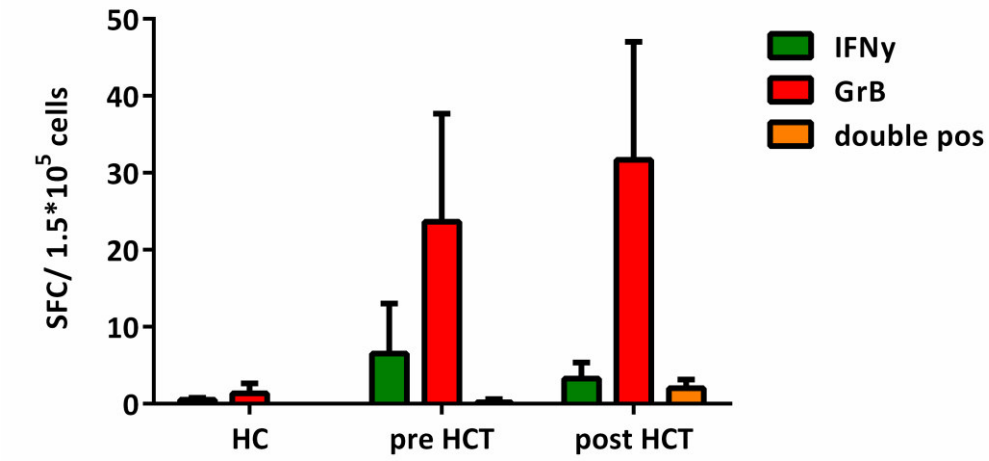


Fig. 17: FluoroSpot analysis of WT1-specific cells secreting IFN-ɣ and granzyme B in healthy controls and AML patients *pre* and *post* transplantation
Pan T cells of healthy controls (HC, n=3) and AML patients pre (n=3) and post (n=22) haematopoietic cell transplantation (HCT) were pre-incubated with WT1 peptide and measured in FluoroSpot assay. Double positive cells emerge from a computerized overlay of IFN-ɣ and granzyme B (GrB) spot images. Data are shown as mean and standard error of the mean.

In summary, ELISpot and FluoroSpot analysis resulted in the detection of WT1-specific cells measured in healthy controls and AML patients, respectively. After transplantation, cells seem to be more active. The FluoroSpot technique is more sensitive than ELISpot due to less background noise (table 17).

3.1.1. Analysis of cytokine secreting cells in relapsed AML patients

The next experiment focuses on the analysis of WT1-specific cells in relapsed patients who could benefit from a WT1-specific immunotherapy. The aim of this experiment was to show if relapse has an impact on WT1 reactive cells in AML patients after HSCT. Therefore, analysis of WT1-specific cells was carried out in eight AML patients after transplantation that were diagnosed with relapse according to standard criteria (Gratwohl, Baldomero et al. 2012). Firstly, the frequency of WT1-specific cells was analysed with ELISpot assay (figure 18).

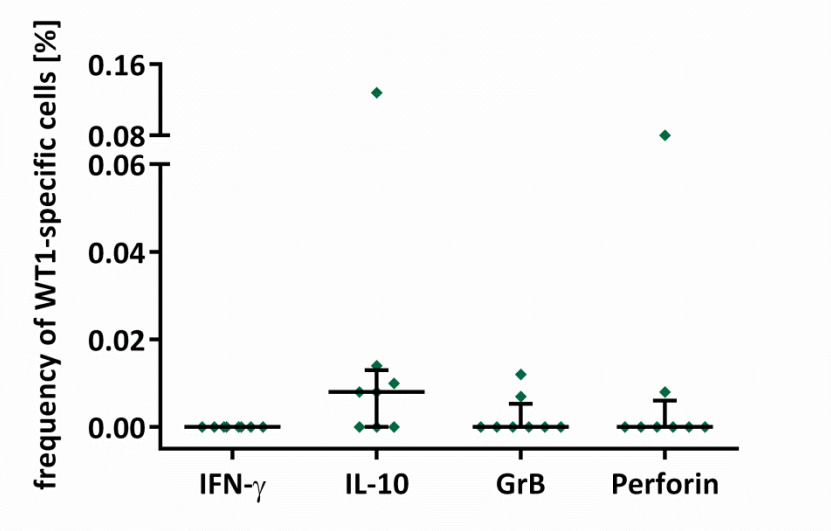


Fig. 18: Median frequencies of WT1-specific cells in relapsed AML patients
Cytokine secretion was measured in AIMV medium. Peripheral blood mononuclear cells of relapsed AML patients post transplantation (n=8) were pre-incubated with WT1 peptide, measured in ELISpot assay and median frequencies of WT1-specific cells were calculated. Data are shown with interquartile ranges.

Low frequencies of WT1-specific cells could also be observed in this group. Results highly depend on the amount of leukemic blasts in the peripheral blood of the patients. The frequency of WT1-specific cells did not correlate with the interval from analysis to relapse diagnosis.

3.1.2. Analysis of WT1 mRNA levels in AML patients *post* transplantation

To further analyse WT1 in AML patients after transplantation, a retrospective analysis was performed. This time, WT1 expression levels were measured by quantitative real time-PCR (qRT-PCR) and values were compared to the interval between sample analysis and relapse diagnosis. WT1 mRNA levels are given in percentage that results of the mRNA expression value of WT1 divided by the expression value of GAPDH used as a housekeeping gene. Results are shown in figure 19.

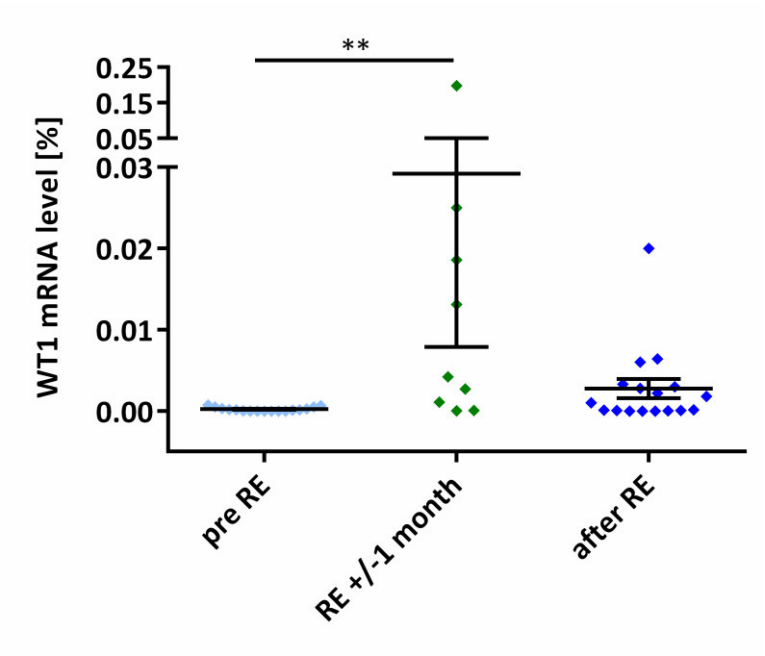


Fig. 19: WT1 mRNA level of AML patients *post* transplantation
*Detection of WT1 mRNA levels by quantitative real time-PCR. Percent mRNA was calculated by dividing specific values by levels of GAPDH as a control. Patient samples were analysed before (pre RE, n=17), during (RE± 1 months, n=9) and after (after RE, n=17) relapse (RE). Mean mRNA levels are shown with standard error of the mean. ** p=0.002*

The cohort was divided into three groups: WT1 levels before relapse (RE), during relapse and up to nine months after relapse. The time of nine months after transplantation is an approximate value where the reconstitution of the cell numbers after transplantation is said to be completed. This however does not refer to the cell's functionality. Innate immunity, including epithelial barriers, monocytes, granulocytes, and NK cells quickly recovers within weeks after transplantation whereas, adaptive immunity by B- and T-cells requires months for recovery (Storek, Geddes et al. 2008). In patient samples prior to relapse (n=17), WT1 levels could not be detected but elevated WT1 levels could be measured during relapse and showed a significant difference with p =0.002 compared to samples pre RE. WT1 levels seemed to be patient-specific during relapse occurrence which is indicated by mRNA values that have a relatively broad detection range. Within this group WT1 mRNA values varied from 0.0002 to 0.2 which is a 10^5-fold difference among these values. Such a difference may be dependent on the severity of tumour burden at relapse and has not been taken into consideration during the measurement. After relapse, levels decreased and were nearly comparable to a WT1 state before relapse. Thus, WT1 mRNA levels are high when relapse occurs.

3.1.3. Expansion of low-frequent WT1-specific T cells

Adoptive immunotherapy for relapse prevention requires the generation of highly specific cells. Prior to adoptive transfer cells have to be expanded *in vitro* to reach therapeutically sufficient numbers. In general, expansion conditions of effector cells should be as 'natural' as possible which means that culturing methods should include cells and other components that reflect the normal environment in the body. Effector cell activation at least requires two signals. One signal is given by antigen-presenting cells that present a peptide antigen via the MHC complex. This peptide is recognized by the effector cell via the T cell antigen-specific receptor (TCR). Due to antigen stimulation, T cells secrete cytokines as measured with ELISpot and FluoroSpot assay in this project. For full activation, the T cell also requires co-stimulatory molecules such as CD3 and CD28. If the signals are transmitted to the nucleus, clonal expansion is initiated and differentiation into effector cells takes place (Smith-Garvin, Koretzky et al. 2010).

Due to the close cooperation with Miltenyi Biotech, MACSi beads coated with co-stimulatory molecules CD3 and CD28 were selected as artificial antigen presenters. In parallel, autologous feeder cells used as antigen presenting cells were lethally irradiated. However, irradiation of feeder cells may lead to apoptotic cell bodies (depending on irradiation dose) with a lower capacity of promoting T cell proliferation because gamma irradiation reduces expression of surface molecules needed for T cell activation. Moreover, feeder cells do not actively secrete cytokines (Cao, Chen et al. 2004). By the use of autologous feeder cells and MACSi beads, the co-stimulatory signals are administered. Effector cells were stimulated with peptide overnight and then magnetically separated by negative selection into untouched Pan T cells or $CD8^+$ T cells. Since one goal of this project is the expansion of WT1-specific T cells, expansion aims at isolating cells in a high purity and number. The purity and the immune state before cell isolation was regularly analysed by flow cytometry. Mononuclear cells were separated by Ficoll gradient and labelled with the standard panel of antibodies for immune state determination. The basic markers include: CD3-FITC for T cells, CD4-APC for T helper cells, CD8-PE for cytotoxic T cells, CD14-APC for monocytes, CD19-APC for B cells and CD16/56-PE for NK cells. The corresponding gating strategy is shown in figure 20 for three different cell populations.

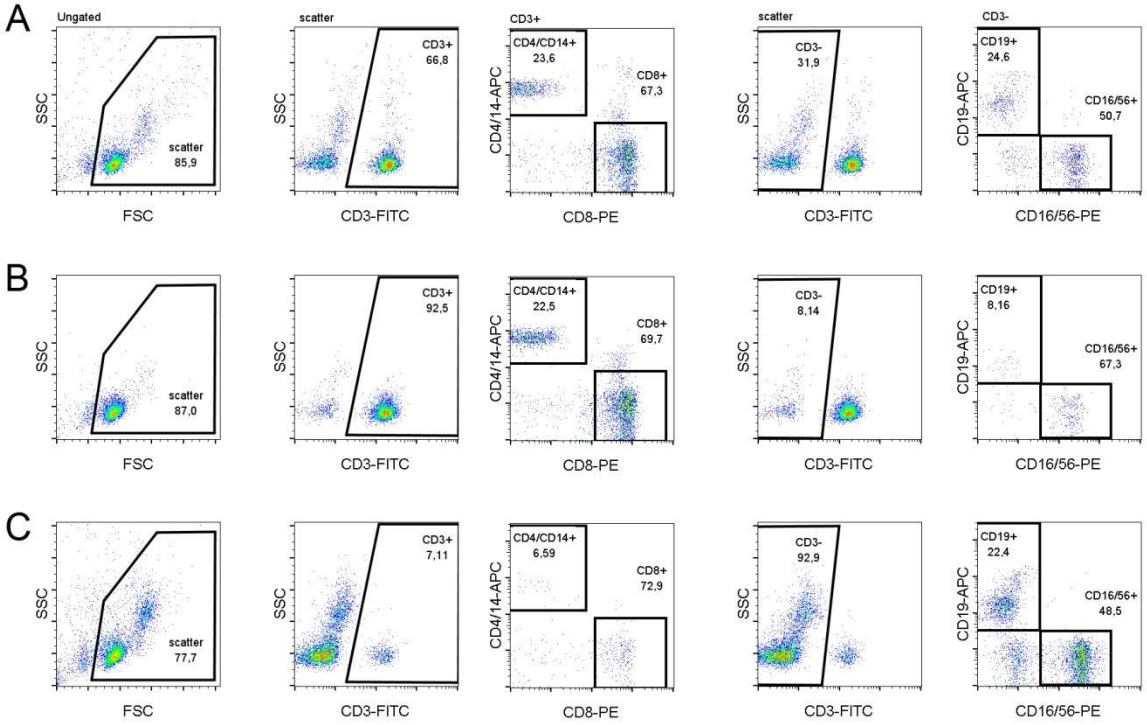


Fig. 20: Gating strategy of freshly isolated PBMCs and Pan T cells of AML patients
Flow cytometric analysis of PBMCs (panel A) and isolated Pan T cells fractions (Pan positive fraction in Panel B, Pan negative fraction in Panel C) of an AML patient. Cells were incubated with monoclonal antibodies anti-CD3-FITC, anti-CD8-PE, anti-CD4/14-APC, anti-CD19-APC and anti-CD16/56-PE. At least 10,000 events were measured per sample. Data were acquired in a FACS Aria I and analysed using FlowJo vX 0.6 software.

Panel **A** shows the PBMC fraction before magnetic separation. Panel **B** displays the $CD3^+$ (= Pan positive, untouched) fraction whereas Panel **C** represents the $CD3^-$ cells (= Pan negative fraction enriched for $CD14^+$, $CD16/56^+$, $CD\ 19^+$ cells). For analysis, magnetic separation was considered pure when the enriched cell population was above 96 %. In this example $CD3^+$ T cells could be enriched up to 92.5 % (figure 20 B). In Panel B, $CD8^+$ T cells accounted for 70 % of $CD3^+$ T cells and $CD4^+$ T cells for 23 %. This $CD4^+$ T cell fraction also contained $CD4/CD14^+$ monocytes. For expansion it is necessary to know if there is already cytolytic potential in the starting material, which means that a high amount of $CD8^+$ T cells is preferred. The amount of $CD16/CD56^+$ and $CD\ 19^+$ cells was gated in $CD3^-$ cell fraction. 7 % of $CD3^+$ T cells remained in the $CD3^-$ fraction (figure 20 C). Results of following expansion approaches are summarized in tables 18 A and B. Section A shows the antibody setting consisting of CD3-FITC, CD4/14-APC and CD8-PE, section B summarizes data of a second setting including CD3-FITC, CD16/56-PE and CD19-APC.

Tab. 18: Summary of flow cytometric data from different expansion experiments in AML patients

[A]	living cells	$CD3^+$	$CD4^+/CD14^-$	$CD8^+$
E1 PBMCs	68.7	35.8	65.1	29.1
E1 Pan^+	78.8	72.9	66.2	29.7
E1 Pan^-	66.2	2.76	15.8	61.2
E2 PBMCs	87.6	68.3	31	65.4
E2 Pan^+	88.6	81.7	36.3	61.3
E2 Pan^-	76.3	24.4	6.11	88.4
E3 PBMCs	85.9	66.8	23.6	67.3
E3 Pan^+	87.0	92.5	22.5	69.7
E3 Pan^-	77.7	7.11	6.59	72.9

[B]	living cells	$CD3^-$	$CD16^+/CD56^+$	$CD19^+$
E1 PBMCs	65.2	63.6	27.9	32.2
E1 Pan^+	74.0	26.1	56.4	8.45
E1 Pan^-	63.1	97.4	19.1	26.3
E2 PBMCs	82.7	31.7	39.9	14.7
E2 Pan^+	86.1	17.8	36.0	2.14
E2 Pan^-	71.7	76.7	39.6	18.5
E3 PBMCs	81.3	31.9	50.7	24.6
E3 Pan^+	81.8	8.14	67.3	8.16
E3 Pan^-	75.2	92.9	48.5	22.4

Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) and isolated Pan T cells fractions (Pan^+=Pan positive fraction, Pan^-=Pan negative fraction) of AML patients (E1-3, n=3) before expansion (E).Cells were incubated with monoclonal antibodies anti-CD3-FITC, anti-CD8-PE, anti-CD4/14-APC, anti-CD19-APC and anti-CD16/56-PE in two different settings ([A] T cell/monocytes setting, [B] NK/B cell setting). Results display the percentage of cells. Data were acquired with a FACS Aria I and analysed using FlowJo vX 0.6 software.

In literature, different expansion strategies have been described that mainly deal with high-frequent cell populations. However, WT1-specific frequencies are comparably low and therefore, no appropriate expansion protocol has been established yet. In order to find a suitable expansion procedure, CMV-positive donors have been used for establishment of different expansion strategies. This approach has the advantage that not only sufficient starting material is present but also the frequency of CMV-positive cells is relatively high. After isolation of subpopulations, cultures were enriched for $CD137^+$ T cells before taking them into expansion. CD137 is a co-stimulatory molecule that was identified as a potent supporter $CD8^+$ T-cell expansion. CD137 expression by T cells is activation dependent which requires *ex vivo* stimulation with defined tumor antigen such as WT1. CD137 signalling

regulates T-cell proliferation and survival, particularly within the T-cell memory pool (Ye, Song et al. 2014). The enrichment of $CD137^+$ T cells from healthy donors can be used for identification and isolation of WT1-specific T cells. However, AML patients may not have enough activated cells. Cultures roughly started with the same cell number of 800,000 cells/ culture for healthy donors which is already considered a low starting cell number (figure 21).

For the first expansion approach two different conditions were tested. Firstly, it was analysed what starting material is most suitable for expansion. Secondly, cells were expanded with or without artificial bead addition. In order to compare results from a common bulk culture, CMV stimulated T cells of a healthy donor were isolated into Pan T cells and $CD8^+$ T cells and expanded in TexMACS medium supplemented with a cocktail of growth-promoting cytokines. The cytokine cocktail was adopted from other expansion protocols (Zeng, Spolski et al. 2005, Kinter, Godbout et al. 2008, Wölfl, Merker et al. 2011) and especially selected for T cell proliferation. It consists of a combination of interleukins such as IL-2, a potent T cell-stimulator that is secreted by central memory T cells upon activation and promotes growth and differentiation of other T cells (Wölfl, Merker et al. 2011), IL-15 and IL-21 that act synergistically to support proliferation of memory $CD8^+$ T cells (Zeng, Spolski et al. 2005) and IL-7 that is a anti-apoptotic survival factor and T cell growth factor for memory cells (Kinter, Godbout et al. 2008). Owing to the low initial frequency of WT1-specific cells, protocols (see 2.2.6) involve repeated WT1 stimulation and the addition of the cytokine cocktail that drives cells into a central-memory phenotype. During expansion, cultures with MACSi beads as an additional stimulation signal showed higher cell numbers than cultures without bead addition as demonstrated in the figure 21. However, higher cell numbers could only be observed after restimulation of cells, here on day 10 and 17.

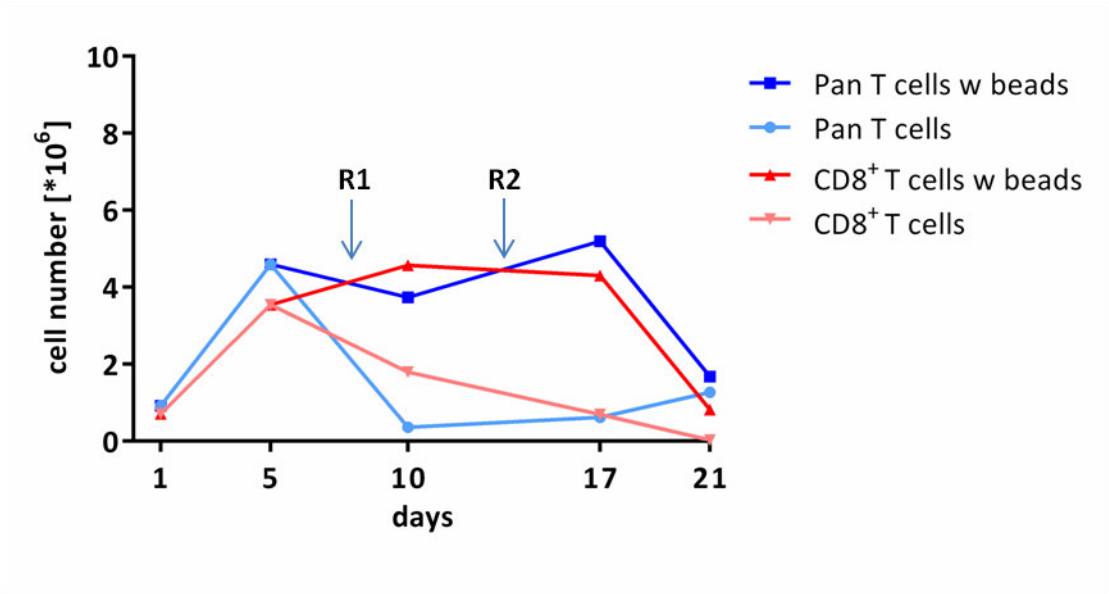


Fig. 21: Expansion of CMV-specific Pan T cells and CD8$^+$ T cells without or with artificial MACSi beads

CMV stimulated T cells of a healthy control were magnetically separated into T cell subpopulations (Pan T cells and CD8$^+$) and expanded in TexMACS medium for 3 weeks without or with (w) artificial MACSi beads for unspecific stimulation. T cell cultures were restimulated (R1 at day 8, R2 at day 15) weekly with a cytokine cocktail of IL-2, IL-7, IL-15 and IL-21 and the CMV peptide pool.

In T cell cultures without bead addition cells seemed to be exhausted much quicker than in cultures with the unspecific stimulation signal. Even after two rounds of restimulation, T cell numbers did not increase. In conclusion, expansion courses indicate that artificial antigen presentation and co-stimulatory signal transduction by the addition of MACSi beads promotes T cell expansion. T cell growth and proliferation could also be observed during expansion by microscopic monitoring of cultures. For monitoring, an exemplary expansion culture extract is depicted in figure 22.

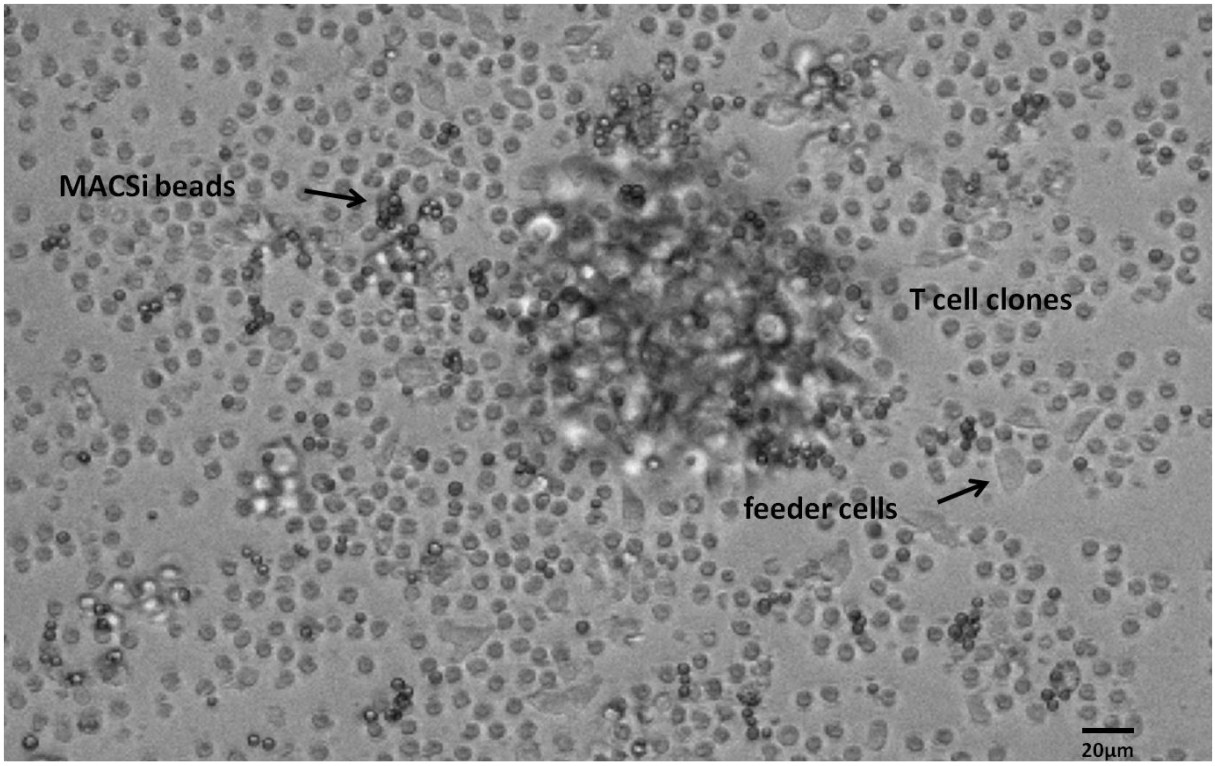


Fig. 22: Expansion culture of Pan T cells with feeder cells and MACSi beads
T cell clones were observed with Zeiss Axio Observer Z1at 400x total magnification.

By regularly monitoring of cell cultures, one can also assess the generation of T cell clones. Formation of T cell clones is proof of concept for expansion strategies. If clone formation is present, T cells proliferate. In order to know which conditions are suitable for T cell proliferation, microscopic observation was also done for bead stimulation. Results of culture conditions with artificial MACSi beads are shown in figures 21, 22 and 23. Figure 23 A shows a Pan T cell culture stimulated with WT1 peptide and MACSi beads at day 2 (upper panel). After restimulation with WT1 peptide and cytokines on day 8 (lower panel), colony formation of T cell clones can be observed as 'black' cell clusters at 100x total magnification. In contrast, figure 23 B shows T cell stimulation with WT1 and cytokines but without the addition of artificial MACSi beads which results in much lower colony formation (lower panel) even after restimulation of the expansion culture with antigen and cytokines. In summary, addition of MACSi beads increased T cell colony formation.

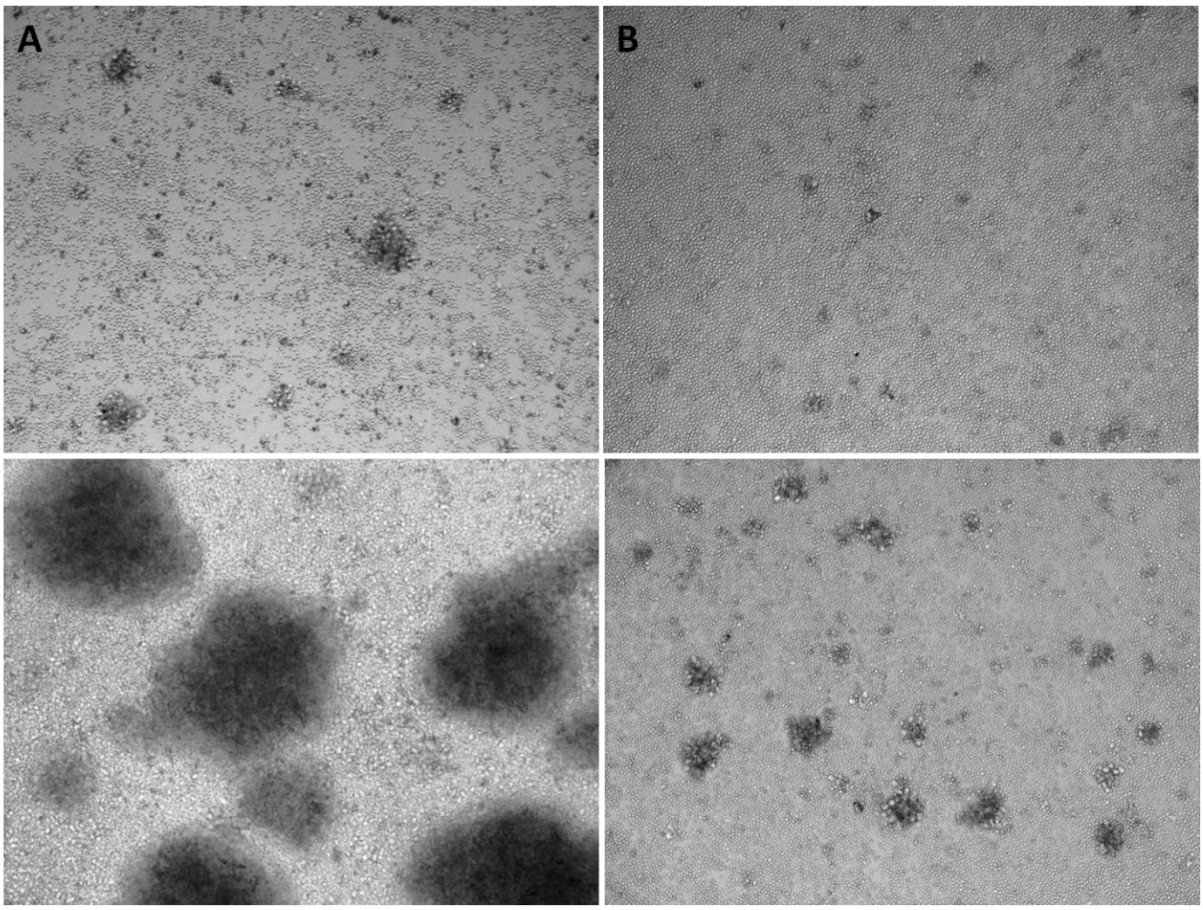


Fig. 23: Expansion progress of antigen-specific Pan T cells with (A) or without (B) artificial MACSi beads

WT1-stimulated T cells were magnetically separated and expanded in TexMACS medium for 3 weeks with (panel A) or without (panel B) artificial MACSi beads for unspecific stimulation. Panel A shows a T cell culture with artificial MACSi beads before (day 2, upper panel) and after first restimulation with WT1 peptide and cytokines IL-2, IL-7, IL-15 and IL-21 (day 8, lower panel). Panel B shows the corresponding T cell culture without bead stimulation. Colonies were observed with Zeiss Axio Observer Z1 at 100x total magnification.

In the next experimental set up, it was tested which cell subpopulation after magnetic isolation would be optimal as starting culture. Owing to the fact that the percentage of $CD3^+$ T cells (Pan T cells) in the mononuclear cell fraction is higher than the percentage of $CD8^+$ T cells as a T cell subpopulation, Pan T cell isolation generally resulted in higher cell numbers as starting culture but also in a heterogeneous T cell population. Expansion of $CD8^+$ T cells resulted in pure starting cultures. However, expansion protocols of other researchers (Mason and Simmonds 1988, Shedlock and Shen 2003) suggest that $CD4^+$ T cells positively influence $CD8^+$ T cell expansion supporting Pan T cell cultures as a starting material. Sufficient numbers of Pan T cells or $CD8^+$ T cells as starting material was not problematic for establishing expansion conditions because material of healthy donors was used (figure 21). For the next experiment, PBMCs of an AML patient after transplantation were isolated and

divided into T cell subgroups for direct comparison. Here, cell cultures started with approximately 400,000 cells per culture (figure 24) Cultures of AML patients were not enriched with CD137 isolation because of limited patient material. In figure 24, the Pan T cell culture expanded 6 fold on day 5 in contrast to the 3 fold expansion of $CD8^+$ T cell cultures. After first (day 10) and second (day 17) restimulation, cells became exhausted and cell numbers decreased.

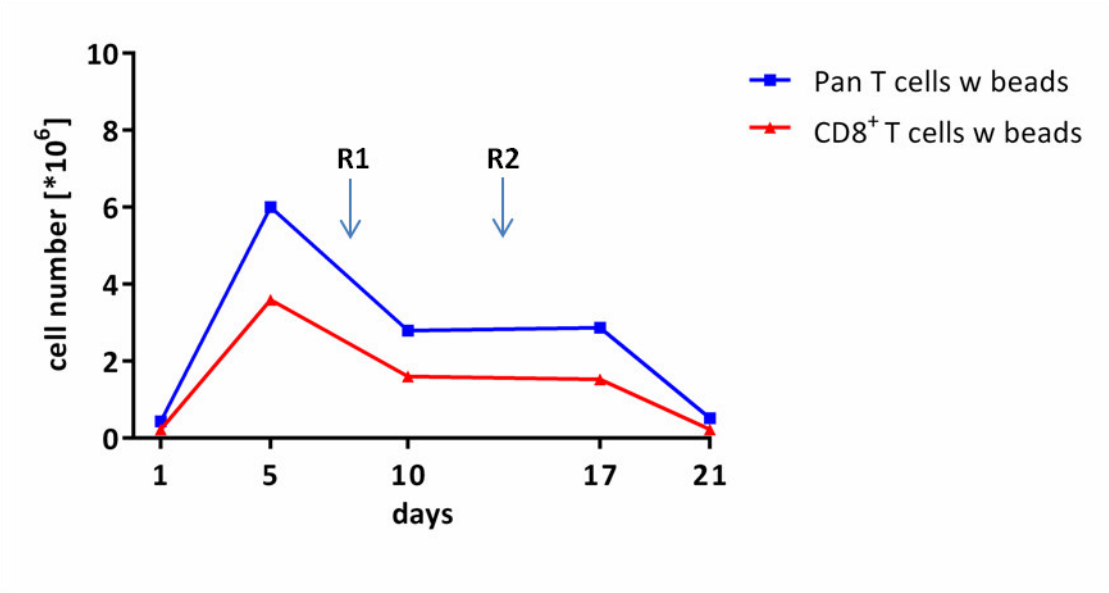


Fig. 24: Expansion of WT1-specific Pan T cells and $CD8^+$ T cells with artificial MACSi beads

After magnetic separation of PBMCs into T cell subpopulations, unlabelled Pan T and $CD8^+$T cells of an AML patient post transplantation were cultured in TexMACS expansion medium with (w) the addition of artificial MACSi beads, a cytokine cocktail of IL-2, IL-7, IL-15 and IL-21 and WT1 peptide. T cell cultures were weekly restimulated with beads, cytokines and peptide (R1 at day 8, R2 at day 15).

By comparing different cell populations, it was concluded, that Pan T cell cultures as starting material proliferated more than $CD8^+$ T cell cultures in a shorter time. Further investigation concentrated on the autologous feeder cell culture that was used to obtain a culture condition as natural as possible. Under normal *in vivo* conditions T cell survival is dependent on cell to cell interactions. Expansion, however, aims at expanding specific cells with a high purity which unfolds the problem of how to get rid of feeder cells for adoptive immunotherapy. For expansion in this project, autologous feeder cells were produced from the negative fraction of magnetic separation, i.e. Pan^- T cells or $CD8^-$ T cells. These cultures were lethally irradiated and administered to the expansion cultures mostly in a ratio of 5:1 (feeder to effector cells). Figure 25 shows the two different expansion strategies of WT1 stimulated Pan T cells in the presence of artificial MACSi beads but with (A) or without (B) autologous feeder cells.

Starting cell numbers differed because of different yields of cells resulting from isolation of PBMCs from the same donor.

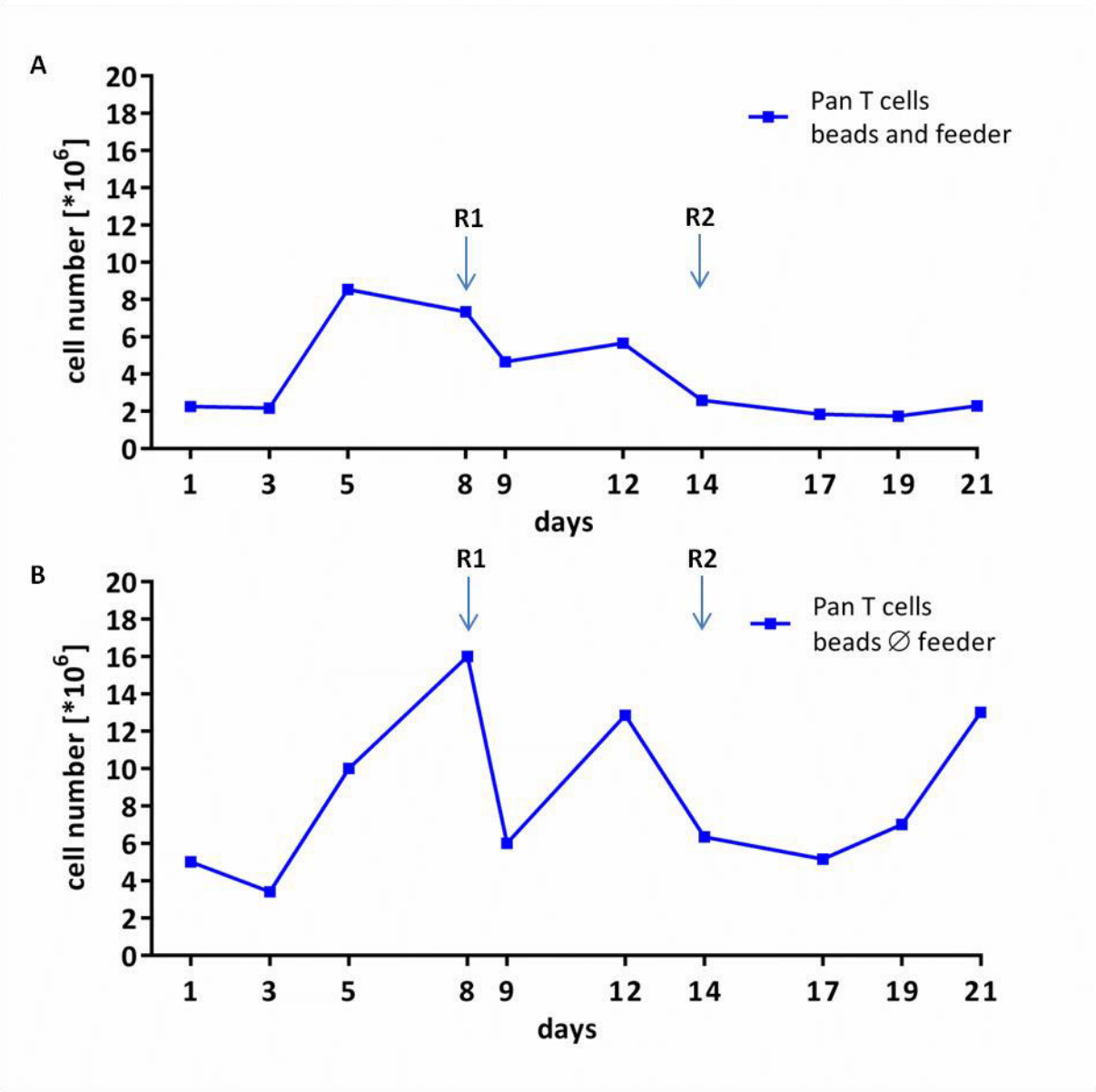


Fig. 25: Expansion of WT1-specific Pan T cells with artificial MACSi beads in presence or absence of autologous feeder cells

After magnetic separation of PBMCs, Pan T cells of an AML patient post transplantation were cultured in TexMACS expansion medium with the addition of artificial MACSi beads, a cytokine cocktail of IL-2, IL-7, IL-15 and IL-21 and WT1 peptide. T cell cultures were expanded with (A) or without (Ø, B) autologous feeder cells. Feeders were irradiated with 30 Gray and T cell cultures were weekly restimulated with beads, cytokines and peptide (R1 at day 8, R2 at day 14).

T cells in the presence of feeder cells (figure 25 A) expanded 4 fold to nearly $9*10^6$ cells per culture after 5 days of incubation. Expansion appeared to decrease after this time point and cells did not proliferate any further during the following restimulation rounds. Regularly microscopic observation confirmed results by little colony formation. Cells were counted by a

cell counting device and the culture was monitored every third day and cultures showed a decrease in cell number. In comparison to these observations, expansion without autologous feeder cells resulted in higher T cell numbers. The course of expansion is presented in figure 25 B shows a 3 fold expansion from day 3 to day 8. Cell quantity changed by 2.8 fold decrease after day 8 when cells were restimulated with WT1 peptide and the cytokine cocktail. On day 9, expansion medium was exchanged for culture medium. Dramatic decrease in cell numbers could be a consequence of cytokine-induced cell death and develops from overreaction of cells to cytokine addition. After second restimulation on day 14, cell numbers recovered and cells proliferated. Proliferation rates were easier to monitor because cell culture without feeder cells were more pure. Even if the initial expansion rate was higher in presence of autologous feeders, the overall cell number at expansion termination was higher for the cell culture without feeder cells (figure 25 B). On day 21, expansion was terminated and cells were analysed in flow cytometry (figure 29), ELISpot and Europium release assay (data not shown). ELISpot analysis resulted in a higher number of WT1-specific spots than spot numbers before expansion. WT1-specific cells tested in Europium release assay did not provoke specific lysis of leukemic cell lines.

This experimental set up was validated with another AML patient sample after transplantation and resulted in a similar outcome. Therefore it was concluded that the presence of autologous feeder cells did not result in higher amounts of specific T cell numbers and moreover contaminated the purity of the expansion culture. It is assumed that unspecific stimulation via artificial MACSi beads and weekly addition of WT1 peptide is sufficient for T cell expansion.

In the following experimental approach the optimised expansion strategy with WT1-stimulated Pan T cells and artificial MACSi beads without autologous feeder cells was used. Because relapse after transplantation is subject of this project, Pan T cells from a relapsed AML patient were expanded for 21 days (figure 26). $18*10^6$ Pan T cells could be isolated and were incubated under optimised conditions. Before expansion, PBMCs were analysed with flow cytometry and resulted in a total $CD3^+$ T cell quantity of 40 % which may indicate that the amount of T cells is decreased because of the presence of leukemic blasts in the patient's peripheral blood. After magnetic separation, Pan T cells were enriched up to 70 %. These 70 % of cells were taken into expansion.

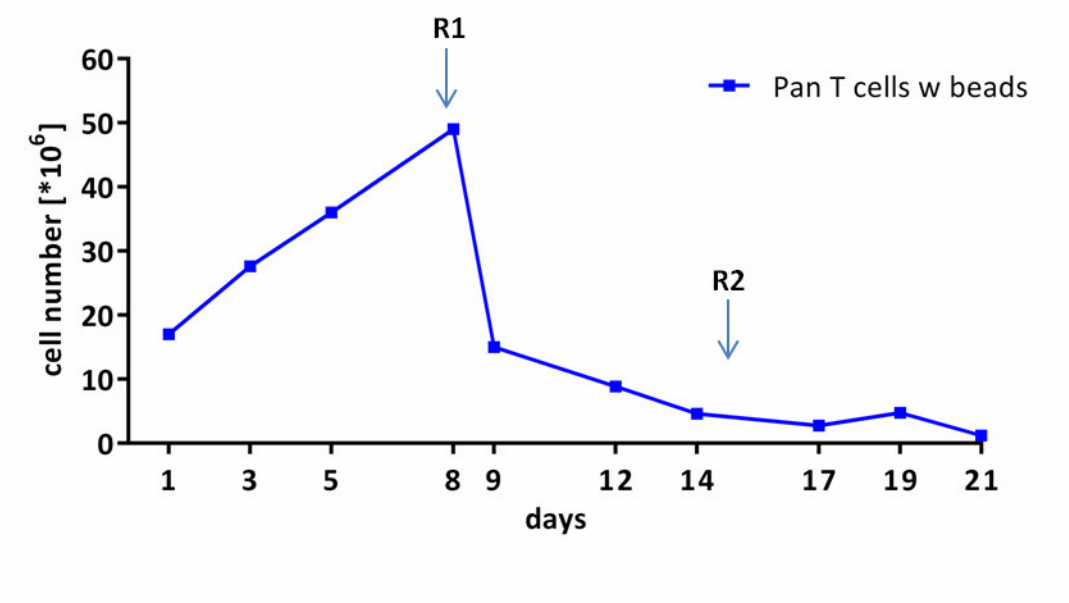


Fig. 26: Expansion of WT1-specific Pan T cells from a relapsed AML patient *post* transplantation

After magnetic separation of PBMCs, Pan T cells were cultured in TexMACS expansion medium with the addition of artificial MACSi beads, a cytokine cocktail of IL-2, IL-7, IL-15 and IL-21 and WT1 peptide. T cell cultures were weekly restimulated with (w) beads, cytokines and peptide (R1 at day 8, R2 at day 15).

As shown in figure 26, the cell number increased up to $5*10^7$ cells in total within the first week of expansion but decreased after restimulation at day 8. According to monitoring of cell number and colony formation, cells did not proliferate after restimulation and decreased in number until termination of the experiment. Expansion of patient cells in relapse is not a good option and is highly dependent on the amount of blasts in the peripheral blood whereby the immune state of the patient has to be examined carefully before expansion.

In another AML patient without relapse, however, cells could be successfully expanded (figure 27). The experimental set up started with WT1-stimulated Pan T cells of an AML patient after transplantation. These cells were expanded with the addition of artificial MACSi beads and the cytokine cocktail but without autologous feeder cells. The course of the expansion depicted in figure 27 showed generally an increase in T cell proliferation. With a starting culture of $1.8*10^6$ cells per culture, T cells could be expanded by a factor of 4 within three weeks expansion.

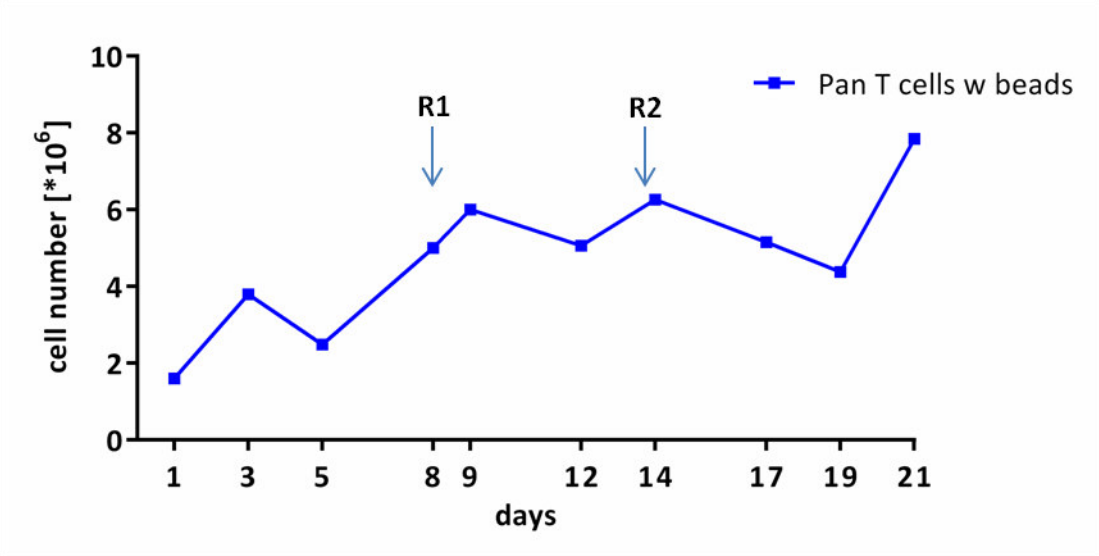


Fig. 27: Representative example of WT1-specific Pan T cell expansion over 3 weeks
After magnetic separation of PBMCs, Pan T cells of an AML patient without relapse were cultured in TexMACS expansion medium with the addition of artificial MACSi beads, a cytokine cocktail of IL-2, IL-7, IL-15 and IL-21 and WT1 peptide. T cell cultures were weekly restimulated with (w) beads, cytokines and peptide (R1 at day 8, R2 at day 14).

T cell viability and proliferation could be confirmed with T cell colony formation, as illustrated in figure 28. The left panel shows one T cell cluster on day 7 of expansion compared to an increased number in colony formation on day 19 after three rounds of restimulation in the right panel.

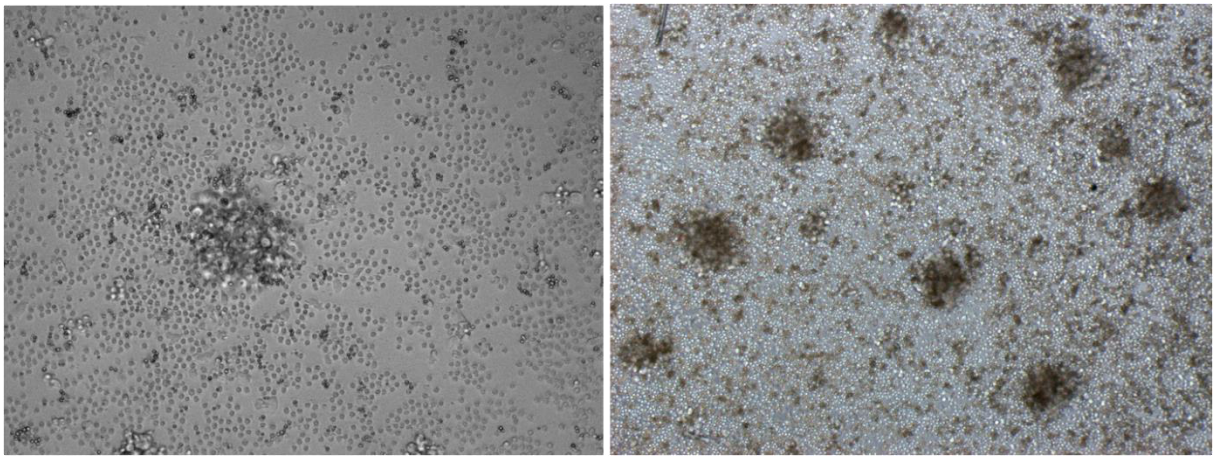


Fig. 28: Colony formation of expanded WT1-specific Pan T cells
WT1-stimulated T cells were magnetically separated and expanded in TexMACS medium for 3 weeks with the addition of artificial MACSi beads, a cytokine cocktail of IL-2, IL-7, IL-15 and IL-21 and WT1 peptide. The pictures show the difference between T cell colony formations on day 7 before restimulation (left panel) and day 19 after three rounds of restimulation (right panel). T cell colonies were observed with Zeiss Axio Observer Z1at 100x total magnification.

Successful expansion has to consider the phenotype of expanded T cells. As a consequence, expanded T cells were analysed for their immunophenotype by flow cytometry.

As already mentioned in the introduction of expansion experiments, the aim of an immunotherapy is the generation of memory T cells that also have cytolytic function. To prove which cell type was expanded, the following gating strategy was used as shown in figure 29.

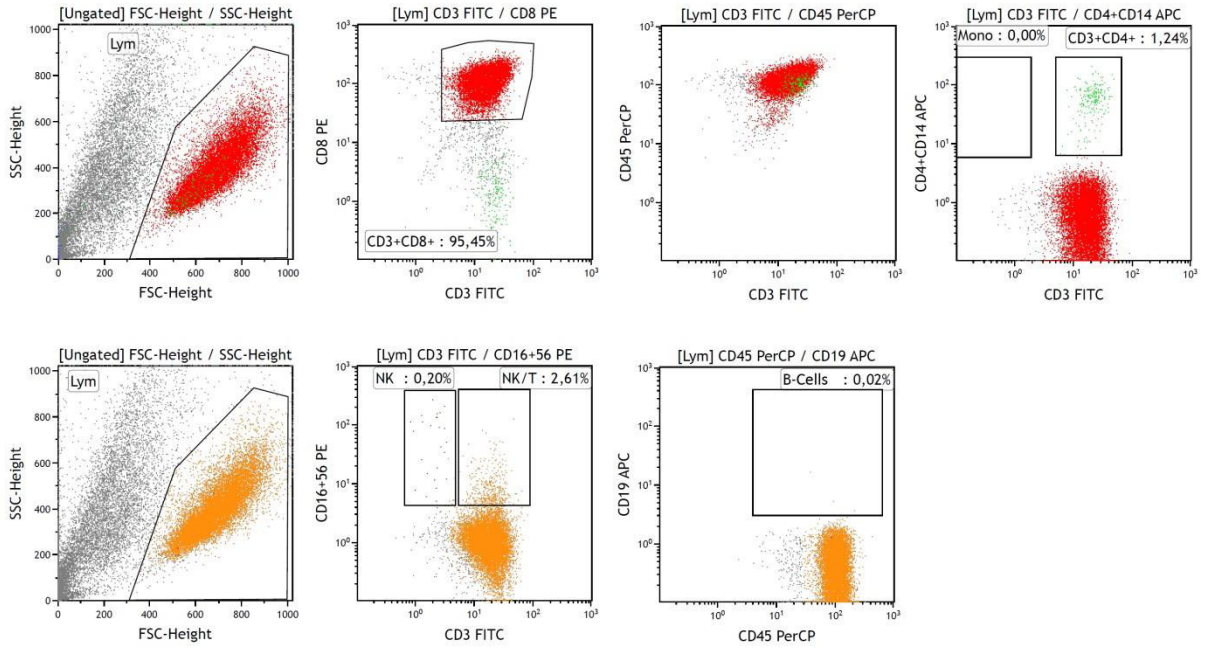


Fig. 29: Flow cytometric analysis of expanded WT1-specific Pan T cells of day 21
WT1-specific T cells and artificial MACSi beads were expanded in TexMACS medium supplemented with a cytokine cocktail of IL-2, IL-7, IL-15 and IL-21 for 21 days and analysed with flow cytometry. Cells were incubated with monoclonal antibodies anti-CD3-FITC, anti-CD8-PE, anti-CD4/14-APC, anti-CD19-APC, anti-CD16/56-PE and anti-CD 45PerCP. Cells were gated on lymphocytes and at least 10,000 events were measured per sample. Data were measured with the FC500 and analysed using Kaluza software.

To analyse the culture, it was first gated on lymphocytes according to their scatter characteristics. The high frequency of events with low forward scatter and/or high side scatter can be explained by the presence of artificial MACSi beads in the culture. In this example, expansion of Pan T cells resulted in 95 % $CD3^+$ $CD8^+$ T cells and 1.3 % $CD3^+$ $CD4^+$ T cells. This representative outcome of a nearly pure $CD3^+$ $CD8^+$ expansion culture is similar to all other expansion approaches measured during this project. Pan T cell positive expansion cultures always developed into a $CD3^+$ $CD8^+$ phenotype whereas $CD3^+$ $CD4^+$ T cells hardly survived. The amount of 0.20 % NK cells and even less B cells could be neglected because they did not interfere with the expansion outcome. In order to see if cells retain a central-

memory-like phenotype, which is driven by the cytokine cocktail, corresponding surface markers like CD45RA, CD45R0 and CD62L have to be analysed. This analysis was carried out by cooperation partners of the Department of Otorhinolaryngology. As a result, cytotoxic $CD8^+$ $CD45RA^+$ $CD62L^+$ effector cells were measured that did not have a memory phenotype (data not shown).

To complement the analysis, WT1 specificity of expanded Pan T cells was measured by Streptamer technology. The Streptamer enables fluorescent staining of WT1-specific $CD8^+$ T cells in an HLA-A2 restricted manner. This restriction makes the generation of WT1-specific effector cells difficult because patient samples are restricted to the HLA-A*02 antigen. Furthermore, Streptamers are restricted to a single peptide sequence of WT1 (Neudorfer, Schmidt et al. 2007). The following gating strategy for WT1-specific cells is summarized in figure 30. Expanded cells were incubated with monoclonal antibodies (anti-CD3-PerCP, anti-CD8-APC, anti-CD4-FITC, anti-WT1-PE and anti-isotype-PE) and gated on viable lymphocytes whereas the lymphocyte negative fraction consisted of MACSi beads and cell debris. Out of the lymphocyte population, 99.9 % of cells were $CD3^+$ T cells. The T cell fraction was further subdivided into $CD8^+$ and $CD4^+$ T cells. A percentage of 77.8 for the $CD8^+$ fraction was measured. For further analysis, cells were gated on the $CD8^+$ subpopulation and the amount of WT1-specific PE-positive cells was determined and compared with the isotype control. PE-positive cells stained with WT1 Streptamer showed an 8-fold increase of 0.27 % compared to the isotype control with 0.033 % PE-positive cells.

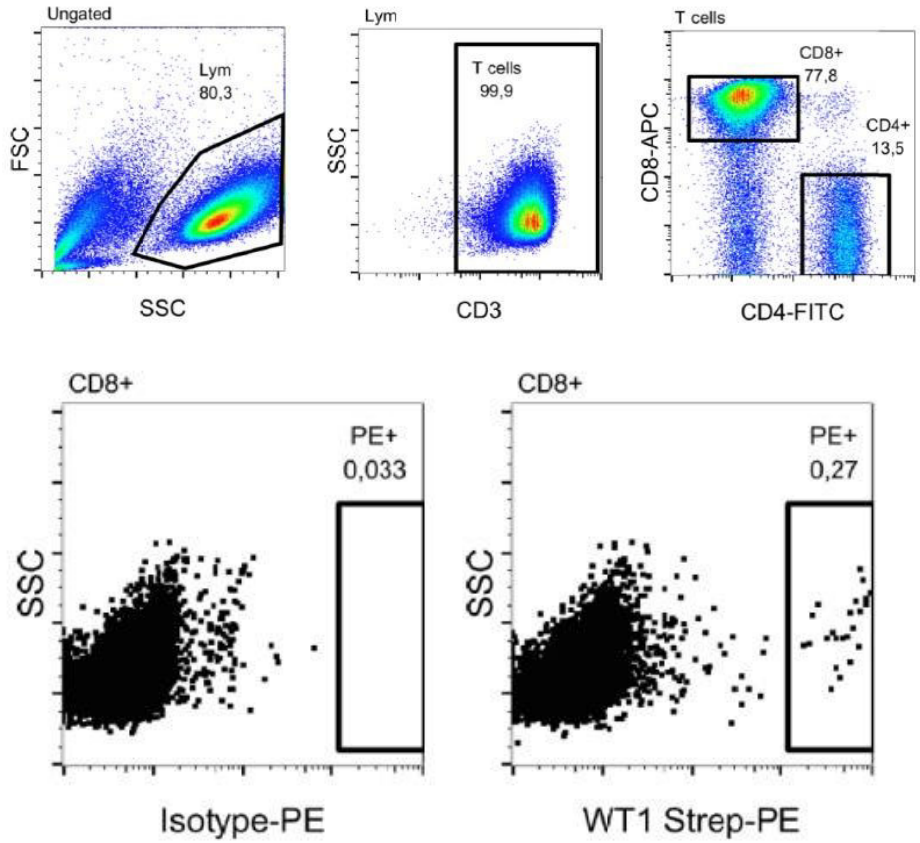


Fig. 30: Representative example of expanded WT1-specific Pan T cells analysed by Streptamer technology

WT1-specific Pan T cells of an AML patient post transplantation were expanded with artificial MACSi beads in TexMACS medium supplemented with a cytokine cocktail for 21 days and analysed with Streptamer technology after termination of expansion (day 21). Cells were incubated with the monoclonal antibodies anti-CD3-PerCP, anti-CD8-APC, anti-CD4-FITC, anti-WT1-PE and anti-IgG-PE as the corresponding isotype control. At least 10,000 events were measured per sample. Data were collected with FACS Aria I and analysed using FlowJo vX 0.6 software.

Three further T cell expansions with the same expansion conditions were also measured by flow cytometry after termination of the experiment (day 21) and cell populations are recorded in table 19. According to measured percentages, it can be concluded that the amount of WT1-specific T cells differs significantly between patients. A patient specific variability could also be observed for WT1 mRNA levels that were measured for AML patients after transplantation (see figure 19). As expected, WT1-specific cells could not be detected in patient sample 1 by HLA-A*02 restricted Streptamer analysis (table 19) because of the HLA type. However, there was no WT1 detection in patient sample 2 even though HLA-A*02 occurred. WT1-specific T cells were detected in patients 3 and 4 with matching HLA-A antigen.

Tab. 19: Immunophenotype of expanded WT1-specific T cells from AML patients *post* HSCT measured in percent frequency

Sample	HLA-A antigen	Lym	[Lym] $CD3^+$	[Lym/$CD3^+$] $CD4^+$	[Lym/$CD3^+$] $CD8^+$	[$CD8^+$] Iso	[$CD8^+$] WT1
Pat 1 Iso		80.3	99.9	31.1	66.1	0.028	
Pat 1 WT1	A*25, A*33	77.4	99.0	32.1	64.8		0.014
Pat 2 Iso		36.4	95.9	12.9	78.5	0.011	
Pat 2 WT1	A*02, A*01	39.6	96.4	3.09	1.22		0.000
Pat 3 Iso		73.9	95.2	69.3	21.7	0.033	
Pat 3 WT1	A*02, A*01	78.5	97.9	73.1	21.9		0.27
Pat 4 Iso		38.5	82.7	35.9	52.2	0.036	
Pat 4 WT1	A*02	38.2	81.6	38.3	50.1		0.054

Flow cytometric analysis of isolated Pan T cells of AML patients (Pat 1-4, n=4) with known human leukocyte antigen (HLA) pattern post transplantation at day 21of expansion. Cells were incubated with the monoclonal antibodies anti-CD3-PerCP, anti-CD4-FITC, anti-CD8-APC, anti-WT1-PE and anti-IgG-PE (Iso). Cells were gated on living lymphocytes (lym). Gates are represented in squared brackets []. Data were acquired in a FACS Aria I and analysed using FlowJo vX 0.6 software.

3.2. Analysis of WT1 in specific target cells

The development of targeted therapies in order to treat AML requires the identification of good target cells that can be used for *in vitro* assays in order to characterize putative effector cells. One approach to identify potential target cells is to analyse the amounts of WT1 protein in leukemic cell lines. As known from the literature, WT1 is overexpressed in AML but only low abundant in healthy tissue. The aim of analysing target cells is to find a candidate which expresses high amounts of WT1 and can be specifically lysed by generated WT1-targeting effector cells. The following experiments tested both lysis potential and WT1 content in leukemic cells lines and AML blasts for comparison.

3.2.1. Europium release (EuTDA) assay

The lytic capacity of cytotoxic cells was tested by Europium release assay. The first experiment was performed using K562 as target cells that were cultured in RPMI 1640 medium supplemented with 10 % HS. Effector cells were isolated from five healthy controls and stimulated with CMV peptide overnight prior to the cell lysis experiment in order to activate the cells. Because of the fact that K562 cells are NK cell targets, the starting effector cells consisted of the whole PBMC fraction and not of isolated T cells which are considered the optimal effector cells for immunotherapy. First of all the best amount of target cells was determined and different ratios of effector cells versus target cells was determined. As a result specific lysis of K562 was measured (figure 31).

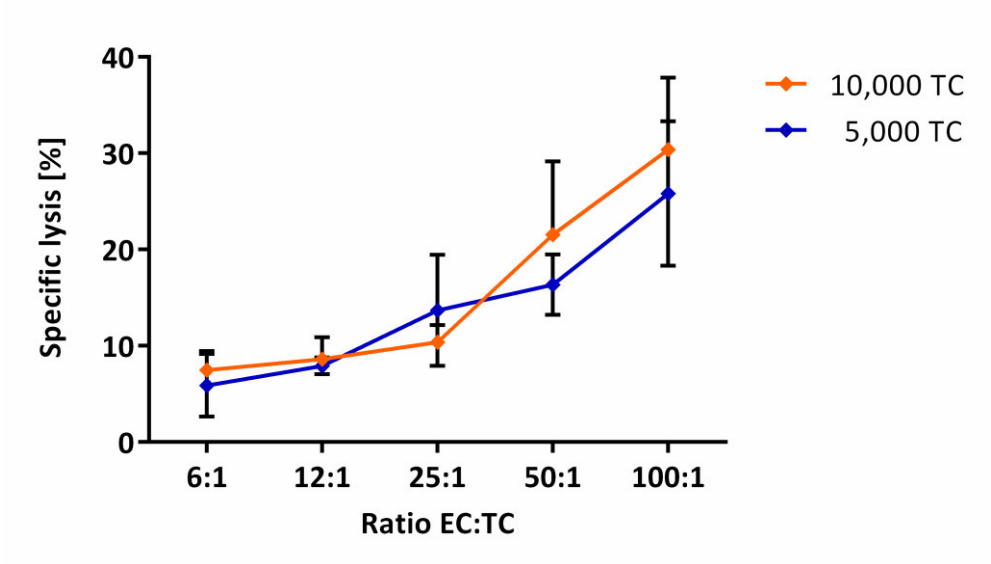


Fig. 31: Specific lysis of K562 by effector cells of healthy controls
Determination of the cell number of the target cell K562 used for following experiments. PBMCs of healthy controls (n=5) were used as effector cells at different effector cell (EC)/target cell (TC) ratios as indicated.

A cell number of 10,000 target cells emerged to be optimal because a higher percentage of specific lysis of K562 could be achieved. The more effector cells were present, the better the lysis of the target cell. For this experimental set up a 100:1 ratio is only feasible in healthy controls. AML patients suffering from panzytopenia do usually not have enough effector cells for isolation and ratios had to be adjusted.

In order to translate conditions into a setting that uses different leukemic target cells, the protocol had to be modified. Thereby, determination of individual growth curves of target cells was necessary. Target cells were only labelled optimally when they were in the log phase. The following experiment was conducted because it is not only important to work out

optimal conditions for effector cells but moreover to define a range of specific release of the TDA ligand. By specific lysis of TDA, the range defines the specific lysis potential of every target cell. As demonstrated in figure 32, ranges can differ substantially.

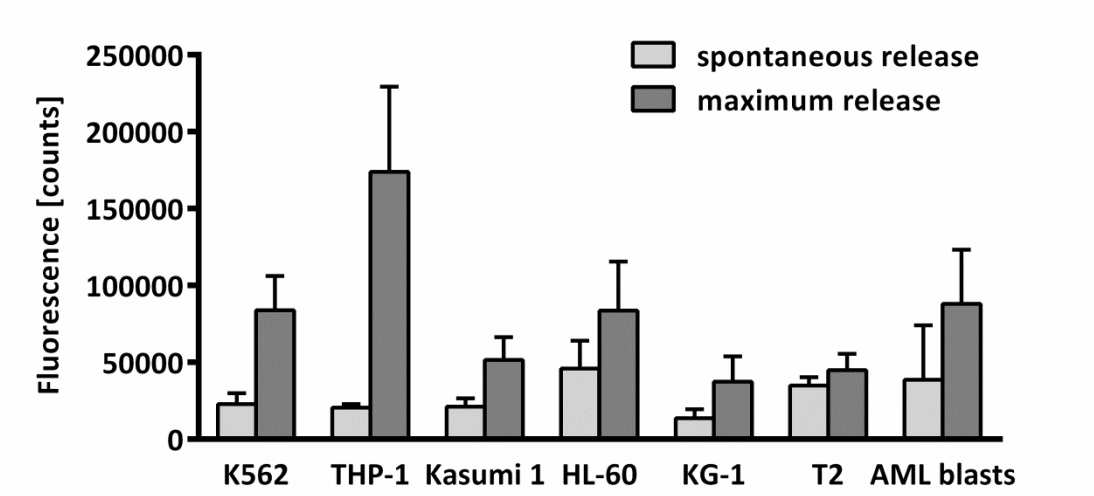


Fig. 32: Spontaneous and maximum release of EuTDA by AML target cell lines and AML blasts

Leukemic target cell lines (K562, THP-1, Kasumi 1, HL-60, KG-1), T2 target cells and leukemic blasts of AML patients pre transplantation were labelled with bis(acetoxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate (BATDA) for 25 min and EuTDA release was determined by fluorescent counts. For maximum release cells were lysed with lysis buffer for 15 min. Results are presented as mean± standard error of the mean of n=3 independent experiments.

The THP-1 cell line appeared as the optimal target cell for measuring specific lysis because of the widest range. The reason for selecting different leukemic cell lines as targets was their various HLA-A antigen profile. With matching HLA antigens unspecific lysis induced by mismatches is less likely. HLA antigens of cell lines are described in the methods section. For measuring TDA release of AML blasts, patient samples were isolated for $CD34^+$ blasts. Blasts from 3 different patients were measured. The specific range for AML blasts was highly dependent on the patient sample.

In a following experiment, T cell activity of $CD8^+$ T cells in presence of different leukemic cell lines was measured. $CD8^+$ T cells were isolated magnetically from healthy controls (n=3) and grown overnight in the presence of recombinant IL-2 for unspecific T cell stimulation. Target and effector cells were incubated for 2 hours before the Europium signal was measured. Due to HLA restriction of $CD8^+$ T cells (HLA-A*02), it was expected that HLA-A*02 positive THP-1 cells were lysed to a higher extend than the other target cell lines

with mismatching HLA antigen expression. In fact only low specific lysis with around 15 % could be measured with THP-1 (figure 33). HLA-A*01 positive HL-60 cells showed a higher percentage of lysis than the THP-1 cell line. Lysis of the HL-60 cell line was, however, not dependent on the number of effector cells. HLA-A*02 positive T2 cells that can be externally loaded with peptide were lysed specifically by $CD8^+$ HLA-A*02 positive effector T cells. Lysis increased with increasing cell ratios. Comparable to THP-1, KG1 cells were not lysed significantly.

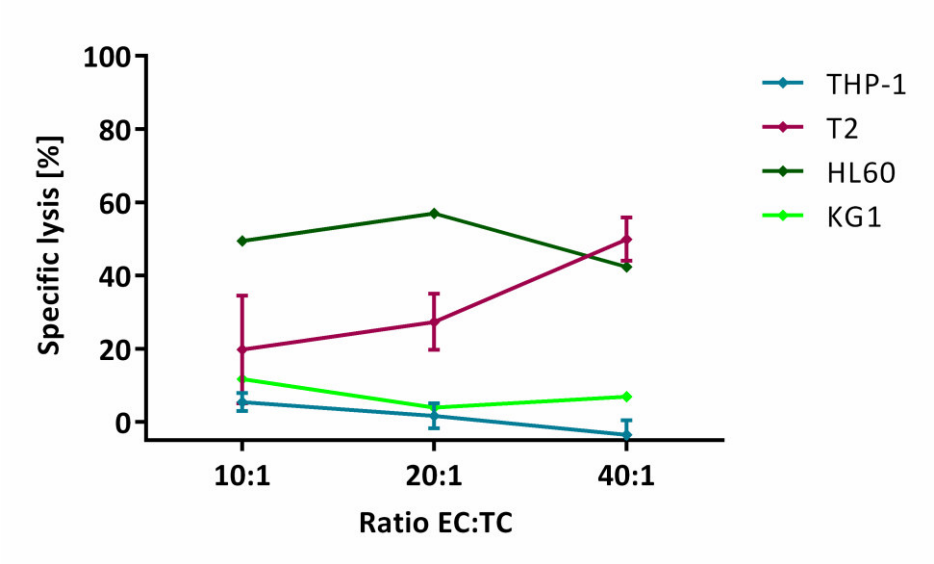


Fig. 33: Specific lysis of AML cell lines by HLA-A*02$^+$ CD8$^+$ T cells
Comparison of cell lysis of AML cell lines (THP-1, HL60, KG-1) and T2 cells by different ratios of effector T cells (EC) to target cells (TC) from healthy controls (n=3). $CD8^+$ T cells were stimulated unspecifically with IL-2 and incubated for 4 days. Target cells were labelled with bis(acetoxymethyl) 2,2':6',2"-terpyridine-6,6"-dicarboxylate (BATDA) for 25 min and specific lysis was calculated.

The concept of an allogeneic setting was analysed in the next experiment. AML blasts from three individual AML patients *pre* transplantation were thawed and incubated with WT1-stimulated $CD8^+$ T cells from corresponding donors. Specific lysis was measured by Europium release assay. All three approaches did not show specific lysis of AML blasts (figure 34). Negative specific lysis for patient-donor match 2 occurred due to high values of spontaneous release. Thus, the frequency of effector cells directed against AML blasts was too low to be detected.

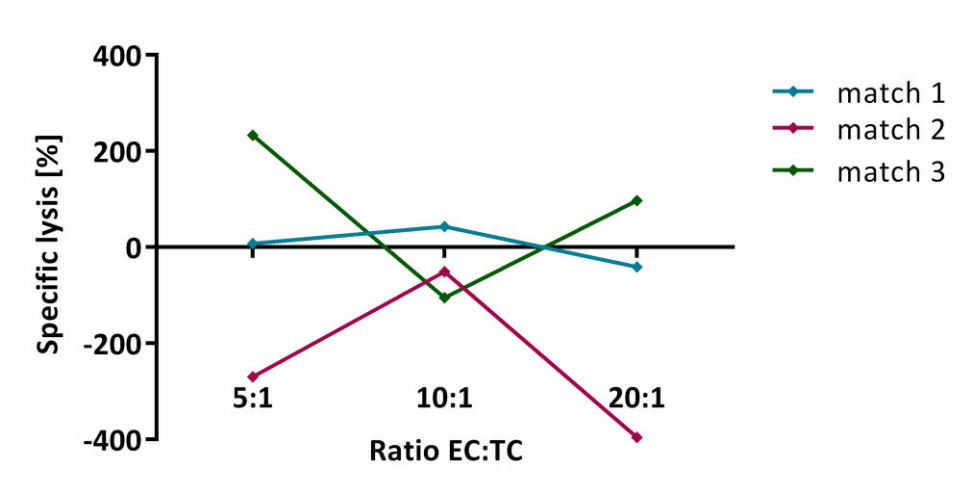


Fig. 34: Specific lysis of CD34$^+$ AML blasts of patients by corresponding donor CD8$^+$ effector T cells

CD34$^+$ AML blasts from AML patients pre haematopoietic cell transplantation (n=3) were incubated with CD8$^+$ T cells of corresponding donors, referred to as match, for 2 hours. Prior to incubation, donor cells were stimulated with WT1 peptide pool for 18 hours. Different effector cell (EC)/target cell (TC) ratios were used as indicated.

The aim of the next experiment was the analysis of HLA specific lysis of leukemic target cells by WT1-specific effector cells. For this experiment, two HLA-A*01 positive AML patients after transplantation were stimulated with WT1 peptide and incubated overnight. Afterwards specific lysis in the presence of HLA-A*01 positive HL-60 cells and WT1-loaded T2 cells was determined. Different effector: target cell ratios were selected as shown in figure 35.

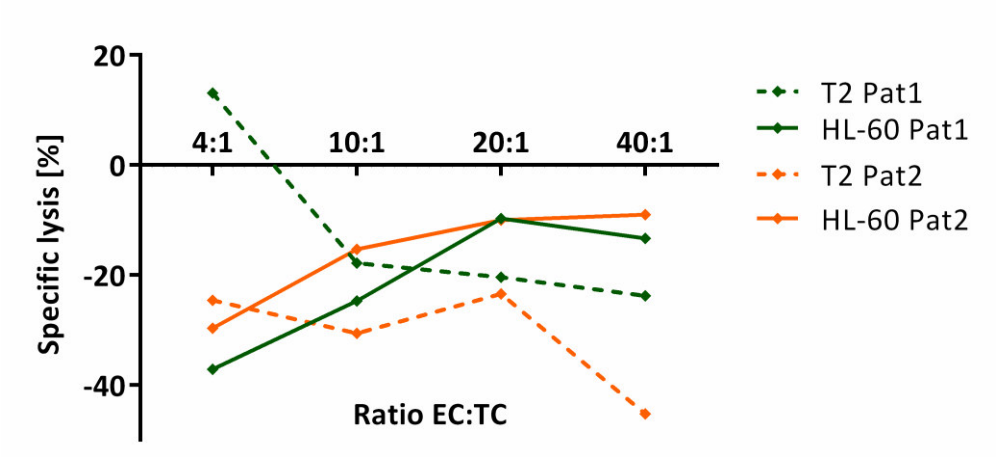


Fig. 35: Specific lysis of cell lines T2 and HL-60 by WT1-specific effector cells of two AML patients *post* HSCT

*Peripheral blood mononuclear cells (PBMCs) from HLA-A*01 positive AML patients (n=2) after transplantation were stimulated with the WT1 peptide pool overnight and further incubated with HLA-A*02 positive T2 and HLA-A*01 positive HL-60 target cells for 2 hours. Target cells were labelled with bis(acetoxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate (BATDA) for 25 min and specific lysis was calculated. Different effector cell (EC)/target cell (TC) ratios were used as indicated.*

Specific lysis due to matching HLA antigen patterns of effector and target cells could not be observed. According to literature, WT1 expression is low or even absent in healthy tissue which means that cells of healthy individuals may need stimulation with WT1 peptide for a long time period.

In the next experiment WT1-specific $CD3^+$ effector T cells of a healthy control were expanded for 7 days. After expansion, cells were analysed microscopically to check viability and proliferation and were tested for effector cell function in EuTDA assay. Prior to analysis, cells were magnetically isolated into $CD3^+$ $CD56^-$ effector cells. The experiment aimed at assessing the capability of WT1 positive effector cells to lyse T2 cells externally loaded with WT1. As controls, the leukemic cell lines K562, THP-1 and HL-60 were used that potentially express WT1 as a target structure on the cell surface. In order to assess the purity of the isolated NK cell negative population ($CD3^+$ $CD56^-$), K562 cells as NK cell targets were used to see if residual NK cell response is present. As a result, leukemic cell lines and T2 cells showed specific lysis (figure 36). As said before, K562 is not a target for effector T cells so that only 20-30 % of lysis could be measured. This percentage could be due to residual NK cells in the culture. The THP-1 cell line also showed lysis up to 20 %. WT1-loaded T2 target cells and HL-60 cells showed increasing levels of specific lysis with increasing cell ratios.

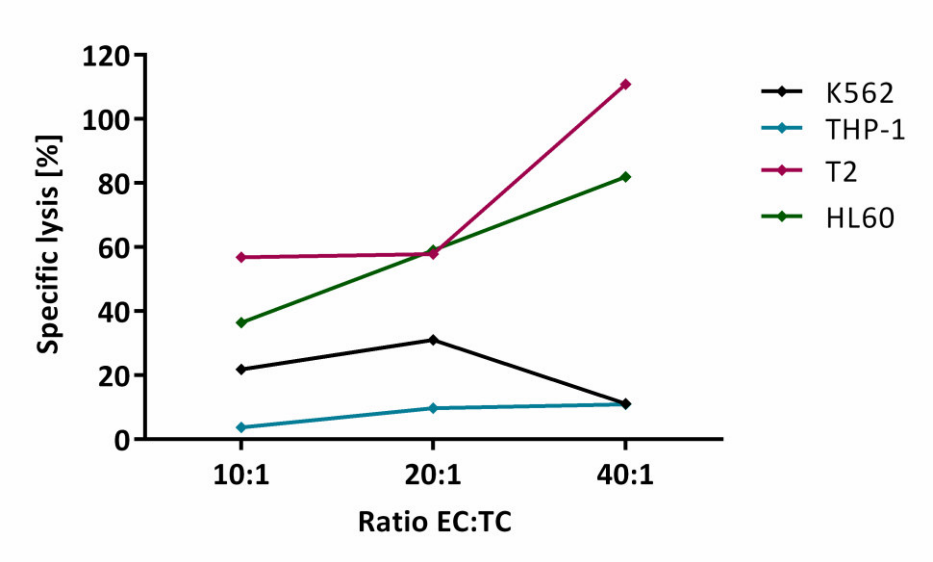


Fig. 36: Specific lysis of AML cell lines by $WT1^+$ $CD3^+$ effector T cells of a healthy control
WT1-specific effector T cells of a healthy control were magnetically separated into $CD3^+$ $CD56^-$ subpopulation and expanded for 7 days. Effector cells (EC) were incubated with different ratios of target cell (TC) lines (K562, THP-1, T2, HL60) for 2 hours in Europium release assay.

In the last lysis experiment, the HLA-A*02 positive THP-1 cell line was incubated with HLA-A*02 positive effector cells that were either non-stimulated or stimulated with CMV and WT1 peptide, respectively. The experiment was conducted in order to see if there were variations in specific lysis when effector cells are stimulated differently. It was assumed that WT1-specific cells were optimal effector cells because the target cell THP-1 overexpresses WT1. As shown in figure 37, WT1-specific lysis was highest compared to the CMV-specific lysis and the unstimulated control.

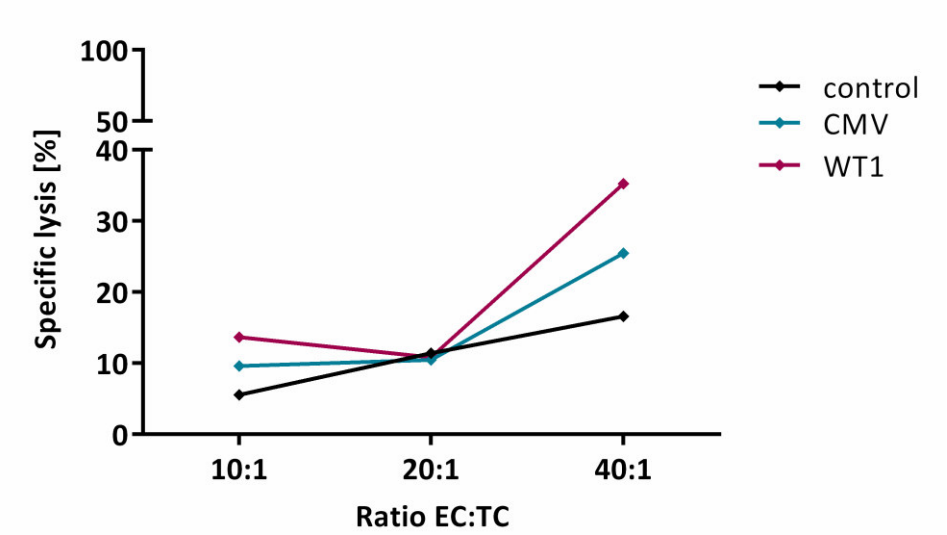


Fig. 37: Specific lysis of THP-1 cells by antigen stimulated HLA-A*02$^+$ T cells after 2 weeks expansion

*Antigen (CMV, WT1)-specific effector T cells from an HLA-A*02 positive healthy donor were measured in EuTDA assay after 2 weeks expansion. During expansion cells were stimulated with antigen and a cocktail of IL-2, IL-7, IL-15 and IL-21. For target cell (TC) lysis, THP-1 was incubated with different ratios of effector cells (EC) for 2 hours.*

In summary, it has been shown that EuTDA assay can be used to detect cytolysis of leukemic cell lines and T2 cells by different types of effector cells. The degree of specific lysis is dependent on various parameters like growth phase and permeability of target cells, cell quality of effector cells and also on handling procedures. These aspects will be further clarified in the discussion.

3.2.2. Cell lysis of T2 target cell line by effector cells

In the following experiments, the lysis of externally loaded T2 cells was analysed under the microscopy. These so called video experiments were analysed for 7.5 hours with Zeiss Axio Observer 1. The microscope was equipped with an incubation chamber allowing for ideal cell culture conditions. For analysis, the target cell line T2 was selected because it can be loaded with peptides as a target structure for effector cells. CMV positive cells were cultured as effector cells for a week under CMV stimulation and the addition of IL-2. During culturing, NK cells were removed from the previous bulk culture. Experiments were conducted with CD3$^+$ effector T cells.

In figure 38, differences in cell sizes can be observed. T2 cells are approximately 20 µm in size, effector cells vary from 7-12 µm. Activated T cells are bigger than resting T cells. T2 is motile and is equipped with flagellae-like structures that can be seen under the microscope.

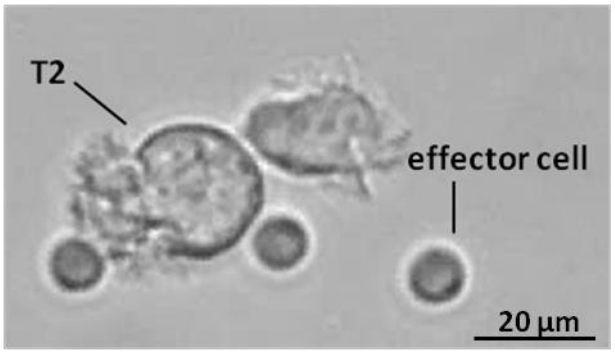


Fig. 38: Illustration of target cell T2 and effector cell

In the first video experiment, CMV loaded T2 cells were incubated with CMV-specific $CD3^+$ effector T cells. Over a time period of 25 min, CMV-specific cells were able to attack target cells. After receiving a signal by CMV-specific $CD3^+$ effector T cells, the target cell undergoes apoptosis (figure 39).

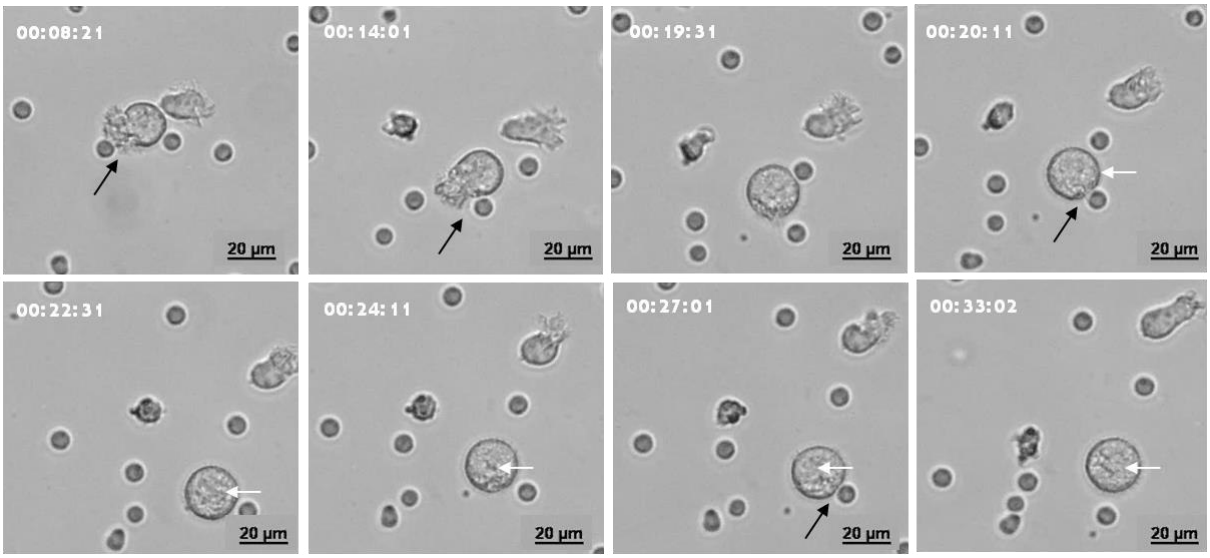


Fig. 39: CMV-positive effector cells drive T2 loaded CMV target cells into apopotosis
Images from time lapse video microscopy showing induction of apoptosis of T2 cells by CMV positive effector cells. Black arrows indicate the direct cell contact of target and effector cell. White arrows show signs of apoptosis in the target cell. Cells were tracked over a real time period of 7.5 h. Scale bar, 20 µm.

The experiment was repeated with similar conditions as used for the first approach. This time, alteration of T2 cells could be observed more clearly. Moreover, it was observed that effector cells were active by moving and changing their morphology. By direct cell contact of effector and target cells, indicated by the black arrow in figure 40, T2 cells seemed to lose their cytoplasmic membrane which is denoted with the white arrow in figure 40. The nucleus of the

target cells showed signs of apoptosis by breaking into smaller nucleosomal units. Therefore it was assumed that specific lysis of T2 cells was initiated. Comparable to the previous experiment, alteration of cells occurred within a time of 30 min.

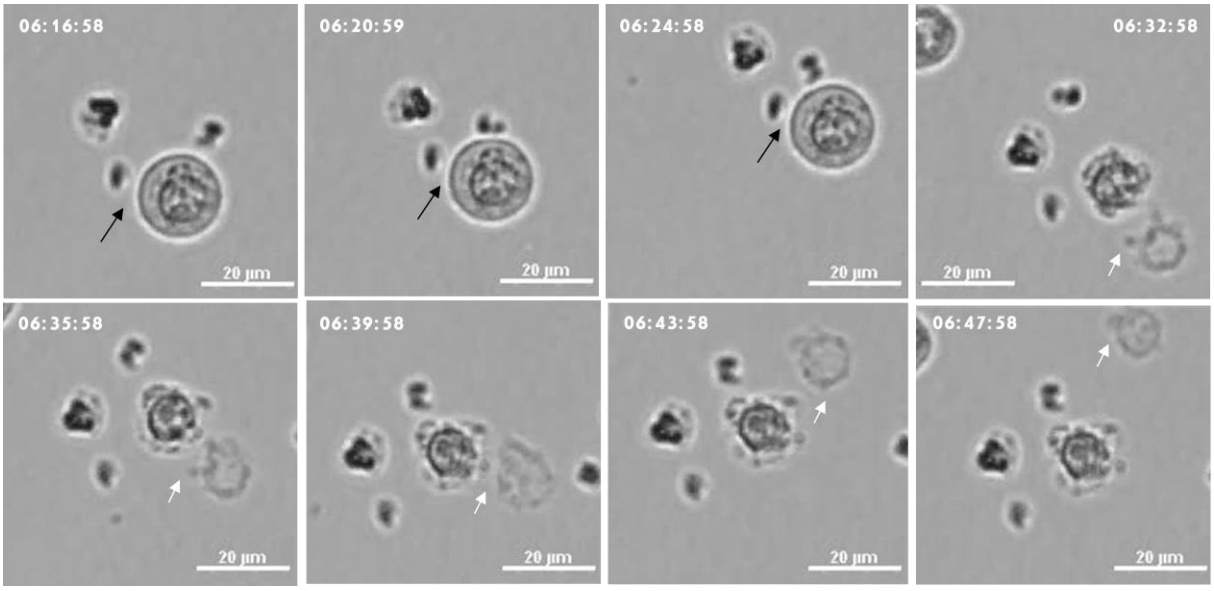


Fig. 40: Cell lysis of CMV-loaded T2 cells by CMV-positive effector cells
Images from time lapse video microscopy show the late stage apoptotic T2 cell triggered by CMV-positive effector cells. Black arrows indicate direct cell contact of target and effector cell. White arrows show apoptotic progression by elimination of the cytoplasm from the nucleus. The time line indicates the real time during microscopy. Cells were tracked over a period of 7.5 h. Scale bar, 20 µm.

Video experiments shown in figure 39 and 40 recorded alterations in the target cell line T2 and showed direct cell contact by effector cells. To confirm that these alterations were signs of apoptotic cell death, the next video experiment was performed. In order to track target cells, T2 was labelled with annexin and propidium iodide to distinguish between apoptosis and necrosis of cells. Soon after initiating apoptosis, cells translocate the phospolipid membrane component called phosphatidylserine (PS) from the inner layer of the membrane to the cell surface. When expressed, PS can be easily detected with annexin which has a high affinity to PS. Propidium iodide which is known from dead cell exclusion in flow cytometry was used for counterstaining to discriminate dead/necrotic cells from apoptotic ones. This discrimination is important because apoptosis is induced by specific target cell lysis whereas necrotic cells are a sign of unspecific cell death (Kanduc, Mittelman et al. 2002).

Apoptosis of target cells could be observed in the next experiment illustrated in figure 41.

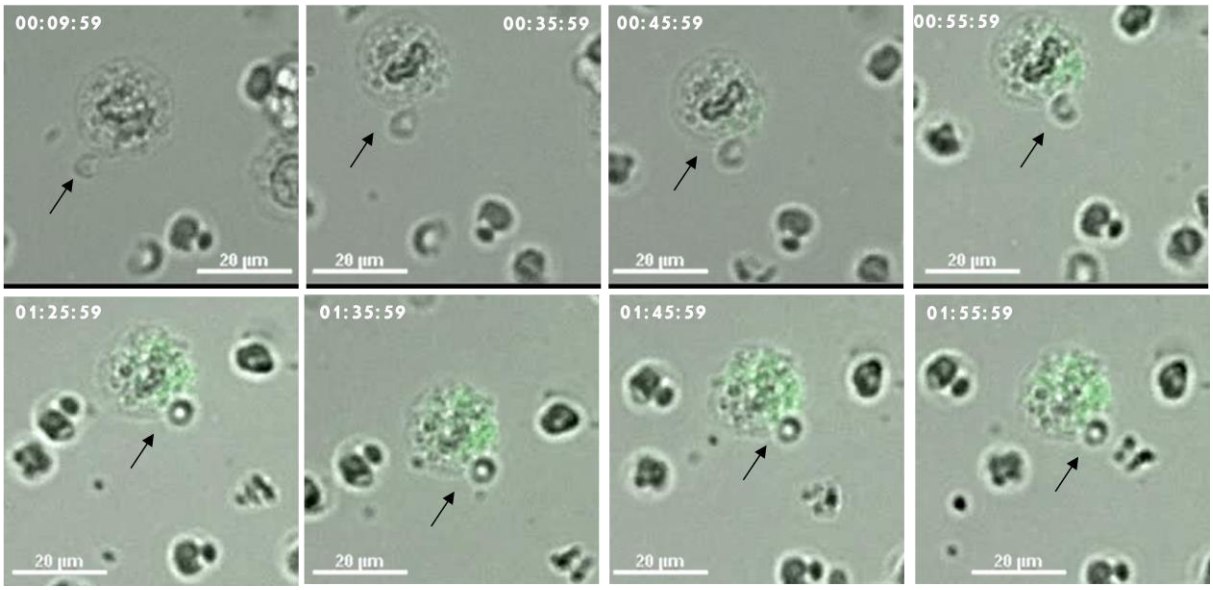


Fig. 41: Apoptosis of CMV-loaded T2 target cells labelled with annexin V by CMV-positive effector cells

Images from time lapse video microscopy show the apoptosis of a CMV-loaded T2 target cell triggered by CMV-positive effector cells in the presence of annexin V (green). Black arrows indicate direct cell contact of target and effector cell. Green cell appearance is a sign of apoptosis induction. The process of apoptosis is shown by membrane blebbing. The time line indicates the real time during microscopy. Cells were tracked over a period of 7.5 h. Scale bar, 20 µm

After 10 min of recording, direct cell contact of target and effector cell could be observed (arrow). Within the next 35 min the first green staining of the target cell membrane was visible. As shown in the upper panel of pictures, a bean shaped nucleus was still visible that broke down to smaller fragments with increasing time. After 1 hour and 55 min the whole cell membrane was stained green as a sign of cell apoptosis. There were no signs of necrotic cells that would transform red by propidium iodide. In these experiments, apoptosis of CMV positive T2 cells was investigated and resulted in an optimized assay that can be conducted alongside other cell lysis experiments. However, selection of functional effector cells is crucial and assay procedure requires time. These experiments were conducted for establishing assay conditions and similar approaches were initiated with WT1-specific effector cells isolated from AML patients. Optimal assay conditions have not been achieved during the project.

3.2.3. Analysis of WT1 mRNA levels in AML cell lines

Analysis of target cell lines was one of the subjects in this project. For setting up *in vitro* assay conditions leukemic cell lines were used as target cells. These cell lines were frozen for several years in liquid nitrogen. As optimal target cells for effector cells, cell lines should overexpress the WT1 antigen. In order to determine the WT1 content, cell lines were sent to our cooperation partners at the Department of Bone Marrow Transplantation, Essen for qRT-PCR analysis. WT1 mRNA levels were determined with WT1-specific primers and resulting data are shown in figure 42.

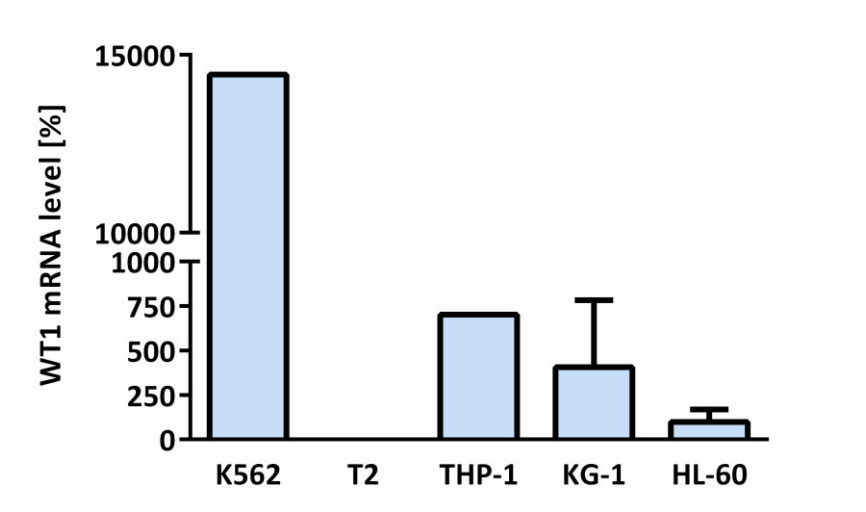


Fig. 42: WT1 mRNA level in AML cell lines
WT1 mRNA levels of leukemic cell lines (K562, THP-1, KG-1, HL-60) and T2 as a control cell line were measured in comparison to GAPDH, a housekeeping gene. The relative expression of WT1 was calculated in percentage by the quotient of WT1 mRNA expression and GAPDH expression, multiplied by 100. Error bars are only shown for KG-1 and HL-60 experiments (n=3). Analysis of K562, T2 and THP-1 was conducted once.

WT1 levels greatly varied between cell lines. K562 had by far the highest levels of WT1 mRNA and served as a positive control for further experiments. T2 cells did not express WT1 mRNA as expected and was used as a negative control. Leukemic cell lines THP-1, KG-1 and HL-60 showed WT1 mRNA expression. Compared to figure 19 where WT1 mRNA was measured in AML patients, AML cell lines possess approximately 1000 times higher mRNA levels. Both patient and cell line mRNA levels were analysed by a standard protocol for qRT-PCR.

However, the amount of mRNA present in the cells does not necessarily correlate with the WT1 antigen that is actually expressed on the cell surface. Therefore, protein concentration was determined by Western Blot followed by polyacrylamide gel electrophoresis which is a

powerful tool providing information about the presence, the size, the charge and the purity of WT1 protein.

3.2.4. WT1 protein detection in AML cell lines by Western Blot

For the first experiment of WT1 protein determination, total protein was isolated from THP-1 cells as a positive control and $CD34^+$ cells from AML patients and healthy donors. In AML patients isolated cells served as AML blasts whereas for healthy controls $CD34^+$ progenitor cells were isolated. The aim of this experiment was to show differences of WT1 protein expression in $CD34^+$ cells and to compare them with AML blasts. The result should support the hypothesis that WT1 protein is overexpressed in AML blasts but only present at a low level in healthy tissue and $CD34^+$ progenitor cells (Schwarzinger, Valent et al. 1990, Inoue, Sugiyama et al. 1994, Bergmann, Maurer et al. 1997, Maurer, Brieger et al. 1997).

Once the cells were lysed, they were boiled to denature any proteases that may interfere with the experiment. In order to compare protein expression, equal amounts of total protein have to be loaded on the electrophoresis gel. Therefore, protein concentration of the extracted protein was quantified using Bradford assay. Concentrations were adjusted to 0.35 µg/µL. In a second approach samples were normalized according to cell number. 500,000 cells/40 µL per sample were used. Samples were loaded in different order for both approaches, however sample numbers are the same. For better understanding, this is shown in table 20.

Tab. 20: Loading scheme of THP-1, cells from AML blasts and $CD34^+$ progenitor cells in Western Blot

1	2	3	4	5	6	7	8	9	10
marker	THP-1	Pat 1	Pat 2	Buffer	Buffer	1_$CD34^+$	1_$CD34^-$	2_$CD34^+$	2_$CD34^-$

5 µL of page ruler™ (marker) and 40µL of THP-1(positive control), AML blasts (Pat1, Pat 2, n=2) and healthy $CD34^+$ progenitor cells (1_CD34, 2_CD34, n=2) were loaded on a polyacrylamide gel for electrophoresis. In lanes 7 and 9: $CD34^+$ fraction after MACS separation, in lanes 8 and 10: $CD34^-$ fraction after MACS separation. Buffer in lane 5 and 6 served as the negative control.

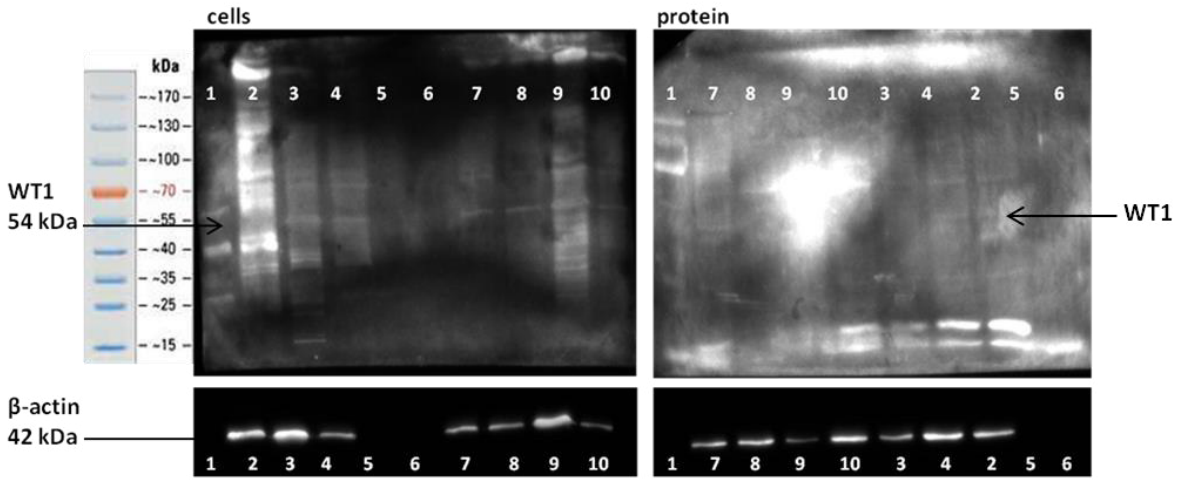


Fig. 43: WT1 protein determination by Western blot analysis in THP-1 and in samples of AML patients and healthy controls

Protein lysates either normalised to cell number (left) or protein concentration (right) were resolved on a 10% polyacrylamide gel and run in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). WT1 was detected by immunoblotting on a polyvinylidene fluoride (PVDF) membrane using a 1:1000 dilution of anti-WT1 goat polyclonal IgG and 1:10,000 dilution of donkey anti-goat polyclonal IgG. Bound antibodies were detected using a secondary antibody, horseradish peroxidase conjugated IgG. Proteins were detected using Femto susbtrate. Actin (42 kDa) as the loading control is shown in the lower panel for both membranes. The used marker (protein ladder) is shown on the left.

As shown in figure 43, protein detection resulted in unspecific protein bands that could hardly be distinguished. In the left picture THP-1 in lane 2 showed the most intense signal corresponding to higher protein content than the other samples. For direct comparison, samples of isolated CD34 cells were loaded as the positive and negative fraction that resulted from magnetic separation. This difference can be seen in lane 9 and 10 loaded with CD34 progenitor cells of a healthy individual. $CD34^+$ cells in lane 9 showed a brighter signal. To improve signal resolution, the membrane was probed with a chemiluminescent enhancer solution. However, signals appeared to be unspecific after treatment with Femto substrate as an enhancer for low-level protein detection. The WT1 protein is approximately 54 kDa in size and a weak band can be seen. This leads to the conclusion that samples did not have high concentrations of WT1. Nearly the same results can be observed in the right picture which displays samples normalized to the total protein concentration. The THP-1 cell line showed the highest signal of proteins even if the corresponding band for WT1 expression could only be estimated. As already stated, addition of Femto substrate led to unspecific signal. In order to interpret the result from any Western blot experiment, the loading control such as β-actin was used throughout this experiment. As a conclusion, Western Blot performance was adequate but experiments had to be repeated with optimized conditions.

Because of the fact that this experiment was conducted with a relatively low cell number of 500.000 cells per sample, the determination of WT1 protein in leukemic cell lines was repeated with $5*10^6$ cells/mL. Depending on the results of mRNA analysis (see figure 42), it was expected that K562 had a high WT1 protein concentration whereas no WT1 protein could be detected in T2. Results are shown in figure 44. WT1 protein could be detected as a band at 54 kDa in all cell lines except for T2, the negative control. Experiments were repeated twice with similar results.

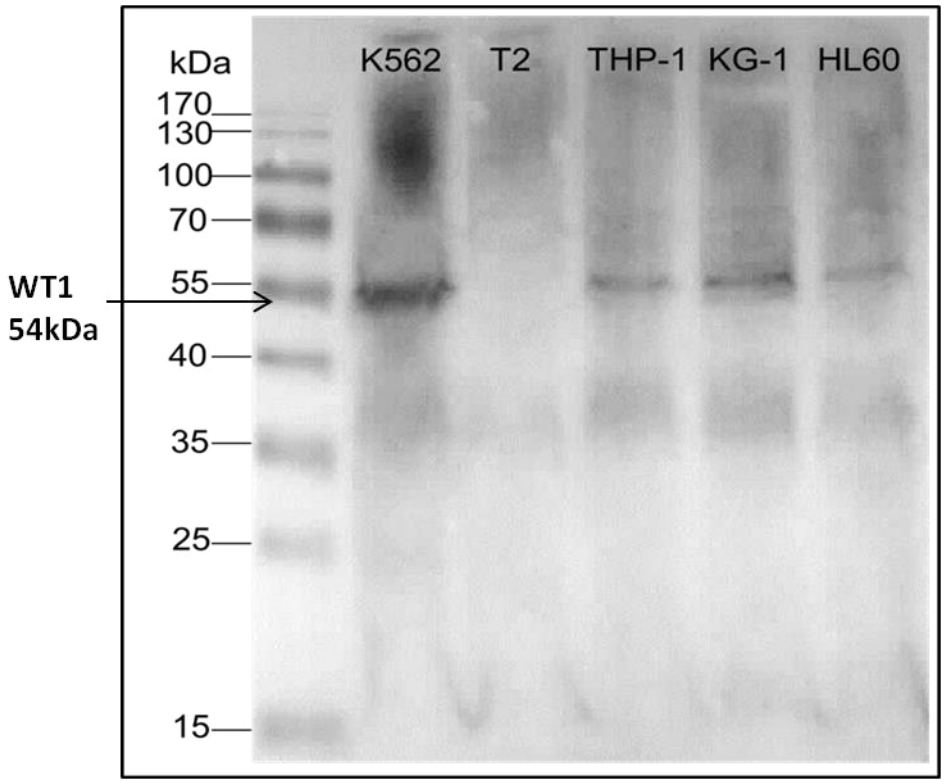


Fig. 44: WT1 protein determination in AML cell lines by Western blot analysis
Protein lysates normalised to cell number were resolved on a 10% polyacrylamide gel and run in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). WT1 was detected by immunoblotting on a polyvinylidene fluoride (PVDF) membrane using a 1:1000 dilution of anti-WT1 goat polyclonal IgG and 1:10,000 dilution of donkey anti-goat polyclonal IgG. Bound antibodies were detected using a secondary antibody, horseradish peroxidase conjugated IgG. Proteins were detected using Femto substrate. The used marker (protein ladder) is shown in the first lane.

3.2.5. Intra-/extracellular staining of WT1 in AML cell lines THP-1 and HL-60

Western Blot analysis is a reliable technique to measure the amount of WT1 protein. As described in previous experiments (figure 43 and 44), detection of low-level proteins is challenging and requires a fairly high cell number which might be a problem in AML patient analysis. Alternatively, WT1 localisation can be examined more specific by fluorescent staining. As known from literature, WT1 shuttles between the nucleus and the cytoplasm (Niksic, Slight et al. 2004) so that WT1 localisation was analysed extra- and intra-cellular in the following experiments.

For WT1 localisation target cell lines THP-1 and HL-60 were stained for extra- and intra-cellular WT1 expression. Results are summarized in figure 45 for both cell lines. Experiments were conducted with three different controls that are indicated by a colour code of the shown histograms. The unstained samples consisting of the living cells only are represented by the grey peaks. In the next measurement cells that were only stained with the secondary antibody coupled with PE are represented by the red peak. The green peak shows the isotype control and the blue peak depicts the actual detection of WT1 protein with WT1 antibody. For each experiment, cells were gated on living cells and histograms were compared.

THP-1 analysis shown in panel A resulted in nearly no extra-cellular WT1 localisation but rather in intra-cellular localisation. These results come about when observing a shift from the blue peak (WT1) compared to the unstained control in grey. This shift cannot be observed for extra-cellular localisation in the upper lane but for intra-cellular localisation. By calculating the delta mean fluorescence intensity (Δ MFI) of the isotype subtracted from the WT1, the expression levels can be compared. MFI values are given in the table 21.

Tab. 21: Delta mean fluorescence intensities of THP-1 and HL-60

Δ MFI	THP-1	HL-60
Extra-cellular WT1	0.01	0.09
Intra-cellular WT1	1.66	0.83

WT1 expression and localization in HL-60 cell line resulted similar outcome as described for THP-1.

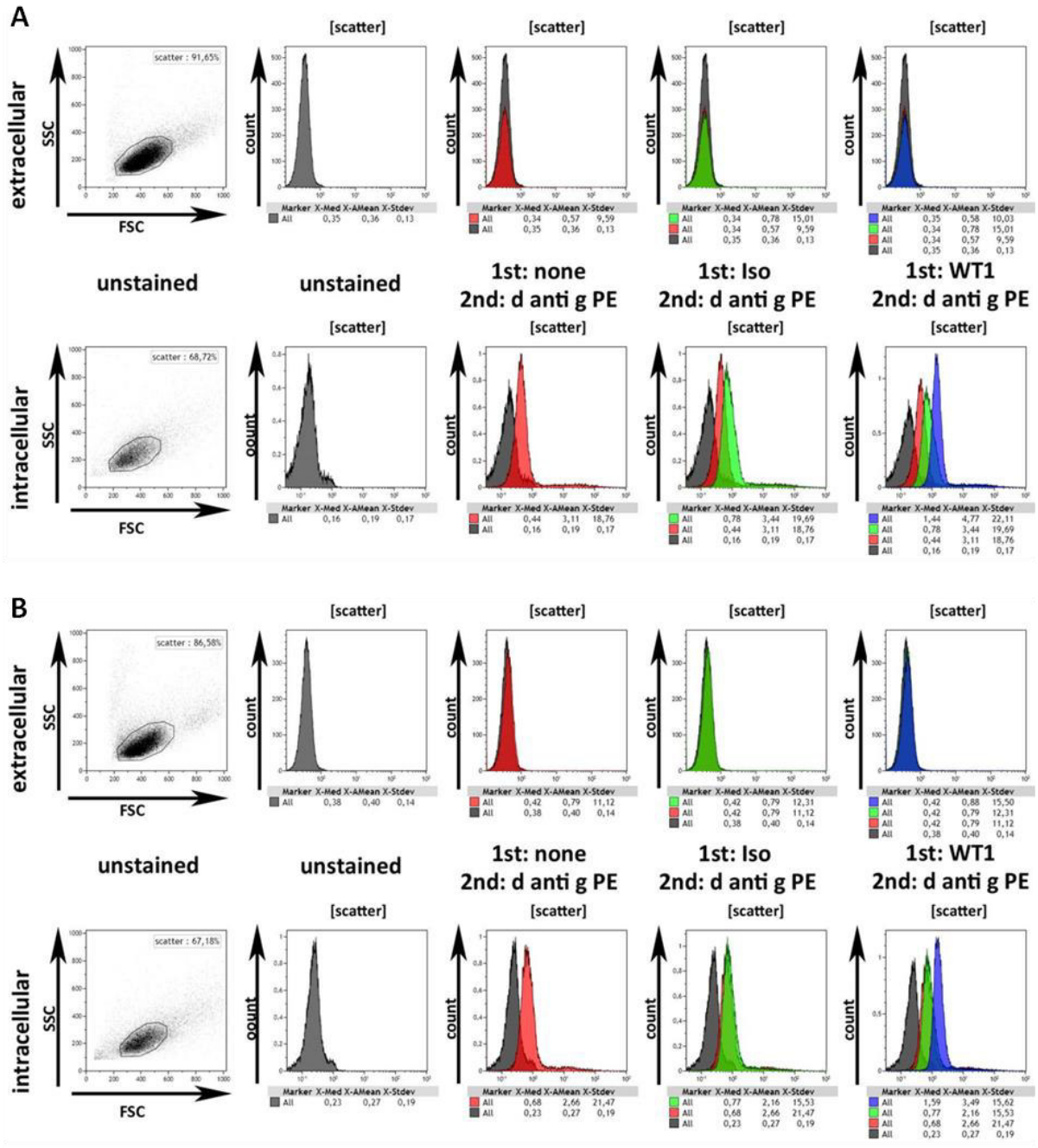


Fig. 45: Extra- and intra-cellular staining of WT1 in THP-1 and HL-60 cells

AML cells THP-1 (Panel A) and HL-60 (Panel B) were analysed for WT1 expression extra- and intra-cellular and gated on forward/side scatter (FSC/SSC). Control samples consisting of unstained AML cells (grey), cells stained with the secondary antibody [donkey anti goat-PE (d anti g PE), red] only and cells stained with the isotype control [Isotype control (Iso), donkey anti goat-PE (d anti g P), green] were compared to WT1 expression in cell lines (blue). The mean fluorescence intensity (X-A.mean) of the isotype expression (green box) was subtracted from WT1 expression (blue box).

In summary, WT1 expression could be detected on mRNA level in K562, THP-1, HL-60 and KG-1 cells. Western Blot analysis resulted in WT1 protein expression in mentioned cell lines. The HLA-A*02 positive cell line THP-1 was considered an optimal target cell for effector cell analysis because it showed the widest range for measuring specific release in EuTDA

assay. Moreover, WT1 was expressed by THP-1 on a transcriptional and translational level but could not be detected on the cell surface.

4. Discussion

This project investigated a novel approach for an adoptive immunotherapy in order to treat relapsed AML patients after HSCT. The overall aim was the generation of WT1-specific effector cells that are able to specifically lyse AML cells. For establishing an adoptive immunotherapy it is crucial to analyse both effector and target cells in *in vitro* assays. In the following text, characterisation and expansion strategies of potent effector cells and their influence on optimal target cell selection are discussed. Finally, the investigated approach will be compared to current immunotherapies that are administered to AML patients in relapse.

4.1. Analysis of WT1-specific cells as potent effectors for adoptive immunotherapy

The first aim of this project was the characterisation of WT1-specific cells used for the generation of an adoptive immunotherapy. To examine effector cell function of WT1-specific cells, cells were characterised according to their frequency and specificity, their proliferative potential for expansion and their potential to lyse AML target cells. For determination of WT1-specific cell frequencies, PBMCs of healthy controls and AML patients *pre* and *post* transplantation were isolated and stimulated with a WT1 peptide pool. This peptide pool consists of overlapping peptide sequences covering the whole WT1 protein. The WT1 peptide pool activates T cell subpopulations by the stimulation of CD4 and CD8 T cells resulting in pro- and anti-inflammatory cytokine secretion of WT1-specific cells (figure 8 and 9) (Krishnadas, Stamer et al. 2011, Miltenyi Biotech 2014). Whereas pro-inflammatory cytokines such as IFN-ɣ are important for long-term proliferation of activated T cells (Ranieri, Popescu et al. 2014), cytolytic enzymes (granzyme B and perforin) secreted by cytotoxic cells mediate a boost response directly after secretion (Wensink, Hack et al. 2015). Both responses are favourable for an adoptive immunotherapy since effector cells should directly attack leukemic blasts and develop memory function for prolonged survival. Immune-suppressive cytokines such as IL-10 are essential for regulatory function. IL-10 is produced by both monocytes/macrophages and T cells belonging to the T helper cell subsets suppressing the production of pro-inflammatory cytokines (Yssel, De Waal Malefyt et al. 1992). Based on these characteristics, it has been suggested that IL-10 might be involved in preventing GvHD and inducing T cell tolerance in the setting of HSCT. Since regulatory cells are thought to attenuate T cell reactivity against leukemic blasts, IL-10 secretion counteracts a successful immunotherapy. However, high secretion levels have been reported to be

associated with fatal outcome in transplanted patients (Hempel, Korholz et al. 1997, Schulz, Munker et al. 2001, Tsimberidou, Estey et al. 2008, Szczepanski, Szajnik et al. 2009, Sanchez-Correa, Bergua et al. 2013) and suggests the removal of IL-10-secreting cells such as monocytes (figure 9) for optimal expansion conditions. In order to analyse different immune responses by WT1 stimulated cells, IFN-ɣ, IL-10, granzyme B and perforin were selected for WT1-specific cell responses in further experiments. In healthy controls, the cytokine secretion profile of WT1-specific cells resulted in relatively low median frequencies for all measured cytokines (figure 10). High WT1-specific frequencies are not expected in healthy controls since WT1 is only expressed at a low level or even absent in normal tissue (Yang, Han et al. 2007, Toska and Roberts 2014). Similar results were observed for WT1-specific frequencies in AML patients, *pre* and *post* transplantation (figure 11 and 12). As a preparation for HSCT, AML patients *pre* transplantation undergo induction and consolidation therapy aiming at the destruction of AML blasts but also healthy cells contributing to a functional immune system so that WT1-specific cells may be also impaired in function. AML patients *post* transplantation are thought to have active cells arising from the reconstitution of the patient's bone marrow. Although median frequencies of WT1-specific cells appeared to be similar in the cohorts (table 16), interquartile ranges were different among the groups. Whereas WT1-specific frequencies were at a similar range in all healthy controls, WT1-specific frequencies in AML patients were detected at a broader range, especially for IL-10 secretion. Since interquartile ranges display single frequencies, it seems that there is a patient-specific variability in WT1-specific frequencies. In single patients cells after transplantation seemed to be more active. It has been reported that patients have different initial frequencies of WT1 precursors which are influenced by various parameters such as disease severity, AML subtype, treatment and time between transplantation and sample analysis (for AML patients *post* transplantations) (Wölfl, Merker et al. 2011). However, cytokine levels in AML patients after transplantation (figure 13) did not significantly increase with time after transplantation. Analysis of cytokine levels during the *post* transplantation period is important for the selection of potent effector cells to administer after transplantation as a supportive therapy.

In order to further characterize WT1-specific cells, FluoroSpot was used. The novel FluoroSpot technique, a fluorescence-coupled ELISpot assay, measures IFN-ɣ and granzyme B secretion simultaneously and was run in parallel with the ELISpot assay. According to other working groups (Hallengard, Haller et al. 2011, Kesa, Larsson et al. 2012, Essone, Kalsdorf et al. 2014) assays were reported to be comparable which could be confirmed in this project. For both IFN-ɣ and granzyme B secreting T cells, responses to WT1 were equally

correlated between the two assays which makes the techniques comparable. Although correlated with ELISpot, FluoroSpot generally resulted in slightly higher WT1-specific frequencies (table 14). Additionally background noise of the unstimulated controls was lower in FluoroSpot (table 17, figure 15) indicating that this assay is even more sensitive and specific. Other groups observed similar frequencies as we observed in the FluoroSpot. But in contrast to our experiments, they used anti-CD28 for co-stimulation (Casey, Blumenkrantz et al. 2010, Han, Bagheri et al. 2012). While having all the advantages of an ELISpot assay, FluoroSpot analysis compresses the different kinetics of cytokine release. The production of cytokines by immune cells rather results from sequential than from simultaneous release. This means that cells predominantly release one cytokine after another (Han, Bagheri et al. 2012). In this FluoroSpot setting, IFN-ɣ and granzyme B are said to be secreted simultaneously. However, by comparing kinetics of cytokine release, granzyme B is secreted within a relatively short time (from 20 min up to 4 hours) to induce a boost for the immune system whereas IFN-ɣ is released after hours (Shafer-Weaver, Sayers et al. 2004). With an incubation time of 24-48 hours, FluoroSpot analysis combines this secretion difference. ELISpot assays could be adjusted for every single cytokine which is not possible for a combined analysis. FluoroSpot also needs less cell material which is a big advantage for AML patient samples having usually low specific cell numbers. Therefore, FluoroSpot assay was identified as the method of choice when analysing WT1-specific frequencies. Simultaneous measurement of IFN-ɣ and granzyme B in AML patients *post* transplantation (figure 16) identified three functional WT1-specific T cell subsets which reflect their memory phenotype and cytotoxic potential, a phenotype which is preferred for adoptive immunotherapy. IFN-ɣ-secreting effector cells define active T cells that could develop into long-lived memory cells. Effector cells secreting granzyme B are known to have cytolytic function and will result in a short but strong immune response. This boost will not hold for long because cytotoxic T cells without memory function are only short-lived (Uttenthal, Martinez-Davila et al. 2014). This short life-span would not be beneficial for adoptive immunotherapy. Effector cells with cytolytic function that secrete both IFN-ɣ and granzyme B simultaneously might be the best starting material for *ex vivo* generation because they basically display both immune responses. However, double positive T cells secreting both cytokines simultaneously resulted in a very low frequency of WT1-specific cells (figure 17) compared to single cytokine secretion so that all three subpopulations would be used for expansion.

Relapsed AML patients who could benefit from a WT1-specific immunotherapy were analysed by ELISpot according to their WT1-specific frequency (figure 18). Because WT1 is

highly expressed in more than 80 % of AML cells (Malagola, Skert et al. 2014), it is considered a prognostic marker for residual leukemic blasts in a relapse situation. However, WT1-specific frequencies were relatively low for relapsed AML patients which might refer to the fact that blasts contaminating the patients mononuclear cells dampened the cytokine secretion in ELISpot assay. During leukomogenesis leukemic blasts in the bone marrow are likely to migrate into the peripheral blood replacing functional mononuclear cells. The more blast cells were detected in the blood, the less functional T cells could be analysed in ELISpot assay. Compared to non-relapsed AML patients *pre* and *pos*t transplantation (figure 11 and 12), WT1-specific frequencies of relapsed AML patients did not differ significantly. To assess the prognostic potential of WT1, WT1 mRNA levels were measured before, during and up to 8 months after AML relapse (figure 19). Data show that WT1 mRNA levels highly increase during relapse and equal zero before and after relapse, an expression level that is similar to healthy controls. Monitoring WT1 levels after transplantation seems to be relevant for further relapse prediction. According to many studies, elevated WT1 mRNA levels accompany with poor prognosis (Yang, Han et al. 2007). Most of the studies on relapse monitoring employ bone marrow as the source of leukemic cells which is not comparable to peripheral blood sources used for this analysis (Malagola, Skert et al. 2014). However, monitoring of WT1 derived from AML blasts in this project showed a significant increase in mRNA levels at the time of relapse and can be used as a prognostic relapse marker. Since WT1 mRNA is elevated during relapse, Elmaagacli and colleagues asked the question if silencing of the *WT1* gene by small interfering RNAs (siRNA) might induce anti-leukemic effects. siRNAs are known to initiate sequence-specific degradation of targeted mRNA with regard to cell proliferation and induction of apoptosis (Karami, Baradaran et al. 2014, Sioud 2014). WT1-specific siRNA analysis showed significant decrease in WT1 expression levels in leukemic cell lines and AML blasts (Elmaagacli, Koldehoff et al. 2005). However, WT1 mRNA levels do not necessarily correlate with protein amount as transcription of mRNA only describes WT1 gene expression but not translation of WT1 proteins. Therefore experiments do not give any information about WT1 expression on the cell surface of AML blasts.

Current immunotherapies are diverse in expansion strategies that are more or less successful. Successful expansion moreover refers to sufficient cell numbers that can be generated rapidly. Clinically relevant protocols are based on the generation of dendritic cells (DC) as antigen presenters. Most protocols involving DC generation are time-consuming. Different groups describe generation times of more than 10 days for potent DCs and generation is fairly difficult in AML patients having a non-functional immune system (Wolfl, Kuball et al. 2007).

Alternatively, strategies make use of magnetic selection of antigen-specific T cells using Streptamers which is only applicable in a high-frequent T cell population and is limited to patients that express the corresponding HLA antigen. Patient cohorts are limited by the initial frequency of effector cells which plays a major role in finding the optimal expansion. In general, various expansion strategies for high-frequent cells have been described in literature especially for the generation of CMV-specific effector cells. CMV has been studied immensely because viral infections are responsible for morbidity and mortality in patients after HSCT (Bruminhent and Razonable 2014, Santos, Brennan et al. 2014, Sousa, Boutolleau et al. 2014, Van Craenenbroeck, Smits et al. 2015). As investigated by cooperation partners of the Department of Otorhinolaryngology and confirmed in this project (figure 21), expansion of high-frequent CMV-specific T cells is possible to a certain extend with the selected expansion strategy described in 2.2.6 and was taken as a basis for WT1-specific T cell expansion. However, after a T cell expansion of approximately 2 weeks, cell numbers dramatically decreased which might be a consequence of activation-induced cell death (Arakaki, Yamada et al. 2014, Dalla Santa, Merlo et al. 2014, Moreno, Negrotto et al. 2014). This situation is initiated by the over-stimulation of cells by the cytokine cocktail which was regularly administered to the expansion culture. Applying the same expansion protocol to low-frequent WT1-specific T cells resulted in a different outcome. Even if sufficient numbers of around $2*10^6$ Pan T cells were present in the starting culture (figure 25 and 27), expansion of WT1-specific cells did not exceed a 4 fold increase. Since DLIs transfused at dosages of $1\text{-}5*10^6$ T cells/kg are found to be a feasible therapeutic strategy after transplantation (Gratwohl, Baldomero et al. 2012), it is highly unlikely that this expansion strategy generates sufficient cell numbers for an adoptive immunotherapy. From the applied expansion protocol, it can be concluded that expanded WT1-specific T cells resulted in a highly pure culture of cytotoxic $CD8^+$ T cell (figure 29) confirmed by flow cytometry even if Pan T cells were selected as the initial culture (figures 24, 25 and 27). Transformation of cytotoxic $CD8^+$ T cells to effector memory cells as an aim of expansion is crucial for increasing antitumor efficacy. Although expanded $CD8^+$ T cells acquired cytotoxic activity in form of granzyme B release by WT1 stimulation, antitumor effects could be absent in adoptive immunotherapy. This refers to the fact that $CD8^+$ T cells secreting granzyme B lose the ability of IL-2 secretion, an important cytokine for resisting apoptosis. Consequently, differentiation of $CD8^+$ T cells into effector memory cells is inversely related to their proliferative potential (Wölfl, Merker et al. 2011, Restifo, Dudley et al. 2012). The more the cells are differentiated after expansion, the lower is their proliferative capacity. Reinfusion of WT1-specific $CD8^+$ T cells derived from less differentiated populations such as central memory T cells has shown to be

successful by Chapuis and colleagues. They managed to generate functional, long-term detectable $CD8^+$ T cells with an expansion strategy similar to the one in this project. In their study, HLA-A*0201-restricted $CD8^+$ T cells expressing CD27, CD28 and CD127 (phenotype of central memory T cells) (Appay 2008) mediated anti-leukemic activity which indicates that expansion of a highly specific, single T cell subpopulation is in general possible (Chapuis, Ragnarsson et al. 2013). Wölfl and colleagues suggested that depletion of $CD4^+$ T cells prior to expansion enhanced T cell proliferation (Li and Yee 2008, Wölfl, Merker et al. 2011). Because expansion of Pan T cell cultures in this setting only resulted in a $CD8^+$ T cell culture, $CD4^+$ T cells were probably only needed for initiating the expansion as 'co-stimulators' but not for the entire expansion phase. However it has been found by Restifo and colleagues that $CD8^+$ and $CD4^+$ T cells secreting IFN-ɣ after expansion initiate the upregulation of MHC class I and II on AML blasts (Restifo, Dudley et al. 2012) which underlines the importance of measuring the cytokine secretion profile by ELISpot assay. WT1-specific cells resulted in a higher number when expanded without autologous feeder cells (figure 25). Even if feeder cells were used to retain *in vivo* conditions, they contaminated the purity of the effector cell culture and could not be removed after expansion. Novel expansion strategies are mostly feeder-free by using particle-based approaches (Oyer, Igarashi et al. 2015). Artificial MACSi beads only deliver the co-stimulatory signal for T cell activation but do not function as antigen presenters to the effector cells (Onlamoon, Boonchan et al. 2013). Therefore, weekly administration of WT1 peptide to the cultures was necessary. Additional growth stimulation by the cytokine cocktail did not result in the generation of long-lived effector memory cells which was tested by phenotypic analysis in flow cytometry for an expansion culture (figure 29). To complement expansion outcome, WT1 specificity of effector cells was proven by cytokine secretion of WT1-specific cells in ELISpot assay and by Streptamer binding in flow cytometry. ELISpot analysis resulted in higher WT1-specific frequencies for IFN-ɣ and granzyme B compared to the initial starting culture. Frequencies of T cells after expansion tested on other stimuli such as PHA, CEF or *Candida albicans* was not as successful as for WT1, indicating WT1 specificity of expanded T cells. However, WT1-specific frequencies could not be compared to unstimulated cells as negative controls. Expansion of unstimulated cells is not possible because cells are not activated and die after 2-3 days even if treated with the same growth supplements (cytokine cocktail). The same results of WT1-specific frequencies were observed for Fluorospot analysis. Whereas initial frequencies of WT1-specific cells before expansion resulted in 0.001 %, a 10-fold increase in expanded WT1-specific cell frequencies could be observed after 21 days. Streptamer technology confirmed rather low frequencies of WT1-specific cells after expansion. However, frequencies were

10 times higher than for ELISpot assay, ranging from 0.054-0.27 % WT1 positive cells. Higher frequencies after Streptamer analysis are expected as this technique only measures binding of WT1 peptides and not function (cytokine secretion). Streptamer staining would be a good option for direct *ex vivo* WT1 isolation. A major drawback of this technique is that Streptamer staining of WT1-specific cells is HLA-A*02 or HLA-A*24 restricted and therefore only applicable for a small cohort of patients. ELISpot analysis is not HLA dependent and therefore broadly applicable. Moreover, Streptamer technology is also restricted to one WT1 peptide sequence whereas in ELISpot assay cells are stimulated with the entire WT1 peptide repertoire. In conclusion, expansion of low frequencies of WT1-specific precursors requires multiple rounds of stimulation which is a time-consuming approach.

The development of WT1 targeted therapies in order to treat relapsed AML does not only require sufficient cell numbers but also functional cells that eradicate residual AML blasts. Cytotoxicity is the most important effector function of cells generated for an adoptive immunotherapy. The cytolytic capacity of effector cells was tested in Europium release assay and 'video experiments' where specific lysis of AML cell lines was investigated. The aim of this assay was not only the functional characterisation of WT1-specific effector cells but also the identification of optimal target cells that express high amounts of WT1 on the cell surface as a target structure. The EuTDA cytotoxicity assay was invented as a non-radioactive alternative to the well known Cr^{51} release assay (Blomberg, Hautala et al. 1996). It has been shown that this assay works for K562 lysis by human PBMCs (Vikström, Lähde et al. 2000). K562 was used for establishing assay conditions, cell lysis experiments were conducted with AML blasts and AML cell lines. Effector cell function was tested in different settings including AML cell line or AML blast lysis by $CD8^+$ T cells of AML patients, AML cell lysis by healthy control cells and lysis of the AML cell line THP-1 by WT1-specific expanded T cells. Efficient cell lysis of AML targets highly depended on HLA antigens presented on both effector and target cells. Specific lysis could be detected for HLA matched antigens as long as no other HLA antigens were present. Activation of HLA-A*02 positive $CD8^+$ T cells by unspecific IL-2 stimulation (figure 33) did not result in significant lysis of AML cell lines expressing different HLA antigens. Increased specific lysis up to 50 % could only be observed in T2 cells which were exogenously loaded with WT1 peptide pool prior to analysis. Because effector cells originated from healthy donors, it was not expected that effector cells were WT1-specific. In another experiment (figure 34), donor-recipient pairs were analysed that were selected as an ideal match for HSCT. Donor derived effector cells were tested for

specific lysis of patient blasts but were not able to lyse target cells. These cells were only stimulated with WT1 over night and were not expanded or enriched for WT1 indicating that initial frequencies of WT1-specific cells could be too low for effector cell function. Evidence was given by another lysis approach with HLA-A*02 positive THP-1 cells and expanded HLA-A*02 positive T cells stimulated with WT1 and CMV that specifically lysed target cells (figure 37). The T2 cell line seems to be an optimal target since this cell line is defective in antigen presentation and only expresses HLA-A*02 antigen on its surface (Wei and Cresswell 1992, Luft, Rizkalla et al. 2001). Expanded WT1-specific effector cells were able to specifically lyse WT1 loaded T2 cells up to 100 % (figure 36). Cytolytic activity of WT1-specific effector cells is not only dependent on high frequencies of specific cells but also on WT1 presentation of target cells. Moreover, target cell activity was highly influenced by growth phases of AML cells and was tested to be optimal when cells were in log phase (data not shown). Also, it was not clear how efficiently target cells could be labelled with BATDA. Since the ligand penetrates the cell membrane, loading efficiency differed between the cell lines. Specific lysis is only measured indirectly by the release of TDA that is set free upon effector cell lysis. Even if this assay benefits from being non-radioactive and fast (< 4 hours) (Lengagne R, Gnatjic S et al. 1998) labelling of target cells as well as assay performance is highly dependent on HLA antigen matching and therefore requires a number of suitable target cells with a broad range of HLA antigens. In AML blasts a variety of HLA antigens is found which may cause problems in this assay. The cytolytic potential of effector cells was also analysed in video experiments making use of the T2 cell line that was identified as an optimal target cell in Europium release assay. By labelling target cells with annexin and propidium iodide induction of apopotosis could be observed. Since apoptosis is a consequence of specific cell lysis (Kanduc, Mittelman et al. 2002) effector cell function could be analysed. Induction of apoptosis by CMV-positive effector cells was confirmed for CMV loaded T2 cells (figure 41). This setting with T2 target cells can be conducted alongside with other lysis experiments but requires careful selection of functional effector cells. As shown for EuTDA assay, HLA restriction is predetermined by HLA-A*02 specificity of T2 cells and apoptosis is only initiated by sufficient frequencies of specific effector cells. Therefore it was not possible to establish optimal conditions for cell lysis by WT1-specific cells.

4.2. Identification of suitable AML target cells to confirm effector cell functionality

The second aim of this project was the analysis of suitable target cells for *in vitro* assays. Target cells were analysed for to their HLA antigen expression, their potential of being lysed by effector cells and their presence of WT1 on mRNA and protein level. According to the impact of HLA antigens in transplantation (Horowitz, Gale et al. 1990, Jacobsohn and Vogelsang 2007, Okumura, Yamaguchi et al. 2007), effector and target cells should match in their HLA antigen pattern for cytotoxicity assay to exclude unspecific lysis of effector cells by HLA mismatches, as already discussed for Europium release assay. AML blasts could not be identified as suitable targets because they neither showed a broad range for specific release of TDA (figure 32) nor specifically interacted with effector cells (figure 34). It is known that AML blasts downregulate MHC class I molecules as an immune evasion mechanism and therefore do not express WT1. This could explain why WT1-specific effector cells are not activated (Restifo, Dudley et al. 2012). Lack of killing presumably resulted from insufficient expression of WT1 target peptide on the cell surface of AML cell lines. Moreover, the amount of peptide plays a role in recognition and could be a limiting factor in this experiment. Therefore, following experiments investigated if leukemic cell lines express WT1 as a target structure. WT1 expression in AML cell lines was confirmed by qRT-PCR, Western Blot and flow cytometry.

WT1 mRNA levels measured by qRT-PCR in AML patients were either very low or even undetectable (figure 19) but greatly varied in AML cell lines (figure 42) as also investigated by other groups (Inoue, Sugiyama et al. 1994, Maurer, Weidmann et al. 1997). The WT1 expression levels of normal bone marrow $CD34^+$ hematopoietic progenitors and acute leukemia cells were reported to be the similar (Inoue, Sugiyama et al. 1994, Maurer, Weidmann et al. 1997). But Inoue and colleagues found that the WT1 expression level of normal $CD34^+$ cells in bone marrow was significantly lower than levels of leukemic cells (Inoue, Sugiyama et al. 1994). If the same amount of WT1 would be expressed in normal hematopoietic progenitors and in leukemia cells, the adoptive transfer of WT1-specific effector cells would have a deleterious effect on haematopoietic progenitors. However, qRT-PCR analysis revealed strong WT1 mRNA expression and resulted in a 1000 times higher mRNA levels for leukemic cell lines versus AML blasts. This high content has not been reported by other researchers so far.

To assess the actual protein amount of WT1, Western Blot analysis was conducted with AML cell lines, $CD34^+$ haematopoietic progenitor cells from healthy controls and $CD34^+$ AML blasts from patients (figure 43). WT1 protein detection was only expected at low level in healthy controls since WT1 appears to be a tumor suppressor in haematopoietic progenitors leading to quiescence and growth arrest (Yang, Han et al. 2007). Western Blot for $CD34^+$ cells of AML patients and healthy controls was difficult to establish because too many cells were needed for analysis. It cannot be stated if WT1 expression is only restricted to $CD34^+$ AML blasts and THP-1 cells. Consequently experiments of WT1 protein determination were repeated in AML cell lines only and resulted in specific bands for WT1 in K562, THP-1, KG-1 and HL-60 samples at approximately 54 kDa (figure 44). Both qRT-PCR and Western Blot analysis showed variations in the level of WT1 expression in leukemic cells lines which could have an impact on specific lysis by effector cells. Cytotoxicity assay results raise the possibility that only a small subpopulation of target cells express sufficient WT1 levels. These experiments did not confirm surface expression of WT1. Interestingly, only a few research groups tried the detection of WT1 protein by Western Blot analysis (Gao, Bellantuono et al. 2000, Bellantuono, Gao et al. 2002) which could be dependent on the high amount of $CD34^+$ cells needed for analysis. Also, WT1 detection on protein levels requires a highly specific WT1 antibody that binds to immunodominant WT1 peptide structures on the surface of target cells. Selection of a suitable antibody for Western Blot analysis was fairly difficult because none of the available antibodies was reported to be highly specific by having a high avidity. Even if available antibodies were polyclonal indicating they generally recognize multiple epitopes because they are generated using the entire immunogen, it was not known if immunodominant epitopes were presented by target cells.

To assess WT1 localisation on the target cell surfaces extra- and intracellular staining was conducted and has the advantage that only little material was required. Since WT1 is described as a transcription factor that shuttles between the nucleus and the cytoplasm (Niksic, Slight et al. 2004) it was assumed that high amounts of WT1 can be found intracellularly. This assumption was confirmed by flow cytometry for AML cell lines THP-1 and HL-60 (figure 45, table 21). No extracellular WT1 expression could be detected. In theory, intracellular proteins are expressed on the cell surface via MHC class I as peptide fragments. Upon presentation, peptides are recognized by the TCR on WT1-specific effector cells (Konig 2002, Morris, Hart et al. 2006). As a nuclear protein, WT1 is inaccessible to classical antibody therapy. Most of the monoclonal antibodies that are considered therapeutically useful only recognize whole surface proteins which make targeting of WT1

quite difficult. Due to the inaccessibility to conventional antibody therapies, many groups work on vaccine approaches where WT1-specific cytotoxic T cells are generated that recognize certain WT1 peptides (Bellantuono, Gao et al. 2002, Pinilla-Ibarz, May et al. 2006, May, Dao et al. 2007, Rezvani, Yong et al. 2007, Van Driessche, Berneman et al. 2012). The WT1-specific peptide sequence is the most prominent peptide expressed by AML blasts and therefore used as a target structure for antibody generation (Rezvani, Yong et al. 2008, Rezvani, Yong et al. 2012, Dao, Yan et al. 2013, Veomett, Dao et al. 2014). Recently, a novel antibody, ESK-1, specific for the immunodominant RMFPNAPYL epitope of WT1 has been found to be highly-specific because of its high avidity (Dao, Yan et al. 2013). ESK-1 binds to AML cells in a HLA-A*02 restricted WT1-specific manner but not to normal PBMCs.

Besides intensive research on relapse prevention, there is still an urgent need for supportive immunotherapies to eradicate residual leukemic blasts after conventional therapy. Tumour escape mechanisms are discussed as the most important stimulus for relapse development (Chan and Coussens 2013, Kaluza and Vile 2013, Kottke and Boisgerault 2013, Leone, Shin et al. 2013, Rommelfanger-Konkol, Pulido et al. 2013, Bruttel and Wischhusen 2014, Bryan and Gordon 2015). Generating immunotherapies is only possible when AML blasts express HLA antigens and other surface molecules that serve as recognition sites for functional immune cells such as T and NK cells. However, AML blasts can have alterations in HLA expression leading to a suppressed immune response by effector cells (van Luijn, van den Ancker et al. 2011, Waterhouse, Pfeifer et al. 2011, Hamdi, Cao et al. 2015). This so called genomic instability is common in certain types of leukemia and leads to the fact that leukemic cells manage to escape the immune system. Vago and colleagues analysed HLA expression patterns in AML patients in a relapse situation and found out that HLA antigens can be lost due to leukemic cell escape (Toffalori, Cavattoni et al. 2012, Vago, Toffalori et al. 2012). This led to the problem that donor T cells could not attack leukemic blasts any longer because they did not express the ‘correct’ signal for T cell recognition. Due to such an immune escape mechanism, the generation of immunotherapies directed against residual leukemic blasts and the maintenance of GvL response becomes difficult. Deficient processing and presentation also applies to other surface structures like adhesion molecules involved in effector cell recognition. In addition to these alterations leukemic cells can evade the immune system by defective secretion of co-stimulatory molecules. These molecules are needed for effector T cell activation and stimulation. Furthermore, co-inhibitory molecules such as Programmed Death Receptor Ligand-1 (PD-L1) secreted by AML blasts down-regulate effector cell function and inhibit their proliferation (Berthon, Driss et al. 2010, Zhou, Munger et al. 2010,

Kronig, Kremmler et al. 2014). Another important immune escape mechanism is the secretion of inhibitory cytokines such as IL-10 and TGF-β which lead to T cell anergy and limit effective antigen presentation to cytotoxic lymphocytes (Lippitz 2013, Li, Li et al. 2014, Tjin, Krebbers et al. 2014). Other studies describe anti-apoptotic mechanisms like insufficient expression of FasL (FAS ligand) and TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand), AML cells employ in order to not be eliminated by effector cells (Min, Lee et al. 2004, Tourneur, Delluc et al. 2004, Pordzik, Petrovici et al. 2011).

4.3. The future of WT1-specific adoptive immunotherapy

WT1-specific effector T cells usually appear at very low frequencies in a fairly small portion of AML patients *pre* and *post* transplantation as shown by ELISpot results. Even after expansion, T cells were characterized by a short life span confirmed by their phenotype (figure 29). However, Chapuis and colleagues managed to expand WT1-specific $CD8^+$ T cells with memory function and antileukemic activity (Chapuis, Ragnarsson et al. 2013). This finding of a successful adoptive T cell transfer is an exceptional case because therapies with naturally occurring cells often fail to mediate therapeutic effects due to lack in high T cell affinity. Whereas naturally occurring T cells are restricted in cell number and especially in avidity as described by a number of researchers, genetically engineered T cells do not face these problems. TCR editing and CAR-modified T cells are promising approaches (Mardiros, Brown et al. 2013, Ritchie, Neeson et al. 2013, Tettamanti, Marin et al. 2013, Cartellieri, Koristka et al. 2014, Gill, Tasian et al. 2014, Tettamanti, Biondi et al. 2014) also shown for WT1-specific immunotherapies (Ochi, Fujiwara et al. 2011, Van Driessche, Berneman et al. 2012). For TCR editing, TCRs from T cells of patients with good antitumor response are cloned and inserted into a vector system that is used for infection of other patient cells (Zhang and Morgan 2012). CARs with antibody-like specificities are even more useful because they can recognize surface structures of leukemic target cells in a non-MHC restricted manner (Restifo, Dudley et al. 2012). However, due to the monoclonal specificity of cells, genetically engineered T cells attack only a narrow range of leukemic blasts. This in turn could lead to antigen escape variants of blasts and unexpected toxicities by antigen mimicry leading to cytokine storm and tissue destruction (Restifo, Dudley et al. 2012). A more disastrous effect of transferring genetically modified T cells specific for the MAGE-A3 peptide (a melanoma-associated antigen) resulted in cardiovascular toxicity followed by mortality in two patients

when engineered TCRs recognized an unrelated epitope of normal cardiac tissue instead of the actual target peptide (Linette, Stadtmauer et al. 2013). In another study, adoptive cell therapy with modified TCRs recognizing MAGE-A3/A9/A12 also resulted in death of two patients due to neuronal cell destruction by unrecognized expression of MAGE-A12 in normal brain tissue (Morgan, Chinnasamy et al. 2013).

As described by many researchers, currently available therapies are mostly applicable to only a small patient cohort due to HLA restrictions. A novel approach of *ex vivo* generation of a WT1-specific T cell adoptive immunotherapy for treating relapsed AML investigated in this project was thought to be an alternative to currently available immunotherapies. However, expansion did not result in a rapid generation of highly-specific cell numbers sufficient for therapeutic use and is therefore not considered as clinically applicable. While there is evidence that generation of non-genetically modified, cytotoxic effector cells for adoptive transfer is possible and beneficial (Chapuis, Ragnarsson et al. 2013), improvement in overall survival and relapse prevention has not been demonstrated by many clinical studies. In addition therapies for clinical use cannot be generated in every laboratory because generation is influenced by methodological factors, money and requires GMP conformity.

5. Summary

Relapse after haematopoietic stem cell transplantation remains a major cause of mortality for patients with Acute Myeloid Leukemia (AML). The generation of adoptive immunotherapies for treating relapse in AML patients is an emerging field that already showed promise in a number of clinical trials. The idea of targeting residual leukemic blasts with adoptive transfer of antigen specific T cells developed over the years by improved understanding of T cell activation strategies and target structure recognition. Nevertheless, many obstacles such as insufficient effector cell numbers after expansion, low avidity of specific cells, time-consuming generation and application to only a small number of patients, need to be overcome. Since the WT1 protein has proven to be a promising tumor-associated antigen being highly expressed on leukemic blasts, it has been studied as a target structure for the generation of an adoptive immunotherapy in this project. Consequently, investigation aimed at the identification of putative WT1-specific effector cells by determining WT1-specific frequencies in ELISpot and FluoroSpot assay. Initial frequencies in AML patients were found to be relatively low (0.001- 0.013 %) concluding that WT1-specific cells have to be expanded *ex vivo* in order to generate sufficient cell numbers for an immunotherapy. The proliferative potential of WT1-specific cells was then tested in different expansion strategies that resulted in at most 4-fold expansion of WT1-specific cells under optimised conditions. Compared to expansion protocols for clinical application, generated cell numbers were not sufficient for adoptive transfer yet. Furthermore, expanded cells mainly consisted of cytotoxic $CD8^+$ T cells secreting IFN-ɣ and granzyme B, but not of effector memory cells. To assess effector cell functionality, one aim was the identification of suitable target cells expressing WT1 that could be lysed by WT1-specific effector cells. The lytic activity of effector cells against AML cell lines and AML blasts from patients was tested by Europium release assay. Since lysis does not only depend on WT1 recognition, WT1 levels were determined by qRT-PCR, Western Blot analysis and flow cytometry. Specific lysis of AML targets could only be achieved in AML cell lines but not in AML blasts. Tumor escape influenced by various factors is discussed to be the major stimulus of relapse which could be an explanation for these results. Considering existing approaches with TCR editing and genetically modified T cells as adoptive immunotherapies for relapse prevention, it seems that immunotherapies with non-modified cells cannot keep up with current requirements for rapid generation of highly WT1-specific cells. Although many expansion strategies have proven successful for the generation of WT1-specific effector cells, T cell avidity remains a problem.

Zusammenfassung

Rezidive sind die häufigste Todesursache bei Patienten mit Akuter Myeloischer Leukämie (AML). Deshalb hat sich die Wissenschaft in den letzten Jahren zunehmend mit supportiven Therapieansätzen wie adoptiver Immuntherapie beschäftigt, die als eine Art Prophylaxe nach Stammzelltransplantation verabreicht werden soll, um restliche leukämische Blasten zu zerstören. Vielversprechende Ansätze sind bereits in klinischer Anwendung, allerdings zielen Erfolge immer nur auf relativ kleine Patientengruppen ab. Daraus lässt sich schließen, dass bisher noch keine optimale Immuntherapie gefunden wurde, die breitgefächert einsetzbar ist. Zeitaufwendige Herstellung von Effektorzellen gegen restliche leukämische Blasten sowie mangelnde Spezifität und Immunogenität behindern die Entwicklung optimaler Immuntherapien, die möglichst schnell und spezifisch in der Klinik eingesetzt werden sollen. Nachdem WT1 als vielversprechendes Tumorantigen identifiziert werden konnte, das auf leukämischen Blasten hoch exprimiert wird, diente es in diesem Projekt als Zielstruktur für eine adoptive Immuntherapie. Zu Beginn wurde die initiale Frequenz von WT1-spezifischen Zellen aus AML Patienten mit ELISpot und FluoroSpot gemessen. Die daraus resultierende, niedrig-frequente Zellpopulation mit 0,001-0,013 % WT1-spezifischen Zellen wurde im Folgenden expandiert, um ausreichende Zellmengen für adoptiven Zelltransfer herzustellen. Unterschiedliche Expansionsansätze wurden miteinander verglichen, ergaben aber unter optimalen Bedingungen nur eine 4-fache Vermehrung von WT1 spezifischen Zellen, verglichen mit der Ausgangspopulation. Abhängig von der vorgegebenen Menge an transfundierten Zellen, die für die klinische Anwendung notwendig ist, reicht dieser Expansionsansatz nicht für therapeutische Zwecke. Die expandierten Zellen bestanden im Wesentlichen aus zytotoxischen $CD8^+$ T Zellen, die IFN-ɣ und Granzym B sezernieren, aber nicht aus Effektor Memory Zellen. Um die Funktionalität der Effektorzellen zu testen, war ein weiteres Ziel des Projektes die Etablierung geeigneter Zielzellen, die WT1 präsentieren. Hierfür wurden AML-spezifische Zelllinien und AML Blasten aus Patienten im Europium Assay auf ihre Lyse durch Effektorzellen untersucht. Da Zelllyse nicht nur von funktionellen Effektorzellen abhängig ist, wurde WT1 auf mRNA Ebene mit qRT-PCR und auf Proteinebene mit Western Blot Analyse und mittels Durchflusszytometrie untersucht. Spezifische Lyse konnte nur bei AML Zelllinien und nicht bei Patientenblasten festgestellt werden, was vermutlich auf Tumor Escape Mechanismen von AML Blasten zurückzuführen ist. Berücksichtigt man aktuelle Therapieansätze mit manipulierten T-Zellrezeptoren oder genetisch modifizierten T-Zellen für die Rezidivbehandlung, so scheint die Expansion von nicht-modifizierten, WT1-spezifischen Effektorzellen weniger erfolgreich, da eine hohe Avidität der angereicherten T-Zellen ein Problem darstellt.

6. Appendix

Antibody concentrations of pro-and inflammatory cytokines used for ELISpot and FluoroSpot analysis are given in detail in the tables below.

Tab. 22: ELISpot - primary antibody concentration

Specificity	Concentration	Clone
Granzyme B	15 µg/mL	GB10
IFN-ɣ	10 µg/mL	1-D1K
IFN-α	15 µg/mL	MT1/3/5
IL-10	10 µg/mL	9D7
IL-12	10 µg/mL	IL12-I
IL-13	10 µg/mL	B-B13
IL-17a	10 µg/mL	MT44.6
IL-2	10 µg/mL	Mouse α human IL2
IL-4	10 µg/mL	MP4-25D2
Perforin	30 µg/mL	Pf-80/164
Tumor-necrosis factor-α (TNF-α)	4 µg/mL	TNF ¾

Tab. 23: ELISpot - secondary antibody concentration

Specificity	Concentration	Clone
Granzyme B	1 µg/mL	GB11
IFN-ɣ	2 µg/mL	7-B6-1
IFN-α	1 µg/mL	MT2/4/6
IL-10	1 µg/mL	12G8
IL-12	2 µg/mL	IL12-II-biotin
IL-13	1 µg/mL	B-B13 II-biotin
IL-17a	0.5 µg/mL	MT5046-biotin
IL-2	2 µg/mL	α human biotinylated
IL-4	2 µg/mL	IL4-II-biotin
Perforin	1 µg/mL	Pf-344-biotin
TNF-α	1 µg/mL	TNF 5

Tab. 24: FluoroSpot - primary antibody concentration

Specificity	Concentration	Clone
Granzyme B	15 µg/mL	GB10
IFN-ɣ	15 µg/mL	1-D1K

Tab. 25: FluoroSpot - secondary antibody concentration

Specificity	Concentration	Clone
Granzyme B	2 µg/mL	GB11-biotin
IFN-ɣ	2 µg/mL	7-B6-1-FS-FITC

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