In vitro characterization of human AML-reactive CTL clones generated from the naive subset of healthy donors and adoptive transfer into leukemia-engrafted NSG mice

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1. Abstract

Acute myeloid leukemia (AML) is a very aggressive cancer of the hematopoietic system. Chemotherapy and immunotherapeutical approaches including hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) are the only curative options available. The beneficial graft-versus-leukemia (GVL) effect of cellular immunotherapy is mostly mediated by donor-derived $CD8^{+}$ T lymphocytes that recognize minor histocompatibility antigens (mHags) and leukemia-associated antigens (LAAs) presented on the surface of AML blasts (Falkenburg et al. 2008; Kolb 2008). A main complication is graft-versus-host disease (GVHD) that can be induced when cytotoxic T lymphocytes (CTLs) recognize broadly expressed antigens. To reduce the risk of GVHD, specific allogeneic T-cell therapy inducing selective GVL responses could be an option (Barrett & Le Blanc 2010; Parmar et al. 2011; Smits et al. 2011). This requires efficient *in vitro* strategies to generate AML-reactive T cells with an early differentiation phenotype as well as vigorous effector functions and humanized mouse models to analyze the anti-leukemic potential of adoptively transferred T cells *in vivo*. In this study, AML-reactive CTL clones and oligoclonal lines could be reliably generated from the naive subset of healthy HLA-class I-identical donors by stimulation with primary AML blasts in mini-mixed-lymphocyte / leukemia cultures (MLLCs) in eight different patient / donor pairs. These CTLs were promising candidates for cellular immunotherapy because of their relatively early differentiation phenotype and strong proliferative and lytic capabilities. The addition of the common γ-chain cytokine IL-21 to the stimulation protocol enabled more precursors to develop into potent leukemia-reactive CTLs, presumably by its beneficial effects on cell survival and antigen-specific proliferation during the first weeks of cultures. It also strengthened the early-stage phenotype. Three long-term cultured CTLs exemplarily transferred into leukemia-engrafted immunodeficient NSG mice mediated a significant reduction of the leukemic burden after a single transfusion. These results demonstrate that CTL clones with reactivity to patient-derived AML blasts can be isolated from the naive compartment of healthy donors and show potent anti-leukemic effects *in vivo*. The herein described allo-MLLC approach with *in vitro* “programmed” naive CTL precursors independent of a HSCT setting is a valuable alternative to the conventional method of isolating *in vivo* primed donor CTLs out of patients after transplantation (Kloosterboer et al. 2004; Warren et al. 2010). This would make leukemia-reactive CTLs already available at the time

point of HSCT, when residual leukemia disease is minimal and the chances for complete leukemia eradication are high. Furthermore, leukemia-reactive CTLs effectively expanded by this *in vitro* protocol can be used as screening populations to identify novel candidate LAAs and mHags for antigen-specific immunotherapy.

2. Introduction

2.1 Immunological background

2.1.1 General overview of the immune system

Human blood consists of plasma and cellular components which include erythrocytes, thrombocytes, and leukocytes. Erythrocytes and thrombocytes have their main functions in oxygen transfer and hemostasis, respectively, but they also have a role in immunity. Leukocytes are immune cells involved in the defense against foreign agents like infectious microbes and against malignant cells such as tumor cells. Furthermore, they play a role in the acceptance of self structures. Leukocytes include granulocytes (neutrophils, basophils, and eosinophils), monocytes / macrophages, dendritic cells (DCs) and lymphocytes. In addition to the immune cells, the immune system is a network of lymphoid organs, humoral factors, and cytokines. Immunity is divided into two fundamentally different types of responses:

First, the innate response that provides immediate host defense by mechanisms that are encoded by their major functions in the germline genes of the host. It includes physical barriers, secreted mucus overlaying epithelial cells together with cilia, and membrane-bound receptors that bind molecular pattern expressed on the surface of microbes. Furthermore, soluble proteins and small bioactive molecules such as complement factors and C-reactive protein are involved that are constitutively present in plasma or are released from activated immune cells. One important group of soluble mediators are cytokines. They are secreted by various immune cells, commonly after activation, and induce responses by binding to specific receptors to alter the behavior of cells in an autocrine, paracrine, or endocrine manner. Cytokines have divers biological effects depending on the cytokine and the responding cell, but typically they have an impact on cell activation, division, apoptosis, or movement of cells.

Cellular components are also involved in the innate response. In case of an infection, activated macrophages secrete cytokines that stimulate division of neutrophil precursors in bone marrow. Released neutrophils are recruited to the infection site to eradicate pathogens by phagocytosis. Natural killer (NK) cells destroy infected or malignant cells lacking MHC-class I-molecules on their surface and kill antibody

opsonized target cells. Eosinopils, basophils, and mast cells release enzymes after activation which destroy pathogens.

The second type of response is the adaptive immunity encoded by genes that are assembled by somatic rearrangement of germline gene elements leading to formation of receptors with a high specificity for their targets. The response includes antigen-specific reactions by B and T lymphocytes that undergo clonal expansion after activation. The adaptive immune response mainly takes place in lymphoid organs. Primary lymphoid organs are bone marrow and thymus where lymphocytes develop from progenitor cells. B cells maturate in bone marrow, while T cells migrate at early stages into the thymus to complete their development. As naive cells B and T lymphocytes migrate into secondary lymphatic tissues such as lymph nodes, spleen, tonsils, and mucosa associated tissues to encounter their specific antigen. B cells express antigen-specific immunoglobulins as transmembrane molecules (B-cell receptor) and as soluble proteins (antibodies), whereas T cells only bear their T-cell receptor (TCR) as transmembrane proteins. After activation lymphocytes start to proliferate and differentiate into effector cells which concentrate at infection sites. B cells differentiate into plasma cells that secrete their antibodies (Abs) to opsonize pathogenic structures and present their bound antigens to T cells. One subset of T cells either differentiates into T-helper cells that secrete cytokines and express co-stimulatory molecules to activate and recruit other immune cells or into regulatory T cells that have immunosuppressing functions. Another T-cell population develops into cytotoxic T cells that lyse their target cells and release cytokines.

The innate immunity occurs immediately after infection of the host and provides the first line of defense, whereas the specific adaptive immunity takes several days or weeks to develop. It forms a memory that enables a more vigorous and rapid response to subsequent antigen exposure. For a fully effective immune response both arms of immunity act together. Defects in the immune system can lead to severe infections or to allergy and autoimmunity (Chaplin 2010; Delves & Roitt 2000; Parkin & Cohen 2001).

2.1.2 T lymphocytes

2.1.2.1 T-cell receptor and antigen recognition of T lymphocytes

In the adaptive immunity lymphocytes recognize peptides presented by infectious or malignant cells in order to destroy them. T lymphocytes sense the presence of antigens by expression of highly variable TCRs that are composed of two glycoprotein chains which are in the majority of T cells the α- and ß-chain. Both chains consist of an extracellular variable (v) region and a constant (c) region which are anchored by a transmembrane domain with a short cytoplasmatic tail. The population of T cells that expresses γ- and δ-delta chains in their TCR (Delves & Roitt 2000) is not explained in more detail here since the main focus lies on the T cells expressing the TCR α- and ß-chains. The antigen binding site of the TCR is located in the v region and is highly polymorphic between single T cells to generate an enormous repertoire of antigen receptors. The v region is encoded in three gene segments: the variable (V), diversity (D), and joining (J) segments, which are assembled in developing T cells by somatic DNA recombination. There are multiple copies of each segment and therefore varying v regions can be assembled by ligation of different combinations of these segments. In that process recombinase-activating gene (RAG) recombinases bind and cleave the DNA at recombination signals flanking each gene segment. DNA repair enzymes process and repair the DNA ends what leads to deletion or inversion of segments. To further enhance diversity, the terminal deoxynucleotidyl transferase adds nucleotides randomly to the coding ends of the segments (Grawunder et al. 1998; Schatz & Ji 2011). The complete TCR is expressed at the cell surface in multiple copies. When the antigen is bound to the receptor, accessory chains forming the CD3 complex associate with the receptor and transmit signals into the cell. The co-receptors CD4 for T-helper cells and CD8 in the case of cytotoxic T cells are also needed to form the complete TCR-complex. For full activation of the T cell co-stimulatory signals such as the interaction of CD28 on the T cell with CD80 or CD86 expressed on the APC are required additionally. The cytoplasmatic tail of CD3 contains sequence motifs which are phosphorylated at key tyrosines by receptor associated kinases after antigen binding. This initiates an activation cascade leading to the activation of protein kinases and therefore induces a downstream program of cell activation.

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T cells recognize their antigenic peptide only in context with a self-component to ensure the identification of infected and malignant cells or to recognize antigens that have been taken up by APCs. Therefore, the antigen is presented to the T cells within the binding groove of a self major histocompatibility complex (MHC), called human leukocyte antigen (HLA)-molecules in humans. There are two major types of HLA-molecules: The HLA-class I- and HLA-class II-molecules. $CD4^+$ T cells generally recognize peptides bound by HLA-class II-molecules, whereas $CD8^+$ T cells mainly react to antigens presented by HLA-class I-molecules. The TCR-complex interacts with the HLA-molecule and the bound peptide, while the co-receptor only binds to the HLA-molecule. The contact of the TCR with both, the HLA-molecule and the antigenic peptide is designated as HLA-restriction (Chaplin 2010; Parkin & Cohen 2001). HLA-class I-molecules are expressed by all nucleated cells to allow infected or malignant cells to present their antigens to $CD8^+$ T cells. There are three major classes of HLA-class I-molecules encoded by distinct genes, designated as HLA-A, HLA-B, and HLA-C. The α-chain gene encodes three extra-cellular domains that form the peptide-binding groove and determine the HLA-class. The α-chain associates with β2-microglobulin to form a stable molecule. Peptides presented by HLA-class I-molecules generally derive from intracellular and nuclear proteins. These endogenous peptides are generated in the proteasome by degradation. Afterwards, they are translocated to the endoplasmatic reticulum (ER) by transporter associated with antigen presentation (TAP). In the ER the antigenic peptide is assembled with the HLA-molecule and leaves the ER for presentation at the cell surface via the Golgi apparatus.

The HLA-class II-molecules are expressed on the surface of APCs (i.e. DCs, B cells and activated macrophages) to present their absorbed antigens mainly to $CD4^+$ T-helper cells. There are three major classes of HLA-class II-molecules designated as HLA-DR, HLA-DQ, and HLA-DP. They consist of two transmembrane chains, called α- and β-chain. Exogenous proteins are endocytosed by APCs and are degraded in the endosome by proteases. They are translocated to a late endosomal compartment termed MHC-class II-compartment (MIIC) where they are loaded onto the HLA-class II-molecules. These are previously assembled in the ER and transported to the MIIC through the Golgi apparatus. The HLA-class II-molecules with their bound peptides are finally transported to the plasma membrane.

A link between the HLA-class I- and II-pathway is designated as cross-presentation, whereby exogenous derived peptides are presented by HLA-class I-molecules. Endogenous proteins can also be presented on HLA-class II-molecules when they are degraded by autophagy or other pathways (Neefjes et al. 2011).

2.1.2.2 Development and survival of αß T cells

Mature T cells have undergone several steps of development before they are completely functional cells of the immune system. Precursor cells from the blood that derive from bone marrow stem cells enter the thymus. The differentiated thymus consists of an inner medulla surrounded by an outer cortex with a subcapsular region. T cells can be distinguished in their developmental steps within the thymus by the expression of the TCR-complex-components. Double-negative thymocytes that are $CD3^-$ $CD4^-$ $CD8^-$ first populate the subcapsular region. Here, the rearrangement of the TCR genes starts with the rearrangement of the TCR-ß locus. The successfully rearranged ß-chain pairs with a surrogate α-chain to a pre-TCR that is expressed on the cell surface. This leads to cell proliferation and the expression of CD4 and CD8 to double-positive thymocytes that migrate to the thymic cortex. Cells with out-of-frame TCR-ß rearrangements fail to produce a functional protein and will die. Once the double positive cells stop proliferating, the α-chain locus starts to rearrange. After completion of TCR-α rearrangement, αß thymocytes undergo selection stages. Cells die unless they are rescued in the positive selection by a low-affinity interaction of the TCR with a self-peptide-HLA-complex that is expressed on epithelial cells in the thymus. Surviving thymocytes express high levels of the TCR afterwards and differentiate into mature single-positive T cells expressing only CD4 or CD8, respectively. Single-positive T cells finally can be found in the thymic medulla. Thymocytes also undergo negative selection which eliminates those cells that are capable of responding to self-antigens presented on HLA-molecules on the surface of thymic DCs to avoid auto-reactivity. After selection and maturation, T cells leave the thymus as recent thymic emigrants (RTEs) which express high levels of CD3-TCR next to CD45RA, CCR7, CD27, and CD62L (Hayday & Pennington 2007; Spits 2002). Peripheral RTEs comprise a distinct population from the naive T-cell pool. They undergo further post-thymic maturation stages in the lymphoid periphery including down-regulation of CD3-TCR and up-regulation of CD28 to become mature naive T cells (Fink & Hendricks 2011).

2.1.2.3 Effector mechanisms of CD8$^+$ T cells

T cells have specific effector mechanisms to fight invading pathogens. A main task of CD4$^+$ T cells is the activation and recruitment of other immune cells in the case of an infection but these cells are not described in more detail herein because the present work is focusing on CD8$^+$ T cells. Cytotoxic CD8$^+$ T lymphocytes (CTLs) have an important role in the clearance of virally infected or tumorigenic cells and intracellular bacteria. They kill their target cells after their activation by stimulation of TCR-CD3-complexes and under cytokine influence. Naive T-cell precursors first undergo an activation process to gain cytotoxic activity which requires one to three days until the expression of granule components is induced. CTLs have secretory granules for storage and exocytosis of their cytotoxic contents. These granules are secretory lysosymes and contain many cytotoxic molecules to destroy their target cells including perforin, granulysin, and a group of serine proteases called granzymes (Russell & Ley 2002). After recognition of a target cell the granules move to the junction between two cells, designated as immunological synapse, where the membrane of the lysosomes and the CTL fuses to release the cytotoxic molecules in the synapse. There, perforin forms a pore in the plasma membrane of the target cell leading to necrosis and enables granulysin and granzymes to enter the cell. Granulysin kills microorganisms by increasing the membrane permeability. Granzyme A and B destroy target cells by inducing single-strand nicks in the DNA or by activating molecules of the caspase family to induce apoptosis, respectively (Fan & Zhang 2005). Next to the granule mediated cell death, CTLs can also induce apoptosis through interaction of Fas (CD95) and FasL. FasL is expressed after TCR stimulation over a one to two hours period. Nevertheless, CTLs seem to kill more often by using the granule pathway. Further, they are also able to produce several cytokines after activation such as tumor necrosis factor (TNF) and IFN-γ to boost the immune response (Barry & Bleackley 2002).

2.1.2.4 Differentiation of CD8$^+$ T cells and expression of surface markers

For an effective immune response naive CD8$^+$ T cells (Tn) are activated by antigens and undergo dramatic clonal expansion. They acquire effector functions, migrate to infection sides, and lyse their target cells. These effector cells (Teff) produce cytokines such as IFN-γ and TNF-α. They are thought to have short half-life and little memory potential. T cells also differentiate into long-lived memory cells (Tm) that

respond with strong proliferation and differentiation into effector cells upon antigen re-encounter. Memory T cells are further divided into central memory (Tcm) and effector memory (Tem) cells based on their expression of activation markers, homing-, and chemokine receptors, as well as effector molecules. The homing receptor L-selectin CD62L and the chemokine receptor CCR7 are often used to distinguish both subsets. Tcm express high levels of CD62L and CCR7 to enter lymphoid tissues and have no effector functions, whereas Tem are only low positive for both markers and produce high amounts of cytokines and cytotoxic molecules (Sallusto et al. 1999). Another memory population expressing high amounts of CD62L and CCR7 has been found recently within the $CD8^+$ naive-like subset. The so called memory stem T cells (Tscm) share phenotypic and functional properties with hematopoietic stem cells. Tscm arise after antigenic stimulation and have potential for self-renewal and multipotency to generate all other memory and effector T-cell subsets (Gattinoni et al. 2011). Similarly, a memory compartment within both, the $CD62L^+$ and $CD62L^-$ subset of $CD8^+$ T cells has been identified that shares features with stem cells as well (Turtle et al. 2009). There are several models dealing with the differentiation pathway of effector and memory CTLs. Some models propose a linear pathway with a subsequent differentiation from Tn into Teff and further into Tm, other prefer a differentiation of Teff and Tm from single cell precursors along distinct pathways, and some assume that the differentiation of different subsets derive from a fixed line at secondary stimuli like cytokines (Belz & Kallies 2010). Another theory is that both subsets can convert into each other under certain conditions (Cui & Kaech 2010). An asymmetric cell division during initial T-cell activation leading to different cell fates has been proposed recently where one cell can give rise to effector and several memory populations (Jameson & Masopust 2009). But that issue remains an open question until today.

To describe the different T-cell subsets more closely, the expression of several surface markers is used. Naive $CD8^+$ T cells are highly positive for CCR7, CD62L, and the co-stimulatory molecules CD27 and CD28. A strong expression of the IL-7 receptor CD127 and the chemokine receptor CXCR4 (CD184) are also associated with a naive phenotype. These molecules are down-regulated in different extent with repetitive antigen stimulation, while the glucuronyltransferase CD57 and the death receptor CD95 (Fas) are up-regulated. Another important differentiation marker is the expression of two isoforms of the protein tyrosine phosphatase CD45, named

CD45RA and CD45RO. CD45RA is strongly expressed on the surface of Tn, it is down-regulated after antigen contact, and it is re-expressed on Teff. On the contrary, CD45RO is not present on the surface of Tn but it is expressed after stimulation and remains on the surface afterwards. An overview of the expression of differentiation markers is shown in figure 1 (Appay et al. 2008; Klebanoff et al. 2006; Sallusto et al. 2004; Kobayashi et al. 2004; Krueger et al. 2003).

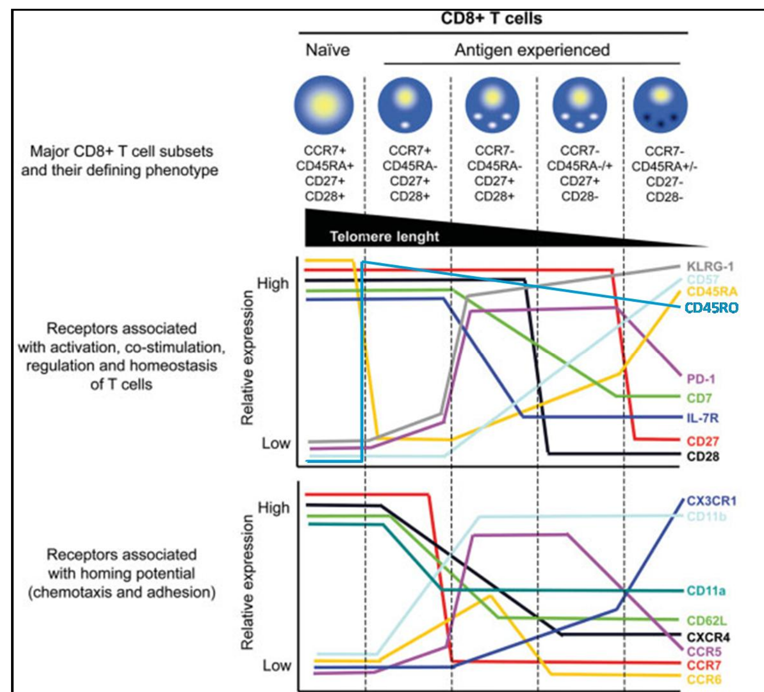

Figure 1: Expression of T-cell markers in CD8^{+} T cells of distinct differentiation stages (adapted and modified from Appay et al. 2008).

2.1.2.5 Co-stimulation of CD8^{+} T cells and cytokine influence

Activation and differentiation of cytotoxic T cells require at least three signals. The first signal is provided by interaction of the TCR-CD8-complex with the antigenic peptide presented by HLA-molecules. The second signal is delivered by co-stimulatory molecules expressed on APCs such as the interaction with CD80 or CD86. The third signal arises from the stimulation of receptors for inflammatory cytokines of the IFN family, particularly IL-12. IL-12 is described to promote proliferation and survival during primary response and to support the development of cytotoxicity and maintenance of Tem (Chowdhury et al. 2011; Obar & Lefrançois 2010; Williams & Bevan 2007). Also other cytokines are important for the differentiation and homeostasis of CTLs. A key role play cytokines of the common γ-chain family which include IL-2, -4, -7, -9, -15, and -21. IL-7 and IL-15 are known to be important in memory formation and viability of CTLs. IL-7 is described to support

cell survival and homeostatic proliferation of naive and memory T cells. IL-15 shall induce basal level of homeostatic proliferation and promotes differentiation to Tcm like phenotype. But both functions are partly overlapping (Obar & Lefrançois 2010; Surh & Sprent 2008). Early signals of IL-2 are needed in the activation phase of CTLs for optimal memory function and the cytokine is described to support proliferation and effector phenotype (Cui & Kaech 2010; Jameson & Masopust 2009). The common γ-chain cytokine which was most recently discovered is IL-21. It appears to promote the development of antigen-specific $CD8^+$ T cells predominantly from the naive subset (Li et al. 2005) and to sustain a Tcm like phenotype of antigen-nonspecific and peptide-specific CTLs in humans (Alves et al. 2005; Kaka et al. 2009). But also synergetic effects of all these cytokines are described.
$CD4^+$ T-helper cells also provide co-stimulatory signals. This can either be indirect by improved activation of APCs or a direct effect by secreted cytokines. But the need for CD4 help remains an open question since there are studies showing CD4 independent CD8 response as well as studies indicating CD4 help as essential (Williams & Bevan 2007).

2.2 Leukemia

2.2.1 General characteristics of leukemia

Leukemias are cancers of the hematopoietic system. In general, they are caused by malignant transformation of leukocyte subtypes leading to immature and not functional cells with uncontrolled proliferation. Leukemic blasts grow out in the bone marrow and pass over to peripheral blood and hematopoietic organs where they displace the normal hematopoiesis. Due to a lack of functional leukocytes and platelets symptoms like fatigue, paleness, weight loss, and a generally higher susceptibility for infections and bleeding occur in patients. Diagnosis is performed by differential blood count and characterization of malignant cells from bone marrow of patients.
Leukemias are further divided according to the degenerated type of leukocytes. Myeloid leukemias derive from myeloid cells, such as granulocytes and monocytes, whereas lymphoid leukemias are caused by transformation of lymphoid cells. Also their clinical cause, acute or chronic, is considered for definition. Acute leukemias have a fast progression with severe symptoms and lead, without treatment, to death

within a few months. Chronic leukemias have a slower course with longer stable phases and often are unnoticed over a period of time.
In Germany leukemias account for 2.4% of all tumors. Per year more than 11,400 people develop this disease with approximal 50% suffering from an acute form. The different subtypes of leukemia can occur in every age but the prevalence of their occurrence are observed more often in certain age groups. Acute lymphatic leukemia (ALL) often occurs in young adults and children, adults in the mid age often develop acute and chronic myeloid leukemia (AML and CML), and chronic lymphatic leukemia (CLL) is rarely seen under the age of 50 (www.krebsgesellschaft.de).

2.2.2 Acute myeloid leukemia (AML)

In the western world AML has an incidence of 3.7 per 100,000 individuals and an age dependent mortality of 2.7 to nearly 18 per 100,000 persons (Deschler & Lübbert 2006). AML is caused by the malignant transformation of myeloblasts, the precursors of myeloid leukocytes. Originally, AML subtypes were classified in the French-American-British Cooperative Group (FAB) system according to cytomorphologic and -chemic criteria (Table 1). Eight different subtypes (M0-M7) are defined and AML is confirmed with a threshold of 30% blasts in bone marrow (Bennett et al. 1985).

Table 1: Classification of AML subtypes by the FAB system (adapted and modified from Bennett et al. 1985; Michl 2005).

FAB subtype		Incidence [%]
M0	myeloblastic leukemia, minimal differentiation	< 5
M1	myeloblastic leukemia, without maturation	20
M2	myeloblastic leukemia, with maturation	30
M3	promyelocytic leukemia	< 5
M4	myelomonocytic leukemia (M4) or myelomonocytic leukemia with eosinophilia (M4eo)	< 30
M5	monoblastic leukemia (M5a) or monocytic leukemia (M5b)	<15
M6	erythroid leukemia	< 5
M7	megakaryoblastic leukemia	< 5

A newer classification of the World Health Organization (WHO) connects the FAB-system with cyto and molecular genetic characteristics (Table 2). Genetic mutations and the resulting prognosis are considered in this system. The threshold of leukemic blasts in the bone marrow is reduced to 20% for diagnosis and certain subtypes of AML with cytogenetic abnormalities such as translocations or inversions

[t(8;21)(q22;q22), inv(16)(p13q22) or t(16;16)(p13;q22) and t(15;17)(q22;q12)] are considered as AML regardless the blast percentage (Vardiman et al. 2002; Vardiman et al. 2009).

Table 2: Classification of AML subtypes by the WHO (adapted and modified from Vardiman et al. 2002; Michl 2005).

	WHO subtype	Examples
1	AML with recurrent genetic abnormalities	t(8;21), t(15;17), inv(16)
2	AML with multilineage dysplasia	following MDS
3	AML and myelodysplastic syndromes, therapy related	alkylating agent / radiation related
4	AML not otherwise categorized	identical to FAB subtype M0 to M7
5	AML of ambiguous lineage	biphenotypic or bilinear

Diagnosis of AML is performed by cytomorphologic and -chemic analyses to identify blasts. In 25% of the cases so called “auer rods” are present which are clumps of azurophilic granular material that forms elongated needles in the cytoplasm of leukemic blasts. Immunophenotyping by multicolor flow cytometry of peripheral blood and bone marrow is used to further subclassify the blasts. Furthermore, cyto- and moleculargenetic analyses are important because chromosomal alterations and molecular abnormalities are prognostic indicators. Three general risk groups are defined by cytogenetic typing: favorable, intermediate, and adverse. Patients belonging to the favorable risk group have blasts with the translocations t(15;17) and t(8;21), respectively, or an inversion on chromosome 16, inv(16). A translocation t(9;11) and a loss of the Y chromosome are associated with intermediate risk. The adverse group includes inversions / translocations on chromosome 3 inv(3) / t(3;3) and complex karyotypes. In older patients frequently multiple chromosomal alterations as described above are detected which implies an extremely poor prognosis. AMLs with normal karyotype are commonly estimated as intermediate risk.

Molecular abnormalities are also used to define prognostic indicators in normal cytogenetic AMLs. Most frequently internal tandem duplications in the FSM-related tyrosine kinase 3 (FLT3-ITD) and myeloid / lymphoid leukemia (MLL) gene, as well as mutations in the coding region of FLT3, nucleophosmin-1 (NPM1), CCAAT / enhancer-binding protein-α (CEBPA), the ras-protein NRAS, and Wilm’s tumor-1 (WT1) genes are detected whereof FLT3-ITD, CEPBA, and NPM1 have the most prognostic significance. The heterogeneous mutations affect processes in

cellular survival, differentiation, and proliferation. The FLT3-ITD mutation occurs in circa 30% of AML patients and is strongly associated with a poor outcome. The gain-of-function mutation activates the molecule and supports proliferation and survival of the cell. Mutations in the NPM1-gene are seen in 50% to 60% of AML patients with normal karyotype and are associated with a good response to chemotherapy. It is a favorable prognosis indicator. Mutations in the transcription factor CEBPA occur in about 15% of cytogenetic normal AMLs and are associated with lower relapse rates and improved overall survival (Betz & Hess 2010).
The main treatment option of AML is chemotherapy. It starts with an induction therapy to achieve complete remission with < 5% blasts in bone marrow and recovery of marrow function. Following, a consolidation and maintenance therapy is necessary to eliminate the residual blasts and to stabilize the remission. Failure to achieve a complete remission suggests a poor prognosis. In this case allogeneic hematopoietic stem cell transplantation (HSCT) of HLA-identical unrelated or sibling donors after total body irradiation is the only available curative option (Burnett et al. 2011). Supportive approaches are donor lymphocyte infusions (DLI) of T cells from the stem cell donor to achieve remissions following relapse after HSCT (Kolb et al. 1995) and adoptive transfer of specific T-cell subsets. Main complications in the phase of immune reconstitution after HSCT are fungal infections and the reactivation of latent viruses especially cytomegalovirus, Epstein-Barr virus, herpes simplex virus, and varicella zoster virus. The graft-versus-host disease caused by donor T cells which attack tissues of the recipient also increases morbidity and mortality. A severe GVHD most often develops in the gut, skin, and liver (Hofmann & Greiner 2011).

2.3 Immunotherapy of AML

2.3.1 Graft-versus-leukemia (GVL) effect and graft-versus-host disease (GVHD)

Immunotherapy as curative option for patients with leukemia is mediated by chemotherapy with irradiation and more importantly by immunological effects of donor cells in the transplant destroying the malignant cells. This GVL effect is mostly attributed to the presence of donor T cells and the existence of histocompatibility differences between donor and recipient since the relapse rate in patients receiving T-cell depleted transplants or transplants from their complete identical twins compared to HLA-identical siblings is enhanced. Often a GVL effect is associated

with the development of GVHD, the lowest relapse rates were observed in patients with severe GVHD. But also a decreased relapse rate in patients without GVHD was observed, suggesting a separated GVL effect (Horowitz et al. 1990; Gratwohl et al. 1995; Ringdén et al. 2000). Both $CD4^+$ and $CD8^+$ T cells are implicated in the cytotoxicity to leukemic cells. Essential for the T-cell mediated GVL effect is the presence of antigens on leukemic cells that can trigger CTL responses. In case of HLA-non-identical transplants, T cells react with strong allo-responses against foreign HLA-complexes or against patient's peptides presented by HLA-molecules. The more mismatched the donor, the more potent should be the GVL response but also the more GVHD is induced which makes it harder for this approach to achieve a success of the therapy. To avoid severe GVHD, patient and donor should be matched in their HLA-ABC and -DR alleles according to high-resolution typing. In HLA-matched allogeneic settings peptides derived from single-nucleotide polymorphisms (SNPs) between donor and recipient give rise to minor histocompatibility antigens (mHags), resulting in immunological non-identity. The allo-response is directed against mHags presented to T cells on the matched HLA-molecules. If mHags are presented ubiquitously on patient's whole body tissues a GVL effect and simultaneously GVHD can be induced. Therefore, hematopoiesis-restricted or even exclusively leukemia-associated antigens (LAAs) could have the advantage of inducing GVL responses without causing GVHD. LAAs include peptides derived from chromosomal aberrations or mutations in malignant cells as well as peptides that are overexpressed or aberrantly expressed in leukemic cells. Also Y chromosome-determined peptides in male patients can be targets for T cells of female donors. Besides $CD4^+$ and $CD8^+$ T cells also NK cells and regulatory T cells have been suggested to play a role in GVL and GVHD (Barrett 2008; Ringdén et al. 2009; Barrett & Le Blanc 2010).

2.3.2 Cellular immunotherapy in AML

Allogeneic HSCT is a curative treatment option in AML. Progenitor cells from HLA-matched related or unrelated donors are the main source for transplants. Hematopoietic stem cells from the donor can be obtained by bone marrow puncture or cells can be mobilized to peripheral blood by injection of granulocyte colony-stimulating factor (G-CSF). If there is no matched donor available umbilical cord blood can be considered as stem cell source. The malignant hematopoietic system of

the patient is destroyed by chemotherapy and irradiation and is replaced by the hematopoiesis of the healthy donor. The therapeutical benefit arises from the GVL effect of donor lymphocytes eradicating leukemic stem cells and blasts. Various clinical studies showed decreased relapse rates and improved leukemia-free survival in patients undergoing allogeneic HSCT (Hill & Copelan 2010; Hofmann & Greiner 2011).

An additional treatment option for patients with relapsing disease or to enhance the GVL effect after HSCT is the donor lymphocyte infusion. Unselected lymphocytes of the transplant donor are infused to achieve re-induction of remission mediated by GVL effects but the risk to induce GVHD remains (Deol & Lum 2010). A report of DLI leading to remissions after relapse of bone marrow transplanted CML patients was first published in 1990 (Kolb et al. 1990). Further studies in the following years reported the different effect and success of the DLI treatment. In AML patients DLI was less successful but in selected patients remissions were achieved (Kolb et al. 1995; Collins et al. 1997). An allogeneic GVL effect against AML was shown in a retrospective study confirming an improved survival in patients who received DLI but only for a small group of patients, mainly those with favorable cytogenetics or low tumor burden at time of relapse (Schmid et al. 2007). Borchers *et al* showed that genetically modified DLI from HSCT donors with infusion of T cells that were transduced with a replication-deficient retrovirus expressing the herpes simplex thymidine kinase and a truncated version of a nerve growth factor receptor for selection and characterization in seven AML and two CML patients also induced a GVL effect. Only one patient developed a mild GVHD and seven of the nine patients were in complete remission after treatment (Borchers et al. 2011). The overall remission rate for patients with relapsed AML following HSCT who received DLI ranges between 15% and 42% with a two year overall survival of approximate 15% to 20%. Chemotherapy prior to DLI to reduce tumor burden appears to be beneficial (Deol & Lum 2010).

A further improvement regarding an increased GVL effect and decreased GVHD is the adoptive transfer of leukemia-specific T cells. Adoptive T-cell therapy includes the *in vitro* generation and expansion of autologous or allogeneic T cells with anti-leukemia reactivity followed by the infusion into the patient. Leukemia-specific T cells can be isolated from the patient directly after HSCT or can be generated by *in vitro* strategies (Bleakley & Riddell 2004; Parmar et al. 2011). The potential of leukemia-

reactive T cells has been demonstrated in mouse models. Human $CD8^+$ T-cell clones could inhibit engraftment of AML cells in immunodeficient mice (Bonnet et al. 1999; Distler et al. 2008; Bleakley et al. 2010). Clinical data for successful AML treatment by adoptive transfer of leukemia-reactive T cells is still rare up to now. Evidence for effective adoptive transfer arises from the treatment of a CML patient at relapse with CTL lines generated *in vitro* by stimulation with primary CML cells. After three T-cell infusions a complete eradication of leukemic cells was achieved (Falkenburg et al. 1999). Anti-leukemia activity was also observed by the same group in three CML and one AML patient with a complete response in two CML patients after transfer of leukemia-reactive CTL lines (Marijt et al. 2007). Recently, CTLs with reactivity against patient's leukemic cells but not fibroblasts were transferred in seven patients with a relapse of MDS or acute leukemia. Five patients received complete morphologic remissions after treatment but relapsed at later time points (Warren et al. 2010). These approaches indicated a general functionality of adoptive T-cell therapy but since only few patients responded long-term with a remission there is still a need to improve the efficacy of this kind of treatment.

2.3.3 Further immunotherapy approaches in AML

Besides cellular immunotherapy other immune modulating approaches have shown anti-leukemia activity. One effort is the antibody therapy where surface molecules with disease-specific expressions are targeted by passively transferred Abs. The efficiency can be further enhanced by attaching radioisotopes or drugs to the Abs that kill the malignant cells. Bispecific Abs that bind simultaneously tumor cells and activate receptors of immune effector cells can also enhance anti-tumor activity. A first proof-of-principal was provided by murine monoclonal antibodies (mAbs) targeting the IL-2 receptor expressed by many T-cell leukemias and lymphomas as well as proliferating normal T cells (Blattman & Greenberg 2004). Several mAbs against surface molecules expressed on AML cells have been investigated till now including CD33, CD45, CD47, CD64, CD66, CD123, and C-type selectin. Most promising results in AML patients have been obtained by the application of an immunoconjugate of an anti-CD33 mAb linked to the potent cytotoxic agent calicheamicin, called gemtuzumab ozogamicin (Barrett & Le Blanc 2010; Mulford 2008). Preclinical investigations with an Fc-optimized antibody against a FLT3 antigen expressed on AML cells showed antibody-mediated cytotoxicity against

FLT3-expressing cells and AML blasts without toxicity to normal cells. This antibody is currently used for treatment of patients (Hofmann et al. 2012).

Another strategy is the application of cytokines into the patient. IL-2 has a potential to boost T cell and NK cell function after induction therapy. Also IL-15, when available for clinical trials, is a promising candidate to expand lymphocytes to reduce relapse rates. Other cytokines of interest could be granulocyte macrophage colony-stimulating factor (GM-CSF) and interferon-γ to increase antigen-presentation by leukemic cells or up-regulate HLA expression on blasts leading to enhanced antigenicity (Barrett & Le Blanc 2010).

A quite promising approach to enhance the leukemia-specific T-cell pool is vaccination. Cell vaccinations with irradiated blasts and cytokines, DC generation out of patients, antigens from AML lysates loaded on DCs, or RNA-transfection for better antigen-presentation have provided only little success rates and data are still preliminary (Barrett & Le Blanc 2010). An improvement could be the antigen-specific vaccination with LAAs expressed on the surface of AML cells. In clinical trials vaccines containing the peptides $WT1_{126}$ and HLA-A24 specific WT1, PR1, as well as RHAMM / CD168 induced immune responses in patients (Schmitt et al. 2009). An overview of current immunotherapeutic strategies mentioned herein is shown in figure 2.

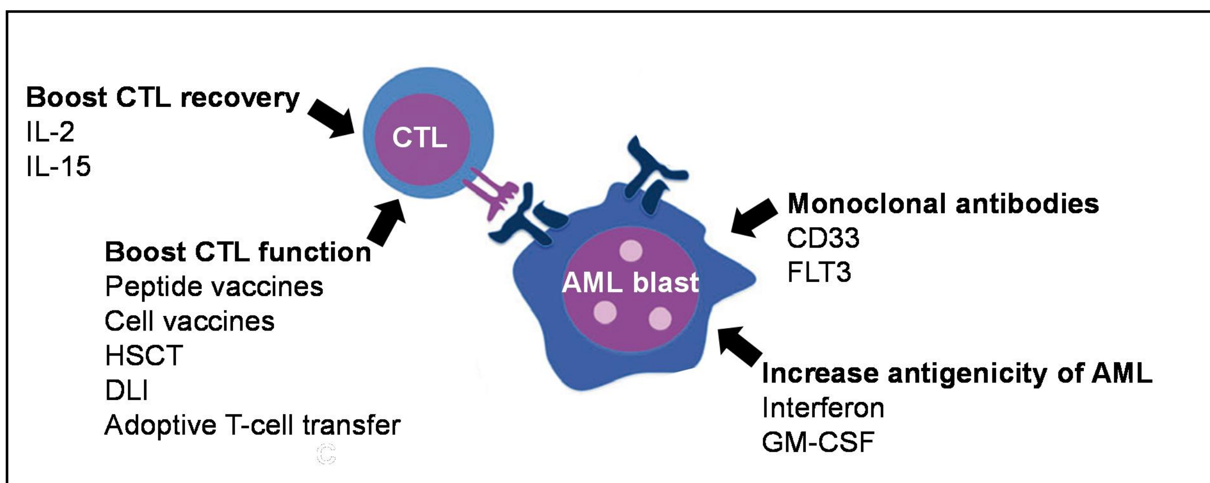

Figure 2: Selected immunotherapeutical strategies for AML treatment (adapted and modified from Barrett & Le Blanc 2010).

2.3.4 Immunoevasion of AML blasts

The immune system can target tumor cells and therefore makes an important contribution to the control of malignant diseases. This has been demonstrated impressively in animal models and leukemia patients by treatment with immunotherapeutic approaches as described above. AML cells are susceptible for immune cells because they express both, HLA-class I- and II-molecules necessary for T-cell recognition and ligands for NK-cell activation (Barrett & Le Blanc 2010). But the immune system not only protects the host from tumor development, it also puts a selection pressure on the malignant cells leading to molecular modifications. This so called “immunoediting” process often causes immunoevasion of cancer cells because malignant cells expressing highly immunogenic antigens are eliminated, whereas cells which have down-regulated molecules important for recognition by immune cells have a survival advantage (Vincent et al. 2011). The lack of important co-stimulatory molecules like CD80 and the down-regulation of complete HLA-molecules have been reported for AML cells (Brouwer et al. 2002; Whiteway et al. 2003), which leads to an insufficient presentation of antigens and therefore to a reduced T-cell recognition. Even the loss of the expression of complete mismatched HLA-molecules after haploidentical HSCT has been observed (Vago et al. 2009). Cancer cells can also produce immunosuppressing molecules. Supernatant from AML blasts has been shown to contain not yet identified inhibitory factors which suppress T-cell function and provide thereby a mechanism for evasion of T cell-mediated killing (Buggins et al. 2001). The expression of only low immunogenic LAAs on leukemic cells which do not stimulate a sufficient immune response is another example how leukemia cells evade recognition by immune cells (Vincent et al. 2011).

2.3.5 Approaches to separate GVL effect of T cells from GVHD

In leukemia patients the curative immune response by immunotherapy mainly relies on leukemia-eliminating T cells that develop from adoptively transferred donor transplants *in vivo* (Falkenburg et al. 2008; Kolb 2008). Although this GVL effect is a frequently observed clinical event, it is strongly associated with unwanted GVHD against non-hematopoietic recipient tissues. Therefore, recent approaches in immunotherapy focus on strategies to separate GVL responses from GVHD and to augment the GVL effect (Barrett 2008). One strategy is the *in vitro* generation and expansion of T cells which are reactive to mHags or LAAs expressed on

hematopoietic or leukemia cells but not on normal recipient tissues. A subset of these special antigens capable to induce T-cell responses has been identified. These include the Wilm's tumor protein (WT-1) overexpressed in many leukemic cells, the serine protease proteinase 3 (PR1) and neutrophil elastase which are overexpressed in AML and CML. Further, the human telomerase reverse transcriptase (hTERT), the apoptosis inhibitor survivin overexpressed in a wide range of malignancies as well as the immature laminin receptor protein (OFA-1 LRP) and the receptor for hyaluronic acid-mediated motility (RHAMM / CD168), both aberrantly expressed in myeloid leukemias (O'Reilly et al. 2010) are described to function as antigens for immunreactive T-cell response. Moreover, the hematopoiesis-restricted mHags HA-1 and HA-2 derived from a SNP in autosomal genes whose immunogenic peptides are presented by HLA-A*02:01, can also trigger CTL responses. Other examples for mHags potentially expressed on AML cells which may be useful targets for segregating GVL effects from GVHD are peptides derived from bcl-2 family members of anti-apoptotic genes BCL2A1 (ACC-1 and ACC-2), presented by HLA-A*24:02 or -B*44:03, and the purineric ATP-gated non-selective cation channel P2X5 (LRH-1), presented by HLA-B*07:02. Donor T cells specific for listed antigens could be amplified from patients after HSCT (Bleakley & Riddell 2011). Furthermore, anti-leukemic effects of CTL clones specific for HA-1 were observed after transfer in NOD / SCID mice that were engrafted with leukemic cells from a CML patient (Hambach et al. 2006). In a phase I study, relapsing leukemia patients were adoptively transferred with T-cell clones specific for hematopoiesis-restricted mHags which were expanded *in vitro*. Transferred T cells infiltrated the bone marrow and showed anti-leukemic activity but the persistence was only short termed. Some patients suffered from pulmonary toxicity because of unexpected expression of the mHags in lung tissue (Warren et al. 2010), giving an example for existing difficulties.

A second strategy includes modification of T cells before application into the recipient. The transfer of genes for tumor- or even LAA-specific TCRs into an appropriate T-cell population may improve safety because of well-characterized reactivity. In a mouse model T cells engineered to express WT-1-specific TCRs were able to protect against the growth of autologous leukemia progenitor cells (Xue et al. 2010). Also the expression of chimeric-antigen receptors (CARs) which link the antigen-binding domain of an antibody with the signaling component of a TCR is a promising approach. T cells electroporated with mRNA for CARs against CD19

expressed on lymphatic lymphomas as well as a subset of AMLs was given to immunodeficient mice bearing xenografted leukemia. T cells rapidly migrated to sites of disease and reduced leukemic burden resulting in a significant prolongation of survival (Barrett et al. 2011). In a clinical study the reinfusion of autologous T cells expressing CARs specific for CD19 in one CLL patient mediated remission for at least ten month after treatment. Engineered cells persisted for six months in the blood and bone marrow and a specific immune response was detected, accompanied by loss of normal B cells and leukemia cells that express CD19 (Porter et al. 2011). Another promising target structure for CARs is the myeloid transmembrane receptor CD33 which is expressed on the surface of leukemic blasts in many AML patients. Administration of modified EBV-specific T cells with CARs targeting CD33 into AML engrafted NOD / SCID mice resulted in antileukemic activity after T cells reached the tumor sites (Dutour et al. 2012). To prevent severe GVHD, a tool to switch-off T cells is the introduction of suicide genes which can be activated with drugs. Some safety trials have shown promising results so far (Ringdén et al. 2009).

A third approach concerns the enrichment of leukemia-reactive T cells or the depletion of alloreactive CTLs from the transplant. To eliminate potentially GVHD inducing CTLs total $CD8^+$ T lymphocytes were depleted from DLI products. Clinical trials with leukemia patients suggested an immune reconstitution and clinical responses after transplantation in association with a lower risk of inducing severe GVHD (Alyea et al. 2004; Meyer et al. 2007; Meyer et al. 2010; Soiffer et al. 2002). A more differentiated approach is the use of distinct T-cell populations for transplantation. By separating T-cell subsets according to their differentiation state it was demonstrated in murine and primate models that most antitumor and antiviral activity was present in the naive / central memory compartment after adoptive transfer (Berger et al. 2008; Hinrichs et al. 2009; Klebanoff et al. 2005). In mouse models AML-reactive CTL clones generated from the naive / central memory compartment could inhibit AML-engraftment by co-incubation prior to AML injection (Bleakley et al. 2010; Distler et al. 2008). Lately, memory stem T cells with a phenotype similar to naive T cells have been described to mediate superior antitumor responses in humanized NSG mice compared to other memory populations after transduction with antitumor-TCRs (Gattinoni et al. 2011). Unfortunately, the precursor frequency of leukemia-reactive T cells is very low in healthy individuals (Alanio et al.

2010; Smit et al. 1998). Amplifying these cells to numbers sufficient for adoptive immunotherapy still requires extensive *in vitro* expansion over several weeks. However, long-term culture of antigen-specific T cells is accompanied by terminal differentiation and functional exhaustion due to replicative senescence. There is strong evidence from animal models and clinical studies that T cells with such a late-stage phenotype fail to persist upon adoptive transfer *in vivo* (Berger et al. 2009; Gattinoni et al. 2005; Klebanoff et al. 2005).

2.4 Motivation and aim of this study

Treatment of chemo-refractory acute myeloid leukemia (AML) has progressed since curative graft-versus-leukemia (GVL) effects can still be induced by allogeneic hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) mainly due to the presence of donor-derived T cells which destroy leukemic cells. However, these therapies are associated with significant treatment-related morbidity and mortality, including graft-versus-host disease (GVHD) which is also induced by donor-derived T cells attacking recipient's healthy tissues. To reduce the severity of GVHD, grafts can be T-cell depleted but this approach is correlated with increased relapse rates because of insufficient GVL responses. Therefore, the need for specific anti-leukemia immunotherapy that directly targets AML cells arises to maintain the GVL effect and reduce the toxicity against healthy tissues (Smits et al. 2011). A method has been previously established in our group to generate $CD8^+$ T cells of healthy donors with patient-specific reactivity to AML blasts under clonal conditions *in vitro*. The use of primary AML blasts with complete HLA-class I-match assures the stimulation of donor T cells against hematopoietic minor histocompatibility antigens (mHags) as well as leukemia-associated antigens (LAAs) presented by recipient AML cells and avoids recognition of mismatched HLA-molecules which are preferred target structures of GVHD (Distler et al. 2008). The aim of this study was to improve the protocol regarding a more efficient generation of AML-reactive $CD8^+$ cytotoxic T lymphocytes (CTLs) to avoid long-term culture and terminal-differentiation of the cells which would be favorable in adoptive transfer approaches. Therefore, a T-cell subset that contains most AML-reactive precursors should be identified to improve the conditions for the outgrowth of leukemia-reactive CTLs. Furthermore, with the use of this effective starting population the impact of the common γ-chain cytokine interleukin (IL)-21, recently described by others to promote the development of

antigen-specific $CD8^+$ T cells (Li et al. 2005) and to sustain a central memory-like phenotype (Alves et al. 2005; Kaka et al. 2009) was thought to be analyzed. Ideally, large numbers of CTLs with sustained effector functions towards AML blasts recognizing hematopoiesis-restricted mHags or LAAs but not normal self-antigens should be expanded. This could allow the induction of GVL effects without causing severe GVHD upon adoptive transfer into the patient. Persistent expression of important markers for co-stimulation and homing on the surface of T cells would be advantageous to ensure survival as well as migration of CTLs into AML infiltrated organs where they should destroy the leukemic blasts. In addition to the requirement for efficient *in vitro* protocols to generate AML-reactive T cells also humanized mouse models are needed to analyze the anti-leukemic potential of adoptively transferred T cells in a pre-clinical setting *in vivo*. Until now, only data demonstrating inhibited engraftment of AML blasts in mice after *in vitro* pre-incubation of CTLs with leukemic cells were published (Bonnet et al. 1999; Distler et al. 2008; Bleakley et al. 2010). In another project of our group the engraftment of primary AML blasts in immunodeficient NOD / SCID / $IL2R\gamma c^{null}$ (NSG) mice was successfully established just recently followed by transfer experiments with HLA-mismatched donor CTL lines (Brunk 2011). Thus, one major aim of this study was to use this mouse model to show the biological significance of the herein generated CTLs in mice with an established leukemic hematopoiesis concerning homing and persistence of adoptively transferred CTLs as well as their anti-leukemic activity *in vivo*.
The successful generation of CTLs recognizing AML antigens in a patient-specific manner and the proof of their anti-leukemic responses *in vivo* would be a valuable basis for the development of therapeutic strategies to stimulate individual anti-leukemia responses in HLA-identical AML patient / healthy donor pairs. This would make leukemia-reactive CTLs already available at the time point of HSCT when patients only have minimal residual disease and the chances for complete leukemia eradication are high. Furthermore, leukemia-reactive CTLs effectively expanded by this *in vitro* protocol can be used as screening populations to identify LAAs and mHags for antigen-specific immunotherapy.

3. Material and Methods

3.1 Cell culture

Culture of eukaryotic cells was performed according to standard procedures under sterile conditions. All cells were cultivated and incubated at 37°C, 5% CO_2, and 100% relative air humidity.

Cell counting was performed in a counting chamber using trypan blue solution. Trypan blue is a diazo dye which is excluded by vital cells due to their intact cell membrane. Vital cells appear bright, while dead cells are blue in phase contrast microscopy.

Centrifugation means a 5 min centrifugation at 470x g at room temperature using the built-in brake, unless otherwise indicated.

3.1.1 Materials for cell culture

3.1.1.1 Substances for cell culture

Substance	Manufacturer
AIM-V medium	Gib*
Cyclosporin A (Sandimmun 50 mg Konzentrat)	Nov
Ciprofloxacin	Nov
Dimethylsulfoxide (DMSO)	Rot
DNase I	Roc
Fetal calf serum (FCS)	PAA
Ficoll lymphocyte separation medium LSM1077	PAA
Heparin-natrium 25000	Rat
Human albumin	Beh
MEMα medium	Gib
Penicillin / Streptomycin (10 000 U / ml Pen+ 10 000 µg / ml Strep)	Gib
Phosphate buffered saline (PBS)	Gib
Phytohaemagglutinin (PHA)	Mur
RPMI 1640 + 1% L-glutamine medium	Gib
Trypsin-EDTA (0.05%)	Gib
Trypan blue	Mer

Human serum was isolated after informed consent from healthy blood donors, pooled, sterile-filtrated (0.22 µm), heat inactivated for 30 min at 56°C, and stored at -80°C.

**All following abbreviations of manufacturers are specified in chapter 3.10*

3.1.1.2 Cell-culture media

T-cell medium	AIM-V 10% human serum
Cell-culture medium	RPMI 1640 10% FCS 1% Pen / Strep
LCL medium	RPMI 1640 20% FCS 1% Pen / Strep
T-blast medium	AIM-V 5% human serum
Fibroblast medium	MEMα 20% FCS 1% Pen / Strep 1% L-glutamine
General freezing medium	AIM-V 8% human albumin 10 IU / ml heparin 10% DMSO (added prior to use)
LP freezing medium	AIM-V 8% human albumin 10 IU / ml heparin 0.1 mg / ml DNase I 10% DMSO (added prior to use)

3.1.1.3 Solutions and buffers for cell culture

Trypan blue solution	2 g / l trypan blue in H_2O, diluted 1:4 with 150 mM saline solution

3.1.1.4 Cytokines and materials for cell culture

Interferon gamma (human IFN-γ)	RD
Interleukin 2 (human IL-2, Proleukin^{TM})	Nov
Counting chamber (Fuchs-Rosenthal)	Mar

3.1.2 Freezing, thawing and storage of cells

For cryo-preservation in liquid nitrogen, cells were frozen in portions of 2 to $10x10^6$ (T cells) or 0.5 to $2x10^8$ (cell lines, PBMCs, AML blasts) in 1 ml of general freezing medium. Cryo tubes were transferred in cryo boxes and stored over night at -80°C before they were placed into the cryobank.

Cryo preservated cells were thawed in AIM-V or RPMI 1640. To remove DMSO, cells were centrifuged, thawing medium was discarded, cells were counted in trypan blue solution, and were seeded in fresh medium or used for further isolation procedures.

3.1.3 Isolation of PBMCs by Ficoll density centrifugation

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors and from leukapheresis products or peripheral blood samples of AML patients. 15 ml ficoll lymphocyte separation medium was preloaded in a 50 ml falcon tube. Blood was diluted with PBS and 35 ml were layered carefully over the separation medium. Ficoll tubes were centrifuged without brake for 20 min at 836x g. The PBMCs concentrate in the interphase between the plasma and the separation solution. This white layer was collected and washed with cold PBS with subsequent centrifugation for 10 min at 680x g. Afterwards another washing step with centrifugation for 5 min at 470x g followed. Before this centrifugation step, an aliquot of the cell solution was used for cell counting. After centrifugation, the supernatant was discarded and PBMCs were frozen in general freezing medium at 0.5-2x10^8 cells per ml in cryo tubes.

3.1.4 Cryo-preservation of leukaphereses

Alternatively to Ficoll density centrifugation, leukapheresis products from patients were frozen without additional separation. An aliquot of the cell suspension was diluted for counting. Leukemic cells were frozen directly in LP freezing medium in approx. 200 portions of 0.5 to 2x10^8 cells per ml in cryo tubes.

3.1.5 Cryo-preservation of bone marrow

Bone marrow from patients was available when aspiration was necessary for diagnostical purpose. Ficoll densitiy centrifugation was performed to isolate PBMCs as described above and cells were frozen in general freezing medium at 0.5 to 2x10^8 cells per ml in cryo tubes.

3.1.6 Overnight culture of AML blasts

AML blasts were thawed and pre-incubated at 1 to 3x10^6 cells per ml in T-cell medium overnight before using them as stimulator or target cells. Reason for this was

a better vitality of cells and in some cases a higher expression of co-stimulatory and HLA-class I-molecules at the surface of the leukemic cells afterwards.

3.1.7 Primary cell culture of patients and healthy donors

Primary AML blasts of patients were isolated from leukaphereses or peripheral blood samples. When available also bone marrow was restored. Patients were newly diagnosed with AML M1, M4, M5, or M5b according to the FAB classification (Bennett et al. 1985). All patients except two (MZ169, MZ728) had a white cell count exceeding 10^5 / µl at primary diagnosis consistent with unfavorable disease prognosis (Delmer et al. 1989). Peripheral blood mononuclear cells (PBMCs) of healthy donors were isolated from buffy coats provided by the Center for Blood Transfusion (University Medical Center of the Johannes Gutenberg University, Mainz) and T cells isolated from these PBMCs were used in *in vitro* experiments as responder cells. Patients and healthy donors were HLA-class I-matched according to high-resolution HLA-typing performed from genomic DNA by PCR with class-I-specific primers (Dr. B. Thiele, Institute for Immunology and Genetics, Kaiserslautern). In the case of a sibling donor, serological typing by the Center for Blood Transfusion (Mainz) was sufficient. The study was approved by the local ethics committee. Informed consent was obtained from participants in accordance with the Declaration of Helsinki.

3.1.8 Generation and cultivation of LCLs

For the generation of Epstein Barr-Virus (EBV)-transformed B lymphoblastoid cell lines (LCLs), 10^7 PBMCs were incubated for 5 h at 37°C with 0.5 ml of EBV-containing supernatant from B95.8 cells. After incubation, 4.5 ml LCL medium was added and cells were transferred in a culture flask. Additionally, the medium was supplemented with cyclosporin A (1 µg / ml) to inhibit T-cell growth and the culture was incubated for 24 h. Afterwards, cells were washed to remove virus particles and cyclosporin A, and cells were seeded in 5 ml LCL medium. Growing LCLs were expanded in cell-culture medium. Cell counts were determined in trypan blue solution twice a week and LCLs were seeded in fresh medium at a density of 0.4×10^6 cells per ml. LCLs were used as target cells in ELISpot- and ^{51}Cr-release assays.

3.1.9 Generation of PHA-activated T blasts

For the generation of phytohemagglutinin (PHA)-activated T blasts (T blasts) $1x10^6$ PMBCs per well in 24-well plates were seeded in 2 ml T-blast medium. PHA (1 µg / ml) and IL-2 (250 IU / ml) were added to stimulate T-cell proliferation. Cells were splitted with fresh IL-2 containing medium when necessary. T blasts were frozen when cells had a blast-like morphology between day 8 and day 12 of culture. Thawed T blasts were used as target cells in ELISpot assays.

3.1.10 Generation of stromal fibroblasts

Stromal fibroblasts were generated from bone marrow aspirates of AML patients according to a previously published protocol (Nonn et al. 2008). Bone marrow was seeded at 5 to $10x10^6$ cells per small sized cell culture flask in fibroblast medium. When necessary, ciprofloxacin (5 µg / ml) was added. After one week of incubation, 50% of the medium was exchanged with fresh medium. In full grown flasks cells were detached using 0.05% Trypsin-EDTA and seeded in large culture flasks. The non-hematopoietic and stromal origin was analyzed by flow cytometry confirming the surface phenotype $CD45^-$, $CD33^-$, $CD14^-$, $CD90^+$, and $D7\text{-}Fib^+$. Fibroblasts also regularly expressed strong levels of HLA-class I-molecules. Pre-treatment of fibroblasts with interferon-γ (500 IU / ml) for 72 h was performed when indicated to further enhance expression of HLA-class I-molecules.

3.1.11 Cell lines

3.1.11.1 K562 cell line

The K562 cell line was originally isolated from a patient with chronic myeloid leukemia (Lozzio & Lozzio 1975) and serves as a NK-cell target because it shows only a very low expression of HLA-class I- and -class II-molecules on the cell surface (Ziegler et al. 1981). It was therefore used as control target for antigen-specific $CD8^+$ T cells to exclude NK-activity. Cells were cultured at $4x10^5$ cells / ml in cell culture medium.

3.1.11.2 T2 cell line

The mutant T2 cell line was derived from a hybrid of the B-cell line 174 and the T-lymphoblastoid cell line CEM (Salter & Cresswell 1986). Cells express only small

amounts of HLA-A02 and other HLA-molecules on their surface due to the lack of a functional TAP-heterodimer (Young et al. 1998). The TAP-deficiency leads to insufficient peptide-loading of the HLA-molecules (Smith & Lutz 1996). Therefore, these cells can be used to exogenously load peptides on their surface. Cells were cultured at $4x10^5$ cells / ml in cell culture medium.

3.2 Magnetic-Activated Cell Sorting (MACS)

3.2.1 Principle of MACS

The isolation of distinct cell populations by MACS™ technology (Mil) is based on magnetic MicroBeads that are coupled to mAbs specific for cell surface molecules. Cells are incubated with the MicroBeads directly or first with biotin-coupled mAbs followed by anti-biotin MicroBeads. Cells are separated on a MACS-column in a magnetic field which retains the labeled cells in the column. Unlabeled cells are washed through. The labeled cell fraction is eluted after removing the column from the magnetic field.

3.2.2 Materials for MACS

3.2.2.1 Substances for MACS

Bovine serum albumin (BSA)	Sig
Ethylendiaminetetraacetic acid (EDTA)	Sig
Phosphate buffered saline (PBS)	Gib

3.2.2.2 Solutions and buffers for MACS

MACS-buffer	5 g / l BSA in PBS 2 mM EDTA

3.2.2.3 Kits and materials for MACS

CD8 MicroBeads	Mil
CD14 MicroBeads	Mil
Naive $CD8^+$ T-Cell Isolation Kit	Mil
LS and MS columns and preseparation filter	Mil
MiniMACS and MidiMACS Separator	Mil
MACS Multistand	Mil

3.2.3 Isolation of naive $CD8^+$ T cells

Naive T cells were isolated from PBMCs by a two-step procedure using the Naive $CD8^+$ T-Cell Isolation Kit (Mil). First non-naive and NK cells expressing $CD45RO^+$, $CD56^+$, $CD57^+$, and $CD244^+$ were depleted with a Biotin-Antibody Cocktail and Anti-Biotin MicroBeads. Afterwards, $CD8^+$ T cells were enriched from the naive unlabeled population by direct labeling with CD8 MicroBeads.

The isolation was performed according to manufactures instructions. Non-naive cells were depleted on LD columns starting with approx. 1 to $1.5x10^8$ PBMCs. Cells were thawed, resuspended in 40 µl MACS-buffer and incubated for 10 min on ice with 10 µl of the Naive CD8 T Cell Biotin-Antibody Cocktail per 10^7 cells. Following, 30 µl MACS-buffer and 20 µl Anti-Biotin MicroBeads per 10^7 cells were added and incubated for 15 min on ice. Cells were washed with 2 ml MACS-buffer per 10^7 cells and were resuspended in 500 µl MACS-buffer up to 10^8 cells. Cells were separated on the column including two washing-steps. The flow through that contains the naive T-cell population was collected. The column-bound non-naive fraction was eluted and was used as feeder-cells in MLLCs.

Next, $CD8^+$ T cells were positively isolated from the naive fraction on LS or MS columns, respectively. Therefore, naive cells were absorbed in 80 µl MACS-buffer per 10^7 cells, were labeled with 20 µl CD8 MicroBeads per 10^7 cells, and were incubated on ice for 15 min. Cells were separated on the column including two washing-steps. The flow through that contains naive $CD8^-$ T cells was collected and used as feeder cells in MLLCs. The column-bound naive $CD8^+$ T cells were eluted and used as responder cells in MLLCs. Purity of isolated naive $CD8^+$ T cells was analyzed by flow cytometry. Additional co-stainings were performed including following markers: CD3, CD4, CD8, CD16, CD27, CD28, CD45RO, CD45RA, CD56, CD57, CD62L, CD95, CD127, CCR7, and CXCR4.

3.2.4 Isolation of total $CD8^+$ cells

$CD8^+$ T cells were isolated from PBMCs by direct labeling with CD8 MicroBeads (Mil) and were separated on LS or MS columns, respectively, as described above. The flow through fraction of $CD8^-$ cells was used as feeder-cells in MLLCs. Purity of isolated $CD8^+$ T cells was analyzed by flow cytometry using mAbs against CD3, CD4, CD8, CD16, and CD56.

3.2.5 Isolation of CD14+ cells

Monocytes were isolated from PBMCs by direct labeling with CD14 MicroBeads (Mil) as recommended in the manufacturer's instruction. Cells were labeled with 20 µl CD14 MicroBeads per 10^7 PMBCs and separated on MS columns. Purity of the isolated monocytes was analyzed by flow cytometry staining against CD14.

3.3 Flow Cytometry (FACS)

3.3.1 Principle of FACS

Flow cytometry was used to characterize cells according to their surface molecules and to detect their viability. The method is based on scattering light of cells and on Abs labeled with fluorophores whose emission light is measured. Simultaneously, relative size, relative granularity, and relative fluorescence intensity can be detected. Cells are labeled with fluorophore-linked mAbs or other fluorescent molecules and are directed along extinction-lasers. The scattered and emitted light is detected and the cells can be classified to populations with distinct phenotypes.

3.3.2 Materials for FACS

3.3.2.1 Substances for FACS

Bovine serum albumin (BSA)	Sig
DNase I	Roc
Ethylendiaminetetraacetic acid (EDTA)	Sig
Formaldehyde (37%)	Mer
Phosphate buffered saline (PBS)	Gib

Human serum was isolated after informed consent from healthy blood donors, pooled, sterile-filtrated (0.22 µm), heat inactivated for 30 min at 56°C, and stored at -80°C.

3.3.2.2 Solutions and buffers for FACS

FACS-buffer	PBS 1 g / l BSA
FACS-fix	PBS 1% formaldehyde
Sort-buffer	PBS 1% human serum 2 mM EDTA

3.3.2.3 Antibodies for FACS

Table 3: Overview of flurophore-conjugated antibodies used to stain cells for flow cytomety.

Marker	Fluorophore	µl / tube	Mfr. (Cat. no.)
CD3	FITC	3	BC (A 07746), BD (555332)
	PE	4	BC (A 07747), BD (555333)
	APC	3	BC (IM 2467), BD (555335)
	Horizon (V450)	1:5, 2	BD (560365)
	PC5	2	BC (A 07749)
CD4	FITC	3	BC (A 07750), BD (555346)
	PE	2	BC (A 07751), BD (555347)
	APC	2	BC (IM 2468), BD (555349)
	PC5	2	BC (A 07752)
CD8	FITC	5	BC (A 07756), BD (551347)
	PE	3	BC (A 07757), BD (555367)
	APC	2	BC (IM 2469), BD (555369)
	Horizon (V450)	1:10, 2	BD (560347)
	PC5	2	BC (A 07758)
	PerCP	8-10	BD (345774)
CD11c	PE	2	BD (555392)
CD14	FITC	2	BC (IM 0645U), BD (555397)
	PE	5	BC (A 07764)
CD16	FITC	5	BC (IM 0814U), BD (555406)
CD25	FITC	10	BD (555431)
CD28	PE	10	BC (IM 2071), BD (555729)
CD33	PE	5	BC (A07775), BD (555450)
CD45	FITC	2	BC (A 07782), BD (555482)
	PE	2	BC (A 07783), BD (555483)
	APC	2	BD (555485)
CD45RA	PE	10	BC (IM 1834U)
	APC	5	BD (550855), Mil (130-092-249)
CD45RO	PE	5	BC (A 07787), BD (555493)
	APC	3	BD (559865)
CD54	PE	5	BD (555511)
CD56	PE	5	BC (A 07788), BD (555516)
CD57	APC	10	Mil (130-092-141)
CD58	FITC	5	BC (IM 1218)
CD62L	PE	10	BC (IM 2214U), BD (555544)
CD69	PE	20	BD (555531)

CD80	FITC	5	BC (IM 1853)
CD83	PE	5	Mil (130-094-181)
CD86	PE	3	BC (IM 2729U)
CD90	PE	5	BC (IM 1840)
CD95	PE	10	BC (IM 1739), BD (555674)
CD127	FITC	20	eB (11-1278-73)
	PE	10	BC (IM 1980U)
CD184 (CXCR4)	APC	10	BD (555976)
CD197 (CCR7)	FITC	5	RD (FAB 197 F)
HLA-ABC	FITC	2	BD (555552)
	PE	5	BD (555553)
HLA-DR	PE	5	BC (IM 1639)
	APC	5	BD (559866)
TCR PAN αβ	PE	10	BD (555548)
TCR PAN γδ	FITC	10	BD (559878)
	APC	10	BD (555718)
Vß1	FITC	10	BC (IM 2406)
	PE	10	BC (IM 2355)
Vß2	PE	10	BC (IM 2213)
Vß3	FITC	10	BC (IM 2372)
Vß4	PE	10	BC (IM 3602)
Vß5.1	PE	10	BC (IM 2285)
Vß5.2	FITC	10	BC (IM 1482)
Vß5.3	PE	10	BC (IM 2002)
Vß7.1	FITC	10	BC (IM 2408)
Vß7.2	PE	10	BC (IM 3604)
Vß8	PE	10	BC (IM 1233)
Vß9	PE	10	BC (IM 2003)
Vß11	FITC	10	BC (IM 1586)
Vß12	PE	10	BC (IM 2291)
Vß13.1	PE	10	BC (IM 2292)
Vß13.2	PE	10	BC (IM 3603)
Vß13.6	FITC	10	BC (IM 1330)
Vß14	FITC	10	BC (IM 1558)
Vß16	FITC	10	BC (IM 1560)
Vß17	PE	10	BC (IM 2048)
Vß18	PE	10	BC (IM 2049)
Vß20	FITC	10	BC (IM 1562)
Vß21.3	FITC	10	BC (IM 1483)
Vß22	PE	10	BC (IM 2051)
Vß23	PE	10	BC (IM2004)
IgG1	FITC		BC (A 07795), BD (555748)
	FITC / PE		BC (A 07794)
	PE		BC (A 07796), BD (555749)
	APC		BC (IM 2475), BD (555751)
	PerCP		BD (345817)
	Horizon (V450)		BD (560373)
IgG2a	FITC		BD (349051)
	PE		BC (A 09142)

Class I iTAg MHC Tetramer (A*02:01, Melan A, ELAGIGILTV)	PE	2	BC (T 01008)
7-AAD		5	BD (559925)

Table 4: Overview of unlabeled antibodies used to stain cells for flow cytometry.

Antibody	Species	Source / Mfr.
w6 / 32	mouse-anti-human	hybridoma supernatant
B1.23.2	mouse-anti-human	hybridoma supernatant
PA2.1	mouse-anti-human	hybridoma supernatant
GAP-A3	mouse-anti-human	hybridoma supernatant
L243 (HB55)	mouse-anti-human	hybridoma supernatant
D7-Fib	mouse-anti-human	BZ (BZL01717)
Secondary mAbs	goat-anti-mouse-FITC, -PE	Imt (IM 1621, IM 0551)

3.3.2.4 Kits and materials for FACS

IOTest® Beta Mark Kit	BC
PE Apoptosis Detection Kit I (Annexin-V)	BD
Falcon 5 ml, polystyrene tubes	BD
FACS Clean Solution	BD
FACS Flow Sheath Fluid	BD
FACS Rinse Solution	BD
FACS Shutdown Solution	BD

3.3.3 Cell surface marker analysis

For direct staining FITC-, PE-, APC-, PC5-, PerCP-, or Horizon (V450)-conjugated mAbs for indicated antigens were used. Cells were washed with FACS-buffer and 0.5 to $1x10^5$ cells per tube were stained for 15 min at 4°C with optimal amount of conjugated mAbs according to titration results as listed above. Tetramer-staining was performed with 2 µl tetramer under the same conditions but with an incubation time of 30 min at room temperature. Afterwards, cells were washed with 1 ml FACS-buffer and were resuspended in 0.2 ml FACS-fix for fixation.

For indirect staining 0.5 to $1x10^5$ washed cells per tube were incubated with 50 µl of hybridoma supernatants containing the respective murine monoclonal antibody for 30 min at 4°C. Cells were washed and stained with secondary FITC- or PE-labeled goat-

anti-mouse IgG (1:200 in PBS) for another 30 min at 4°C. Cells were washed and subsequently fixed in 0.2 ml FACS-fix.

When apoptotic or dead cells were analyzed, 7-AAD and Annexin-V-PE were used after staining cells with conjugated mAbs. Therefore, cells were resuspended in 100 µl Binding-Buffer (1:10 in aqua dest) as recommended in the manufacturer's instruction of PE Apoptosis Detection Kit I (BD). Cells were incubated with 5 µl 7-AAD and 5 µl Annexin-V for 15 min at room temperature. Another 100 µl Binding-Buffer (1:10 in H_2O) were added before measuring immediately.

Analyzes were performed using the flow cytometer BD FACSCanto II. Viable cells were gated in FSC / SSC- Channel and 10^4 events were measured with the BD FACS DIVA software. For further re-analysis EXPO™32 Software (BC) was used. To evaluate expression levels of distinct markers, the relative fluorescence intensity was calculated from median fluorescence intensity (MFI) values of relevant stainings divided by MFI values of the respective IgG isotype control stainings. Statistical data analysis was conducted with SPSS-15 / 17 software. Mann-Whitney *U* test was performed when appropriate.

3.3.4 T-cell receptor Vβ chain analysis

T-cell receptor Vβ chains of antigen-specific $CD8^+$ T cells were determined using mAbs recognizing 24 different Vβ families as listed below. Vß chains were directly stained with single mAbs as described above or the IOTest® Beta Mark Kit (BC) was used. With the kit a detection of 3 Vβ expressions in the same tube is possible by combining three mAbs with two fluorophores. A first mAb is FITC-conjugated, a second one is PE-conjugated, and a third one is a mixture of a PE- and a FITC-conjugated form. Antigen-specific T cells were co-stained with APC- or PC5-conjugated anti-CD8 mAbs to exclusively analyze the Vß-expression on $CD8^+$ T cells. Therefore, 0.5-1x10^5 washed T cells per tube were incubated with anti-CD8 mAbs and simultaneously with 20 µl of each Vß-combination (tube A-H) for 30 min at room temperature in the dark. Subsequently, cells were washed with 1 ml FACS-buffer and were fixed in 0.2 ml FACS-fix. Analyzes were done using a BD FACSCanto II flow cytometer with BD FACS DIVA software. For further re-analysis EXPO™32 Software (BC) was used.

Table 5: Overview of flurophore-conjugated antibodies against different TCR Vß-chains contained in the IOTest® Beta Mark Kit (BC).

Tube	Vβ family mAb	Fluorophore	Isotype (species)
A	Vβ5.3 Vβ7.1 Vβ3	PE FITC / PE FITC	IgG1 (mouse) IgG2a (mouse) IgM (mouse)
B	Vβ9 Vβ17 Vβ16	PE FITC / PE FITC	IgG2a (mouse) IgG1 (mouse) IgG1 (mouse)
C	Vβ18 Vβ5.1 Vβ20	PE FITC / PE FITC	IgG1 (mouse) IgG2a (mouse) IgG (mouse)
D	Vβ13.1 Vβ13.6 Vβ8	PE FITC / PE FITC	IgG2b (mouse) IgG1 (mouse) IgG2a (mouse)
E	Vβ5.2 Vβ2 Vβ12	PE FITC / PE FITC	IgG1 (mouse) IgG1 (mouse) IgG2a (mouse)
F	Vβ23 Vβ1 Vβ21.3	PE FITC / PE FITC	IgG1 (mouse) IgG1 (rat) IgG2a (mouse)
G	Vβ11 Vβ22 Vβ14	PE FITC / PE FITC	IgG2a (mouse) IgG1 (mouse) IgG1 (mouse)
H	Vβ13.2 Vβ4 Vβ7.2	PE FITC / PE FITC	IgG1 (mouse) IgM (rat) IgG2a (mouse)

3.3.5 Flow cytometric cell sorting

To isolate distinct CD8$^+$ T-cell subsets that differ in their expression of surface markers, PBMCs were sorted by FACS. PBMCs were stained with anti-CD3 and anti-CD8 mAbs to gate this T-cell population. Additionally, surface markers to distinguish between CD8$^+$ subsets including CD45RA, CD45RO, CD62L, or CCR7 were stained. Cells were washed with FACS-buffer and were incubated with the appropriate mAbs (50 µl / 10^8 cells) for 15 min on ice. Afterwards, cells were washed and resuspended in sort-buffer. To exclude clump of dead cells the suspension was filtered and when necessary 0.25 to 1 mg / ml DNase I was added. For sorting-procedure 1.5 to 2x10^7 cells per ml was the optimal concentration. Sorting and re-analysis was done using a BD FACSAria cell sorter with BD FACS DIVA software in collaboration with the Flow Cytometry Core Facility (Dep. of Med. III, University Medical Centre Mainz). Sorted cell populations were used as responder cells in MLLCs.

3.4 Enzyme-Linked Immunosorbent Spot (ELISpot) assay

3.4.1 Principle of ELISpot

ELISpot assay was used to show cytokine production of $CD8^+$ T cells in response to stimulation with target cells. Therefore, capture antibodies against an epitope of the designated cytokine are bound to hydrophobic membranes in 96-well ELISpot plates. T cells and stimulator cells are co-incubated in the wells. Released cytokines bind to the immobilized Abs and can be detected using biotinylated detection Abs against a second epitope of the cytokine that interacts with an avidin / horse radish peroxidase (HRP)-complex. The enzyme converts its substrate in a color reaction making a colored spot visible for each cytokine releasing lymphocyte. The number of spots is showing the frequencies of reacting T cells in the culture.

3.4.2 Materials for ELISpot

3.4.2.1 Substances for ELISpot

Acetic acid	Rot
3-amino-9-ethylcarbazole (AEC) tablets	Sig
AIM-V medium	Gib
Bovine serum albumin (BSA)	Sig
N,N-dimethylformamid (DMF)	Rot
Ethanol (>99%, EtOH)	Rot
Hydrogen peroxide (30%, H_2O_2)	Sig
Natrium acetat	Sig
Phosphate buffered saline (PBS)	Gib
PBS dry chemical “Instamed”	Bic
Tween20	App

Human serum was isolated after informed consent from healthy blood donors, pooled, sterile-filtrated (0.22 µm), heat inactivated for 30 min at 56°C, and stored at -80°C.

3.4.2.2 Solutions and buffers for ELISpot

Avidin / HRP-complex solution	10 ml PBS 0.01% Tween20 1 drop reagent A (Vectastain® Elite Kit) 1 drop reagent B (Vectastain® Elite Kit)

Acetat-buffer	Aqua bidest 2.9 g / l natrium acetat 1.5% acetic acid
ACE solution	10 AEC tablets dissolved in 25 ml DMF ad 500 ml acetat-buffer 0.5 µl / mL 30% H_2O_2 (added prior to use)
Wash-buffer	PBS 0.05% Tween20

3.4.2.3 Kits and materials for ELISpot

Anti-hIFN-γ-antibody #1-D1K (capture Ab)	Mat
Anti-hIFN-γ-antibody #7-B6-1 (detection Ab)	Mat
Multiscreen HST™ IP 96well filtration plate	Mp
Vectastain® Elite Kit	Vec

3.4.2.4 HLA-specific mAbs for ELISpot

Table 6: Overview of antibodies isolated from culture supernatant and used in saturating concentrations for HLA-blocking experiments in ELISpot.

Antibody	Specificity	Isotype	Reference
w6 / 32	total HLA-A, -B, and -C	mouse IgG2a	(Barnstable et al. 1978)
B1.23.2	HLA-B and -C, HLA-A09, HLA-A32(19)	mouse IgG2a	(Rebai & Malissen 1983)
PA2.1	HLA-A02 and Aw69	mouse IgG1	(Parham & Bodmer 1978)
SFR8-B6	HLA-Bw6	mouse / rat fusion IgG2b	(Radka et al. 1982)
GAP-A3	HLA-A3	mouse IgG2a	(Berger et al. 1982)
L243 (HB55)	HLA-DR	mouse IgG2a	(Lampson & Levy 1980)

3.4.2.5 HLA-A02 presented T-cell epitopes for ELISpot

Table 7: Overview of antigenic-peptides used for loading APCs in ELISpot assays.

Protein	Name	Sequence (position)	Typ	Function	Reference
WT-1	Wilms tumor protein	RMFPNAPYL (p126-134)	LAA	transcription factor	(Oka et al. 2000)
PR1	Proteinase-3	VLQELNVTV (p169-177)	LAA	serine protease	(Molldrem et al. 1996)
PRAME	Preferentially expressed ag of melanoma	VLDGLDVLL (p100-108)	TAA	transcription repressor	(Kessler et al. 2001)
HA-1	Minor H antigen	VLHDDLLEA (p166-174)	mHag	Rho-like GTPase-activating protein	(den Haan et al. 1998)
Melan A	Melanoma-associated antigen	ELAGIGILTV (p26-35)	TAA	melanocyte differentiation antigen	(Maeurer et al. 2002)

3.4.3 IFN-γ ELISpot assay of AML-reactive CD8$^+$ T cells

Interferon-γ secretion of T cells in response to different target cells including AML blasts and LCLs were analyzed by ELISpot assay. Membranes were first equilibrated with 35% EtOH and after washing with PBS, ELISpot plates were coated with capture Abs (10 µg / ml in PBS, 60 µl / well) over night at 4°C. Unbound Abs were washed with PBS and membranes were blocked with serum containing AIM-V. T cells were seeded as duplicates at 0.5 to 2x10^4 cells / well in AIM-V supplemented with 10% human serum. Target cells were added at 1.5x10^4 cells / well to a final volume of 100 µl. T cells without target cells were used as controls for spontaneous cytokine release. Equally, a spontaneous cytokine release of target cells was detected.

Split-well IFN-γ ELISpots were performed with all mini-MLLCs to analyze reactivity against AML blasts at day 19 or 26 of culture, respectively. Therefore, a mean number of 2x10^4 cells from each mini-culture was used in the assay. Responding cells were further expanded.

To investigate reactivity of AML-reactive CTLs to known LAAs and mHags, HLA-A*02:01-restricted CTLs were tested against T2 cells loaded with peptides derived from the following proteins: WT-1 p123-134, PR1 p169-177, PRAME p100-108, or HA-1 p166-174. Peptides (20 µg / ml) were incubated with the target cells for 2 h at 37°C. Afterwards, cells were seeded at 1.5x10^4 cells / well and were co-incubated with T cells (2x10^4 cells / well, 100 µl / well) with a final concentration of 10 µg / ml peptide.

To identify restriction elements of T cells, target cells were pre-incubated with HLA-class I blocking Abs for 20 min on ice. The following mAbs were added at saturating concentration: w6 / 32, B1.23.2, PA2.1, SFR8-B6, GAP-A3, and L243.
Assays were incubated 18 to 20 h at 37°C. Afterwards, plates were washed with wash-buffer, biotinylated detection Ab (2 µg / ml in PBS + 0.5% BSA, 60 µl / well) was added, and incubated for 2 h at 37°C. After washing, plates were incubated with the avidin / HRP-complex (100 µl / well) for 1 h at room temperature in the dark. The avidin / HRP-complex solution was prepared 30 min in advance. Plates were washed with wash-buffer and following with PBS and, as the final step, the AEC solution (100 µl / well) was added. After 10 min the color reaction was stopped by washing the plates with tap water. Membranes were dried and spots were counted by the computer-assisted image analyzer KS-ELISpot (Zei). Results represent means ± standard deviations (SD) of duplicates. Data were statistically analyzed with the Wilcoxon signed-rank test using SPSS-15 / 17 software.

3.5 Enzyme-Linked Immunosorbent Assay (ELISA)

3.5.1 Principle of ELISA

ELISA was used to detect cytokines in the cell culture supernatant of $CD8^+$ T cells. Primary mAbs against an epitope of the designated cytokine are bound to ELISA plates and cell culture supernatant is incubated. Cytokines contained in the supernatant bind to the immobilized mAbs and can be detected using a secondary biotinylated mAb against a second epitope of the cytokine that interacts with an avidin / HRP-conjugate. The enzyme converts its substrate in a color reaction. This reaction can be measured in a spectrometer and the concentration of the cytokine can be calculated by the use of serial standard dilutions.

3.5.2 Materials for ELISA

3.5.2.1 Substances for ELISA

Fetal calf serum (FCS)	PAA
Phosphate buffered saline (PBS)	Gib
PBS dry chemical “Instamed”	Bic
Sodium bicarbonate ($NaHCO_3$)	Rot

Sodium carbonate (Na_2CO_3)	Rot
Tween20	App

3.5.2.2 Solutions and buffers for ELISA

Assay-buffer (pH 7.0)	PBS 10% FCS
Coating-buffer (pH 9.5)	Aqua dest 8.4 g / l $NaHCO_3$ 3.6 g / l sodium carbonate
Wash-buffer	PBS 0.05% Tween20

3.5.2.3 Kits and materials for ELISA

BD OptEIA^{TM}Set Human TNF	BD
TMB Substrate Reagent Set	BD

3.5.3 TNF-α ELISA of AML-reactive CD8$^+$ T cells

To detect tumor necrosis factor-α (TNF-α) secretion of T cells after stimulation with AML blasts, cell culture supernatant was analyzed by ELISA using BD OptEIA^{TM}Set. Membranes were coated with capture Abs (4 µl / ml in coating-buffer, 50 µl / well) over night at 4°C. Unbound Abs were washed with wash-buffer and membranes were blocked with assay-buffer for 1 h at room temperature. Plates were washed and afterwards 50 µl / well of standards or cell culture supernatant, respectively, were filled in as duplicates. AML blasts in medium used for stimulation served as negative controls, while standards were considered as positive controls. Standards were used in following concentrations: 1.0 ng / ml, 0.5 ng / ml, 0.25 ng / ml, 125 pg / ml, 62.5 pg / ml, 31.25 pg / ml, and 15.63 pg / ml. Assays were incubated for 2 h at room temperature. Plates were washed. Pre-incubated working-detector containing detection mAbs (4 µl / ml with 4 µl / ml avidin / HRP in assay-buffer, 50 µl / well) were added and incubated for 1 h at room temperature. Plates were washed, substrate AB (TMB Substrate Reagent Set, BD) was added (A : B = 1 : 1, 100 µl / well) and incubated for 30 min in the dark at room temperature. Reaction was stopped using 50 µl / well 2 N H_2SO_4 and ODs at 450 nm / 570 nm were measured within 30 min with the ELISA reader (Dyt).

3.6 ^{51}Chromium (^{51}Cr) release assay

3.6.1 Principle of ^{51}Cr release assay

^{51}Cr release assay was used to show cytolytic activity of leukemia-reactive $CD8^+$ T cells against their target cells. Target cells are incubated with ^{51}Cr and take up the radioactively labeled salt which persists intracellulary. When labeled target cells are lysed by CTLs, the radioactive chromate is released to the supernatant and can be measured in a gamma-counter. Specific lysis of the CTLs can be calculated using the spontaneous- and maximum-release of the target cells.

3.6.2 Materials for ^{51}Cr release assay

3.6.2.1 Substances for ^{51}Cr release assay

AIM-V medium	Gib
^{51}Chromium (sodium chromate, $Na_2{}^{51}CrO^4$)	AB
Fetal calf serum (FCS)	PAA
Penicillin / Streptomycin (Pen / Strep)	Gib
Phosphate buffered saline (PBS)	Gib
RPMI 1640 + 1% L-glutamine medium	Gib
TritonX	Sig
Tween20	App

Human serum was isolated after informed consent from healthy blood donors, pooled, sterile-filtrated (0.22 µm), heat inactivated for 30 min at 56°C, and stored at -80°C.

3.6.2.2 Media for ^{51}Cr release assay

T-cell medium	AIM-V 10% human serum
Cell-culture medium	RPMI 1640 10% FCS 1% Pen / Strep

3.6.2.3 Kits and materials for ^{51}Cr release assay

Cytotox tubes (0.6 ml)	Gr

3.6.3 ^{51}Cr release assay of AML-reactive $CD8^+$ T cells

Target cells were labeled with 100 µCi $Na_2{}^{51}CrO_4$ in FCS for 90- 120 min at 37°C. After four washing-steps with cell culture medium, labeled cells were seeded in V-shaped 96-well plates ($1,5x10^3$ cells / well, 80 µl / well). T cells were added as duplicates (80 µl / well) at indicated effector-to-target ratios (E:T) and were incubated for 5 to 6 h at 37°C. When recognition of external loaded peptides was analyzed, labeled target cells were pre-incubated with these peptides for 60 to 90 min at 37°C before T cells were added. Assay medium was T-cell medium. After incubation, plates were centrifuged for 5 min at 282x g without brake and 80 µl supernatant per well was collected. The supernatant was measured in a gamma-counter to detect released ^{51}Cr. Positive-control for maximum ^{51}Cr-release were labeled target cells incubated with PBS+ 1% Tween20 or PBS+ 1% TritonX, respectively, instead of T cells. Negative-control for spontaneous ^{51}Cr-release were labeled target cells in T-cell medium. The percent specific lysis was calculated by the following formula: [(experimental release - spontaneous release) / (maximum release - spontaneous release)] x100. The spontaneous release / maximum release ratios of target cells were generally <25%.

3.7 Mixed-Lymphocyte / Leukemia Culture (MLLC)

Mixed-Lymphocyte / Leukemia Culture was performed under sterile conditions. All cells were cultivated at 37°C, 5% CO_2, and 100% relative air humidity.
Cell counting was performed in a counting chamber using trypan blue solution. Centrifugation means a 5 min centrifugation at 470x g at room temperature using the built-in brake, unless otherwise indicated.

3.7.1 Principle of MLLCs

MLLCs were used to stimulate AML-specific $CD8^+$ T cells from a pool of donor-derived T cells with mixed specificities in a patient-specific manner. To avoid allo-reactivity against foreign HLA-class I-molecules, T-cell donor and patient were completely HLA-class I-matched according to high resolution genomic typing. To stimulate the T cells, cells were incubated with patient's irradiated AML blasts and a cytokine-combination. T cells bearing a TCR recognizing antigens presented on leukemic blasts were activated and started to proliferate, while those T cells lacking a

TCR for leukemic antigens received no stimulating signals. The outgrowth of AML-reactive T cells was analyzed by IFN-γ ELISpot and ^{51}Cr release assay.

3.7.2 Materials

3.7.2.1 Substances for MLLCs

AIM-V medium	Gib
Phosphate buffered saline (PBS)	Gib
Trypan blue	Mer

Human serum was isolated after informed consent from healthy blood donors, pooled, sterile-filtrated (0.22 µm), heat inactivated for 30 min at 56°C, and stored at -80°C.

3.7.2.2 MLLC culture media

T-cell medium	AIM-V 10% human serum

3.7.2.3 Solutions and buffers for MLLCs

Trypan blue solution	2 g / l trypan blue in H_2O, diluted 1:4 with 150 mM saline solution

3.7.2.4 Materials for MLLCs

Melan A-peptide (ELAGIGILTV, p26-35)	Pep
Counting chamber (Fuchs-Rosenthal)	Mar

3.7.2.5 Cytokines for MLLCs

Table 8: Overview of human cytokines used to stimulate MLLCs.

Interleukin	Source	Mfr
IL-2 (Proleukin™)	recombinant human (E. coli)	Nov
IL-7	recombinant human (E. coli)	RD
IL-12	recombinant human (S. frugiperda)	RD
L-15	recombinant human (E. coli)	RD
IL-21	recombinant human (E. coli)	Bm

3.7.3 Mini-MLLCs of AML-reactive $CD8^+$ T cells

Mini-MLLCs were performed to generate leukemia-reactive $CD8^+$ T-cell clones without a cloning by limiting dilution. The protocol was modified from our recently

published protocol (Distler et al. 2008). Mini-MLLCs were started in U-shaped 96-well plates with 10^4 T cells per well what is in accordance with a predicted precursor frequency of leukemia-reactive CTLs in PBMCs of healthy donors that is lower than 10^{-5} (Smit et al. 1998). Responder cells were MACS isolated naive or total $CD8^+$ T cells from healthy donors. When PBMCs were FACS-sorted for $CD8^+$ cells that show a high or low expression of differentiation-markers, all sorted cell populations were used as responders. T cells were stimulated with 10^4 irradiated primary AML blasts and 10^4 irradiated feeder-cells per well in T cell medium. Irradiation dose was 35 to 65 Gray. AML blasts were pre-incubated overnight in T-cell medium at 37°C to achieve a higher expression of HLA-class I- and co-stimulatory molecules (designated as d1 AML blasts). The T-cell culture medium was further supplemented with IL-7 (5 ng / ml), IL-12 (1 ng / ml), and IL-15 (5 ng / ml). To analyze the impact of IL-21 on the generation of AML-reactive CTLs, the cytokine was added (10 to 30 ng / ml) or left out, respectively, in side-by-side mini-MLLCs. T cells were weekly re-stimulated with irradiated d1 AML blasts in fresh cytokine-containing medium. From day 14 on, IL-12 was replaced by IL-2 (50 to 250 IU / ml). The first test to show AML-reactivity of T cells was a Split-well IFN-γ ELISpot at day 19 or 26 of culture. AML-reactive mini-cultures were further re-stimulated in 96-well flat bottom plates and growing-cultures were subsequently expanded in 48-well plates first and finally in 24-well plates (ratio stimulator : responder = 1 : 1).

3.7.4 Maxi-MLLCs of AML-reactive $CD8^+$ T cells

To analyze the AML-reactivity in different $CD8^+$ T cell subsets, bulk-cultures were performed. Responder cells were $CD8^+$ $CD3^+$ T cells which were further separated by FACS-sort according to high or low / negative expression of the differentiation markers CCR7 and CD45RO. The sorted populations were enriched for naive and Tcm cells (i.e. $CCR7^{high}$ and $CD45RO^{low / negative}$) or for Tem and Teff cells (i.e $CCR7^{low / negative}$ and $CD45RO^{high}$). Sorted cells were weekly stimulated with d1 AML blasts in cytokine-containing T-cell medium in 48-well or 24-well plates as described for mini-MLLCs, starting with $0.5x10^6$ or 10^6 T cells per well, respectively. Used cytokines were IL-2 (100 IU / ml, from day 14), IL-7 (5 ng / ml), IL-12 (1 ng / ml, day 0 to day 7), IL-15 (5 ng / ml), and IL-21 (10 ng / ml).

3.7.5 Maxi-Mixed-Lymphocyte / Peptide Cultures (MLPCs) of peptide-specific $CD8^+$ T cells

For adoptive transfer experiments in NSG mice, Melan A-specific (ELAGIGILTV, p26-35) CTLs were used a control cells. These T cells were generated within another project in our group. Briefly, $CD8^+$ T cells from the naive subset of Donor689 isolated by MACS were initiated in bulk-cultures starting with $1x10^6$ T cells per 24-well and were stimulated with peptide-loaded autologous mature DCs and feeder cells in presence of IL-7 (5 ng / ml), IL-15 (5 ng / ml), and IL-21 (10 ng / ml). For following weekly stimulations peptide-loaded autologous PBMCs or T2 cells were used in presence of IL-2 (from day 14 on, 10 IU / ml), IL-7 (5 ng / ml), IL-15 (5 ng / ml), and IL-21 (10 ng / ml). After three weeks of culture, CTLs were enriched by MACS according to their CD137 expression. $CD137^{high}$ cells were strongly Melan A-reactive. These cells were expanded up to day 42 of culture and were frozen. At that day 90% of the T cells were Melan A-tetramer positive. For adoptive transfer T cells were thawed, restimulated, and transplanted three days afterwards.

3.8 Adoptive T-cell transfer in AML-engrafted NSG mice

3.8.1 Principle of adoptive T-cell transfer

The *in vivo* function of AML-reactive CTLs was analyzed in AML-engrafted NSG mice. As therapeutical mouse model, primary AML blasts were injected into the mice. When AML blasts were successfully engrafted to an extent of 1% to 3% blasts in murine bone marrow, T cells were adoptively transferred into the mice. After one week, the presence of human blasts in murine bone marrow, spleen, and peripheral blood was analyzed and compared to control groups that were transferred with T cells not recognizing engrafted AML cells or with no T cells. To see long-term effects of the treatment, mice were also analyzed four weeks post transfer.

3.8.2 Materials for adoptive T-cell transfer

3.8.2.1 Substances for adoptive T-cell transfer

Bovine serum albumin (BSA)	Sig
DMEM medium	Gib
Fetal calf serum (FCS)	PAA

Lysing Buffer (10x)	BD
Penicillin / Streptomycin (10 000 U / ml Pen+ 10 000 µg / ml Strep)	Gib
Phosphate buffered saline (PBS)	Gib

3.8.2.2 Solutions and buffers for adoptive T-cell transfer

Prep-medium	DMEM 1% FCS 1% Pen / Strep
FACS-buffer	PBS 1 g / l BSA
FACS-fix	PBS 1% formaldehyde

3.8.2.3 Cytokines and materials for adoptive T-cell transfer

Table 9: Overview of cytokines injected into the mice in parallel and three days following adoptive T-cell transfer.

Cytokine	Amount / mouse	Mfr.
IL-2 (human IL-2, Proleukin^{TM})	1000 IU	Nov
IL-7 (human Fc-IL-7)	20 µg	Mer
IL-15 (human IL-15)	5 µg	Pep

3.8.2.4 NOD / SCID / IL2Rγc^{null} (NSG) mice

For all *in vivo* experiments NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl} / SzJ, better known as NOD / SCID / IL2Rγc^{null} (NSG) mice were used. Animals (stock number 005557) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice have the severe combined immune deficiency mutation (SCID) and an IL-2 receptor gamma chain knockout which leads to a lack of mature T cells and B cells, functional NK cells, and a deficiency in cytokine signaling. Mice resist to develop lymphoma even after irradiation and show good engraftment rates especially for human HSC but also for acute leukemic blasts (Agliano et al. 2008; Ito et al. 2002; Shultz et al. 2005).
Stock breeding, maintaining, and manipulation of mice were done in the Central Animal Facility of University Medical Center Mainz under specific pathogen free (SPF) conditions. The autoclaved drinking water was supplemented with 0.08 mg / ml Borgal (sulafadoxinum, trimethoprimum). All animal studies were approved by the German state authorities and were performed according to guidelines of the European Union and Germany.

3.8.3 Engraftment of human AML blasts in NSG mice

To improve engraftment of leukemic blasts (Greiner et al. 1998; Shultz et al. 2005), mice (6 to 8 weeks old) were sublethally irradiated through a ^{137}cesium source with 1.5 Gray 16 to 24 h before transplantation. Next day, mice were warmed with infrared light to dilate the veins. Subsequently, mice were injected i.v. in the tail vein with $5x10^5$ primary AML blasts in 200 µl PBS supplemented with 0.5% FCS per mouse.

3.8.4 Adoptive transfer of human AML-reactive CTLs into NSG mice

For therapy approaches, AML-reactive $CD8^+$ T cells generated in mini-MLLCs in HLA-class I-matched settings (see chapter 3.7.3) were used which recognized *in vitro* the AML blasts that were engrafted in mice. CTLs were stimulated with AML blasts in presence of 100 IU / ml IL-2, 5 ng / ml IL-7, 5 ng / ml IL-15, and 10 ng / ml IL-21 three days before transfer. As control T cells, Melan A-specific CTLs not recognizing the AML blasts were used that were stimulated with peptide (Melan A, ELAGIGILTV) loaded T2 cells and the same cytokine combination. At transfer day, AML-engrafted mice were warmed with infrared light and 5 to $10x10^6$ T cells per mouse were injected i.v. in the tail vein in 200 µl PBS supplemented with 0.5% FCS. Additionally, human cytokines (per mouse: 1000 IU IL-2, 20 µg Fc-IL-7, and 5 µg IL-15) were added to improve environment for the T cells. Three days after T-cell transfer, 5 µg IL-15, and when indicated, also 1000 IU IL-2 were injected i.v. in the tail vein in 200 µl PBS.

3.8.5 Isolation and phenotypic analysis of peripheral blood, spleen and bone marrow cells from transplanted NSG mice

Mice were sacrificed by cervical dislocation and were disinfected with 70% ethanol. Subsequently, the chest region was opened and approximately 100 µl peripheral blood was taken from the heart sac and was supplemented with 20 µl heparin. Afterwards, the spleen was harvested, comminuted through a 100 µm cell-strainer, single cells were washed, and stored in prep-medium on ice. To get bone marrow cells, the femur of one hind leg was prepared from tissue. The bone was opened at both ends and the bone marrow was washed out with prep-medium. To separate the cells, bone marrow was filtered through a 100 µm cell-strainer and was stored in prep-medium on ice. Single cell suspensions from spleen and bone marrow were spun down at 470x g for 5 min at 4°C (parameters were used for all following

centrifugation steps). Supernatant was decanted and pellets were incubated for 1 to 2 min in lysing buffer (1:10 in aqua dest). Reaction was stopped by adding prep-medium and cells were spun down again. Pellets were resuspended in FACS-buffer (1 ml for bone marrow, 2 ml for spleen). Per organ, 100 µl cell suspension and 30 µl of peripheral blood were used for each flow cytometric staining. Cells were incubated for 15 min at 4°C with designated antibodies.

Table 10: Staining strategy used to identify human $CD33^+$ AML blasts and $CD8^+$ T cells in murine bone marrow, spleen, and peripheral blood.

	PE	**APC**	**Horizon (V450)**
tube 1	no	no	no
tube 2	CD33	CD45	CD3
tube 3	CD8	CD45	CD3

Spleen and bone marrow cells were washed with 1 ml FACS-buffer and were spun down. Blood cells were supplemented with lysing buffer (1:10 in aqua dest) and incubated for 2 to 3 min. Reaction was stopped with FACS-buffer and blood cells were spun down. Supernatant of all three approaches was discarded, cells were resuspended in 200 to 300 µl FACS-fix and 20 000 events were measured on FACS Canto II (BD). Remaining cells from bone marrow and spleen were frozen.

3.9 Further materials

3.9.1 Laboratory equipment

Cell sorter (FACS Aria)	BD
Centrifuge (Megafuge 1.0R, Multifuge 1S-R)	Her
Cryobank (Espace 331 Gaz)	AL
ELISpot counter (KS-ELISpot 4.9)	Zei
ELISA reader (MRX Relevation)	Dyt
Flow Cytometer (FACS Canto II)	BD
Gamma-counter (Wizard 2)	Per
Gamma-irradiator ^{137}Cs cells (Gammacell 2000)	Mol
Gamma-irradiator ^{137}Cs mice (Typ 0B58 / 905-2)	Bu
Incubator (Hera cell 240)	Her
Microscope (Axiovert 40 C, 25)	Zei
Pipette (2 to 20 µl, 20-200 µl, 100-1000 µl)	Gil
Pipette (1 to 10 µl)	Epp
Pipette multichannel (5 to 100 µl, 30 to 300 µl)	Sta
Pipettor (Pipetboy acu)	Int
Sterile bench (Hera safe HS18)	Her
Water deionization machine (Purelab classic)	Egl

3.9.2 Plastic material

Cryo box (Mr. Frosty)	Nal
Cryo tubes (Cryo-S)	Gr
Cell culture flask (Cellstar 25 cm^2, 75 cm^2, 175 cm^2)	Gr
Cell culture plates (24 well, 48 well, 96-V-, 96-U-, 96-flat-well)	Gr
Cell strainer 100 µm	BD
Falcon Tubes (15 ml, 50 ml)	Gr
Petri dish (35 ml, 60 ml, 95 ml)	Gr
Pipette tips (TipOne 10 µl, 200 µl, 1000 µl)	Sta
Pipettes single use (Cellstar 2 ml, 5 ml, 10 ml, 25 ml, 50 ml)	Gr
Reaction tubes (0.5 ml, 1.5 ml, 2 ml)	Epp
Syringes / canulaes	Bra
Sterile filter (Stericup Express plus 0.22 µm, Stericup Durapore 0.45 µm)	Mp
Tubes with frit (Leucosep 50 ml)	Gr

3.10 Abbreviations of Manufacturers

AB	Amersham Bioscience, GE Healthcare Europe, Freiburg
AL	Air Liquide, Düsseldorf
App	AppliChem, Darmstadt
BC	Beckmann Coulter, Karlsruhe
BD	BD Parmigen / Biosciences, Heidelberg
Beh	CSL Behring, Marburg
Bic	Biochrom KG, Berlin
Bm	Biomol, Hamburg
Bra	Braun, Melsungen
Bu	GE Healthcare Buchler, Braunschweig
BZ	BIOZOL, Eching
Dyt	Dynex Technologies
eB	eBioscience, Frankfurt am Main
Egl	Elga Lab Water, Celle
Epp	Eppendorf, Hamburg
Her	Heraeus, Hanau
Imt	Immunotech, Marseille, France
Int	Integra Biosciences, Fernwald
Gib	Gibco / Invitrogen, Karlsruhe
Gil	Gilson, Limburg
Gr	Greiner bio-one, Frickenhausen
Mar	Marienfeld, Lauda Königshofen
Mat	Mabtech AB, Nacka Strand, Stockholm, Sweden
Mer	Merck, Darmstadt
Mil	Miltenyi Biotec, Bergisch-Gladbach
Mol	Moolsgard Medical, Gansloe, Denmark
Mp	Millipore, Eschborn
Mur	Murex Biotech, Kent, UK
Nal	Nalgene Nunc, Wiesbaden
Nov	Novartis, Nürnberg
PAA	PAA, Pasching, Austria
Pep	PeproTech, Hamburg
Per	Perkin-Elmer, Rodgau

Rat	Ratiopharm, Ulm
RD	R&D systems, Wiesbaden-Nordenstedt
Roc	Roche Applied Science, Mannheim
Rot	Carl Roth AG, Karlsruhe
Sig	Sigma Aldrich, Steinheim
Sta	Starlab, Ahrensburg
Vec	Vector Laboratories, Burlingame, USA
Zei	Zeiss, Göttingen

4. Results

4.1 Characterization of HLA-class I-matched patient / donor pairs

4.1.1 MZ169-AML

Patient MZ169-AML was diagnosed with acute myeloid leukemia (AML) FAB M4 in August 2008. He had an initial white blood cell count (WBC) of $3.5x10^4$ / µl. After chemotherapy he was in remission. In January 2011 the AML relapsed and the patient received an allogeneic hematopoietic stem cell transplantation (HSCT) from an HLA-class I-matched unrelated donor after chemotherapy. Since then he has been in complete remission. For *in vitro* experiments buffy coats from a HLA-class I-matched sibling donor were available (Table 11).

4.1.2 MZ201-AML

Patient MZ201-AML was diagnosed with AML FAB M5b in August 2004. She had an initial WBC of $2x10^5$ / µl. The patient had a progressive disease that was refractory to chemotherapy and died nine months after diagnosis. Buffy coats of two different healthy unrelated HLA-class I-identical donors could be used for *in vitro* experiments from the Center for Blood Transfusion at the University Medical Center Mainz (Table 11), all following buffy coats of healthy donors were provided by this institution as well).

4.1.3 MZ369-AML

Patient MZ369-AML was diagnosed with AML FAB M4 in November 2005. She had an initial WBC of $1.5x10^5$ / µl. After chemotherapy the patient was in remission and received an allogeneic HSCT from a HLA-class I-matched sibling donor. Afterwards, the disease relapsed three times and was refractory to therapy. The patient died one year after diagnosis due to sepsis. From the sibling donor peripheral blood mononuclear cells (PBMCs) were available for *in vitro* experiments (Table 11).

4.1.4 MZ529-AML

Patient MZ529-AML was diagnosed with AML FAB M1 in March 2007. She had an initial WBC of $1.9x10^5$ / µl. She responded to chemotherapy and received an allogeneic HSCT from an HLA-matched unrelated donor. After two relapses the

patient died 13 months after diagnosis due to sepsis and kidney failure. A buffy coat from a HLA-class I-identical healthy donor (a different from the one who donated the transplant) could be used for *in vitro* experiments (Table 11).

4.1.5 MZ580-AML

Patient MZ580-AML was diagnosed in February 2007 with AML FAB M4 and had a WBC of $4x10^5$ / µl. The patient first showed blast persistence during chemotherapy. After reconstitution, he received an allogeneic HSCT from an HLA-matched unrelated donor but developed an early relapse afterwards and died seven months after diagnosis. A buffy coat from a HLA-class I-identical healthy donor (a different from the one who donated the transplant) could be used for *in vitro* experiments (Table 11).

4.1.6 MZ653-AML

Patient MZ653-AML was diagnosed with AML FAB M5 in January 2009 with a WBC of more than $2x10^5$ / µl. She responded inert to chemotherapy and died one week after diagnosis due to AML and kidney failure. Buffy coats from a HLA-class I-matched healthy donor could be obtained for *in vitro* experiments (Table 11).

4.1.7 MZ667-AML

Patient MZ667-AML was diagnosed with AML FAB M1 in May 2007 with a high WBC. The patient first showed blast persistence after chemotherapy. After reconstitution, she received an allogeneic HSCT from an HLA-matched unrelated donor. One day after transplantation the patient died (Table 11).

4.1.8 MZ728-AML

Patient MZ728-AML was diagnosed with AML FAB M4 in January 2009. She had an initial WBC of $7.8x10^4$ / µl. She was treated with curative induction- and salvage therapy but showed blast persistence. Following chemotherapy to treat a new increase of blasts, she died due to pneumonia and respiratory insufficiency. For *in vitro* experiments PBMCs from a HLA-class I-identical healthy unrelated donor could be used (Table 11).

4.1.9 MZ987-AML

Patient MZ987-AML was diagnosed in September 2009 with AML FAB M4 as secondary disease from a MDS. The initial WBC was $1.5x10^5$ / µl. After chemotherapy the patient had a persistent disease. He got an allogeneic HSCT from an HLA-class I-matched but HLA-DRB1-different unrelated donor. In September 2010 the disease relapsed. Following, the patient died due to the relapse, sepsis, and GVHD with superinfection. For *in vitro* experiments PBMCs from a HLA-class I-identical healthy unrelated donor could be used (Table 11).

Table 11: Overview of patient / donor pairs used as experimental models.

Model	Age/Sex	FAB Subtype	HLA-A	HLA-B	HLA-C	Karyotype	Mutations
MZ169-AML	44 / m	M4	01, 24	15(62), 41	03, 17	46, XY / 45, X-Y	t(8;21)
SIB Donor			01, 24	15(62), 41	03, 17		
MZ201-AML	74 / f	M5b	*01:01, *02:01	*08:01, *56:01	*01:02, *07:01	46, XX	-
Donor168			*01:01, *02:01	*08:01, *56:01	*01:02, *07:01		
Donor650			*01:01, *02:01	*08:01, *56:01	*01:02, *07:01		
MZ369-AML	30 / f	M4	*01:01, *24:02	*18:01, *38:01	*07:02, *12:03	46, XX	-
SIB Donor			*01:01, *24:02	*18:01, *38:01	*07:02, *12:03		
MZ529-AML	36 / f	M1	*03:01, *11:01	*15:01, *35:01	*03:04, *04:01	46, XX	Flt3-ITD
Donor730			*03:01, *11:01	*15:01, *35:01	*03:04, *04:01		
MZ580-AML	54 / m	M4	*01:01	*57:01	*06:02	46, XY	Flt3-ITD, NPM1
Donor931			*01:01, *02:01	*35:01, *57:01	*04:01, *06:02		
MZ653-AML	75 / f	M5	*01:01, *30:01	*08:01, *13:02	*06:02, *07:01	46, XX	-
Donor069			*01:01, *30:01	*08:01, *13:02	*06:02, *07:01		
MZ667-AML	53 / f	M1	*23:01, *33:01	*14:02, *49:01	*07:01, *08:02	46, XX	Flt3-ITD, NPM1
MZ728-AML	65 / f	M4	*01:01, *02:01	*07:02, *08:01	*07:01, *07:02	46, XX	-
Donor284			*01:01, *02:01	*07:02, *08:01	*07:01, *07:02		
MZ987-AML	55 / m	M4	*02:01	*15:01, *15:17	*03:04, *07:01	46, XY	Flt3-ITD
Donor940			*02:01	*15:01, *15:17	*03:04, *07:01		

Most patients which were included in the studies herein were estimated as high risk patients because of the occurring FLT3-ITD mutation and / or a very high WBC (> $1x10^5$ / µl) at primary diagnosis.

4.2 Phenotypic features of AML blasts after overnight culture

For the generation of AML-reactive $CD8^+$ T cells primary AML blasts from patient's leukapheresis products were directly used as antigen-presenting cells (APCs) to assure responses against hematopoietic leukemia-associated antigens (LAAs) and minor histocompatibility antigens (mHags) presented by the leukemic cells. To

stimulate donor T cells, interactions of their t-cell receptors (TCRs) and co-receptors with surface molecules of AML blasts are required. The expression of HLA-class I- or II-molecules as well as co-stimulatory (CD80, CD86, CD40) and adhesion molecules (CD54, CD58) on the target cells is necessary for a sufficient recognition by T cells (Brouwer et al. 2002; Brouwer et al. 2000). For a preferably high expression of these molecules and improvement of viability of the AML blasts, leukemic cells were thawed and incubated in T-cell medium overnight. The expression of cell surface markers was analyzed by flow cytometry. Exemplarily shown is MZ653-AML, a FAB M5 AML at culture day one (Figure 3).

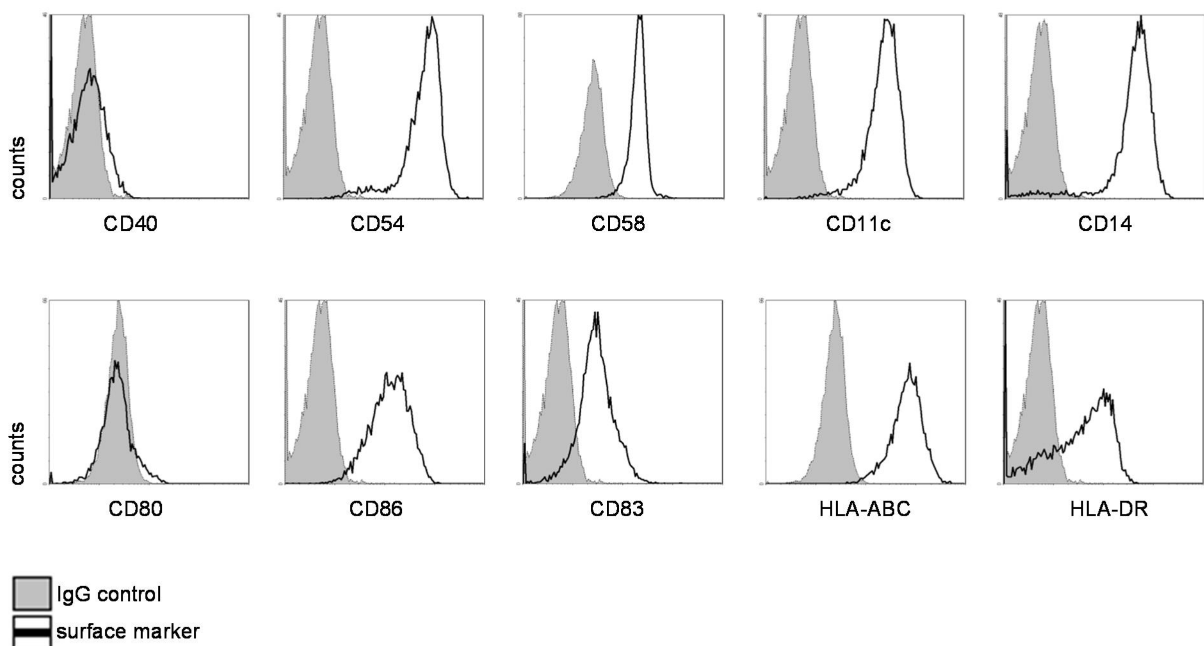

Figure 3: Expression of important co-stimulatory, HLA- and adhesion molecules on the surface of AML blasts analyzed by FACS. The expression pattern of leukemic blasts from patient MZ653 after overnight incubation is exemplarily shown. To identify AML blasts, cells were gated on the myeloid marker CD33 and the hematopoietic marker CD45**.**

Expression of important co-stimulatory molecules could be detected. HLA-class I- and II-molecules were clearly expressed as well as CD54, CD58, CD83, and CD86. Furthermore, monocytic and dendritic markers like CD11c and CD14 were present on the cell surface of this acute monoblastic / monocytic leukemia which were not detectable on leukemic blasts of patients with other FAB subtypes at similar levels (data not shown). An expression of CD40 and CD80 was not detectable.

4.3 Generation of AML-reactive CD8$^+$ T cells from the naive enriched compartment

In hematopoietic stem cell transplantation T cells from the donor mediate both, the desired graft-versus-leukemia (GVL) and the unwanted graft-versus-host disease (GVHD). Current strategies to separate GVL from GVH effects include the adoptive transfer of leukemia reactive T-cell lines and clones isolated from PBMCs of healthy donors by primary *in vitro* stimulation (Bleakley & Riddell 2004). There are studies in animal models showing that CD8$^+$ T cells generated from the naive or central memory compartment are superior in regard to persistence and anti-tumor effect to those derived from their counterpart effector memory fraction upon adoptive transfer (Berger et al. 2008; Gattinoni et al. 2011; Hinrichs et al. 2009). Thus, we investigated the anti-leukemic potential of T cells derived from the naive (Tn) / central memory (Tcm) versus the effector memory (Tem) subset. In order to find an appropriate starting population for the *in vitro* generation of leukemia-reactive cytotoxic T lymphocytes (CTLs) by stimulation with primary leukemic blasts, T cells from both compartments were enriched to compare the precursor frequencies of AML-reactive CD8$^+$ T cells. First, the expression of Tn / Tcm (CCR7, CD45RA, and CD62L) as well as Tcm / Tem (CD45RO) markers on PBMCs from healthy donors was analyzed. In order to investigate the potential of these markers to separate T cells in subpopulations, PBMCs were stained and CD8$^+$ T cells were analyzed by flow cytometry. Marker expressions of two different donors are shown in figure 4.

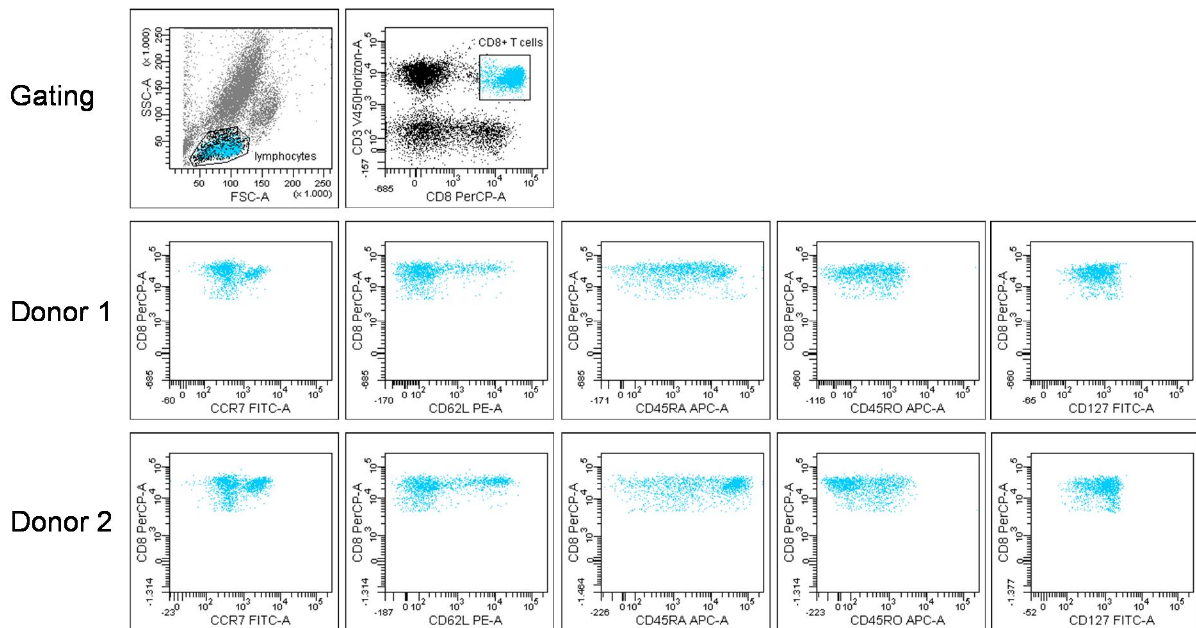

Figure 4: Expression of differentiation markers for distinct T-cell stages on the surface of CD8^{+} T cells in human PBMCs analyzed by FACS. Shown are representative analyses of PBMCs of two different healthy donors. Lymphocytes were gated in FSC / SSC channel and out of this population CD3^{+} CD8^{+} T cells were further gated (upper panel). The expression of CCR7, CD62L, CD127, CD45RA, and CD45RO on the surface of CD8^{+} T cells is illustrated.

The CD8^{+} T-cell population could be divided into distinct subsets with high and low / negative expression of all four surface molecules. In the following experiments, we decided to use CD45RO and CCR7 to investigate the *in vitro* AML-reactivity of T cells. With regard to future clinical applications, single surface molecules were chosen for separation and not a combination of markers to keep the procedure simple. The expression of CD45RO is initiated after antigen contact. The molecule is therefore not present on the surface of Tn what makes it an appropriate marker to enrich naive cells from the CD45RO^{-} and memory cells from the CD45RO^{+} subset (Klebanoff et al. 2006; Sallusto et al. 2004). CCR7 is expressed on the surface of Tn and Tcm and is down-regulated afterwards. Therefore, memory T cells are mainly found in the CCR7^{-} fraction (Appay et al. 2008; Sallusto et al. 2004). The potential of T cells from the subpopulations distinguished by high or low / negative expression of CCR7 and CD45RO to generate anti-leukemia responses was analyzed after separating these subpopulations by flow cytometric cell sorting. As shown in figure 5, the resulting subsets had a high purity (median purity 93%). PBMC donors and AML patients had a complete HLA-class I-match which was determined by high resolution genomic typing. MZ369-AML / SIB Donor, MZ653-AML / Don.069, and MZ987-AML / Don.987 were used as donor / patient pairs for experimental models.

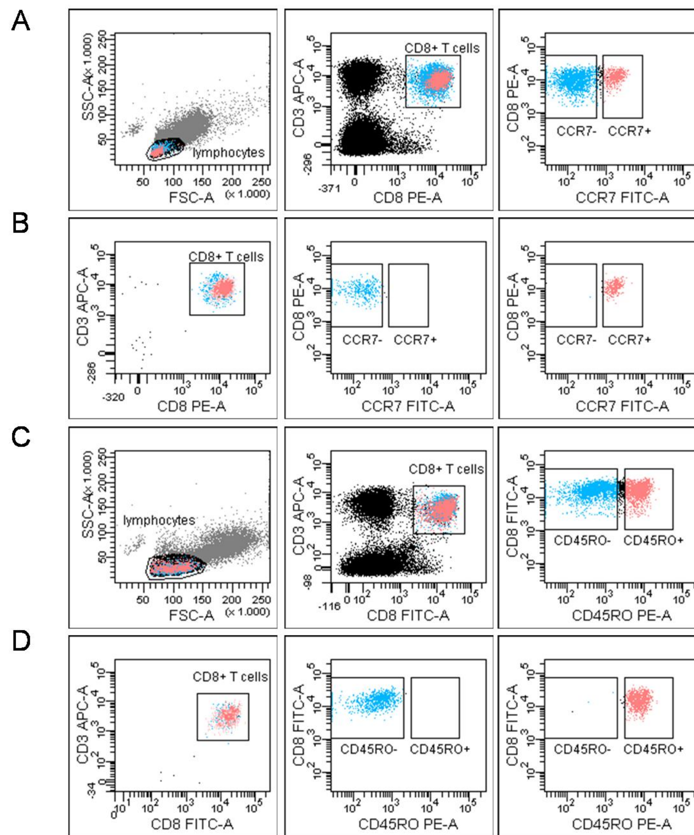

Figure 5: Sorting strategy to separate T cells from the Tn / Tcm or Tem subset by FACS. $CD3^+$ $CD8^+$ T cells were gated out of lymphocytes and T cells were sorted according to low / negative or high expression of (A) CCR7 and (C) CD45RO, respectively. After sorting procedure, the collected populations were reanalyzed (B, D) to verify purity of resulting subpopulations. Exemplarily shown are results from the separation of PBMCs of the sibling donor matched to patient MZ369.

The sorted T-cell populations were used as responder cells in so-called maxi-Mixed-Lymphocyte / Leukemia Cultures (MLLCs). These bulk cultures were initiated with 0.5 to $1x10^6$ T cells per well and were stimulated with equal numbers of primary AML blasts and a cytokine combination including IL-2, IL-7, IL-12, IL-15, and IL-21. T-cell cultures were cultured for three weeks and were restimulated weekly. Functional properties of responding cultures were analyzed by ^{51}Cr-release and IFN-γ ELISpot assay. Furthermore, at day 14 of culture FACS analyses were performed (Figure 6).

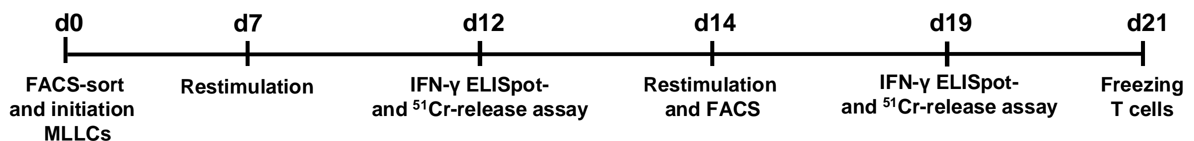

Figure 6: Flow chart of generation and characterization of maxi-MLLCs initiated with $CD8^+$ T cells enriched from the Tn / Tcm ($CCR7^+$ and $CD45RO^-$) or Tem ($CCR7^-$ and $CD45RO^+$) subset by flow cytometric sorting. Bulk cultures were initiated with 0.5-$1x10^6$ T cells per well and were restimulated weekly with primary AML blasts and a cytokine combination including IL-2 (from day 14 on), IL-7, IL-12 (day 0 to 7), IL-15, and IL-21. Functional and phenotypic features were determined during the culture period of three weeks.

At day 12 and day 19 of culture, T-cell cultures were tested for AML-reactivity. Recognition of AML blasts was mainly detected in MLLCs initiated with T cells from subsets enriched for Tn and Tcm ($CCR7^+$ and $CD45RO^-$). T-cell cultures that were initiated with these subsets had a specific lytic activity against patient's AML blasts and Epstein Barr-Virus (EBV)-transformed B lymphoblastoid cell lines (LCLs) which could not be detected in their counterpart fractions. Targets were recognized in a patient specific manner, demonstrated by a lacking reactivity to donor-derived LCLs as control for EBV- and auto-reactivity and K562 as NK-cell target (Figure 7A). Moreover, recognition could be blocked by the use of saturating concentrations of anti-HLA-class I-mAbs (Figure 7B), indicating HLA-class I-restriction of the CTLs.

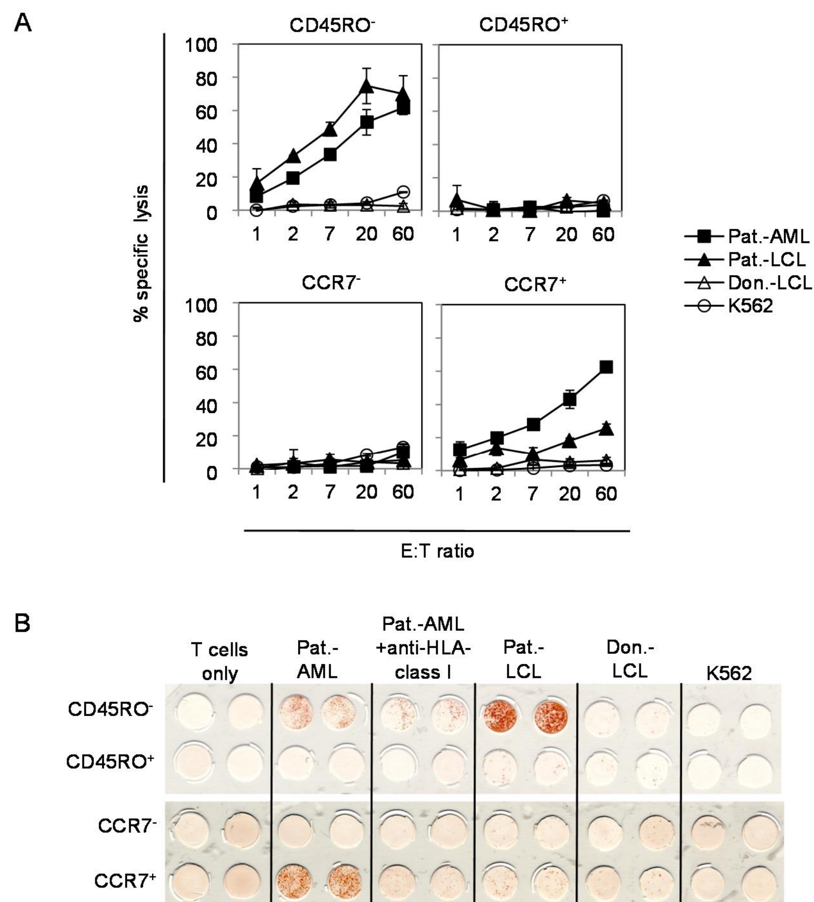

Figure 7: Functional properties of FACS-sorted $CD8^+$ T-cell cultures generated from the $CD45RO^{+/-}$ and $CCR7^{+/-}$ subset investigated by ^{51}Cr-release and IFN-γ ELISpot assay. Exemplarily shown are results from one patient (MZ653) out of three. (A) In a 5 h ^{51}Cr-release assay the lytic activity of T-cell cultures generated from subpopulations enriched for Tn / Tcm or Tem, respectively, against patient's AML blasts and LCLs, as well as donor's LCLs and K562 was tested at indicated effector-to-target ratios. (B) Results were confirmed by measuring IFN-γ secretion in an 18 h ELISpot assay using $1x10^4$ T cells / well and $5x10^4$ target cells / well. Additionally, restriction of T cells was shown by the use HLA-class I-specific mAbs.

Characterization by FACS at day 14 confirmed the $CD3^+$ $CD8^+$ phenotype of MLLCs, as in all cultures 95% to 100% of the cells were positive for both markers. Cultures initiated with T cells from the Tn / Tcm subsets still showed a more “naive phenotype” compared to their counterpart fractions because they had a higher expression of CD45RA, CD62L, CD27, CXCR4 and slightly more CD28 and CCR7 on their surface (data not shown). In summary, these data demonstrated that most T cells expressing t-cell receptors (TCRs) that recognize antigens presented on the surface of AML blasts were derived from the Tn and Tcm subset.

The use of maxi-MLLCs for the generation of leukemia-reactive CTLs included some difficulties: In long-term cultures the reactivity of the T cells was reduced and the proliferation rate decreased. Additionally, the cultures were polyclonal and could contain T cells with other specificities than for AML blasts, for example virus-reactive T cells expanded by the cytokine usage (data not shown). To improve culture conditions for the outgrowth of leukemia-reactive CTLs to better deal with the low precursor frequency of antigen-specific T cells in healthy donors (Alanio et al. 2010; Smith & Lutz 1996), our group has developed a protocol to generate T-cell clones in so called mini-MLLCs. Mini-cultures were initiated with only 10^4 T cells per well to achieve that ideally one leukemia-reactive T-cell precursor is present in one culture unit (Distler et al. 2008). This mini-MLLC approach was used for all following T-cell generations.

As shown above, the naive $CD45RO^-$ fraction contained most precursors of AML-reactive T cells. A MACS Kit (Naive $CD8^+$ T Cell Isolation Kit, Miltenyi) is available that also separates T cells according to their CD45RO expression among others markers which was used for all following isolations of naive T cells. First, non-naive cells expressing CD45RO, CD56, CD57, and CD244 are depleted with this kit. In a second step, $CD8^+$ T cells are isolated from the enriched naive cells. Unseparated $CD8^+$ T cells were isolated from PBMCs of the same donor by MACS for comparison (CD8 MicroBeads, Miltenyi). Isolated populations were analyzed by flow cytometry for expression of naive and memory markers (Figure 8).

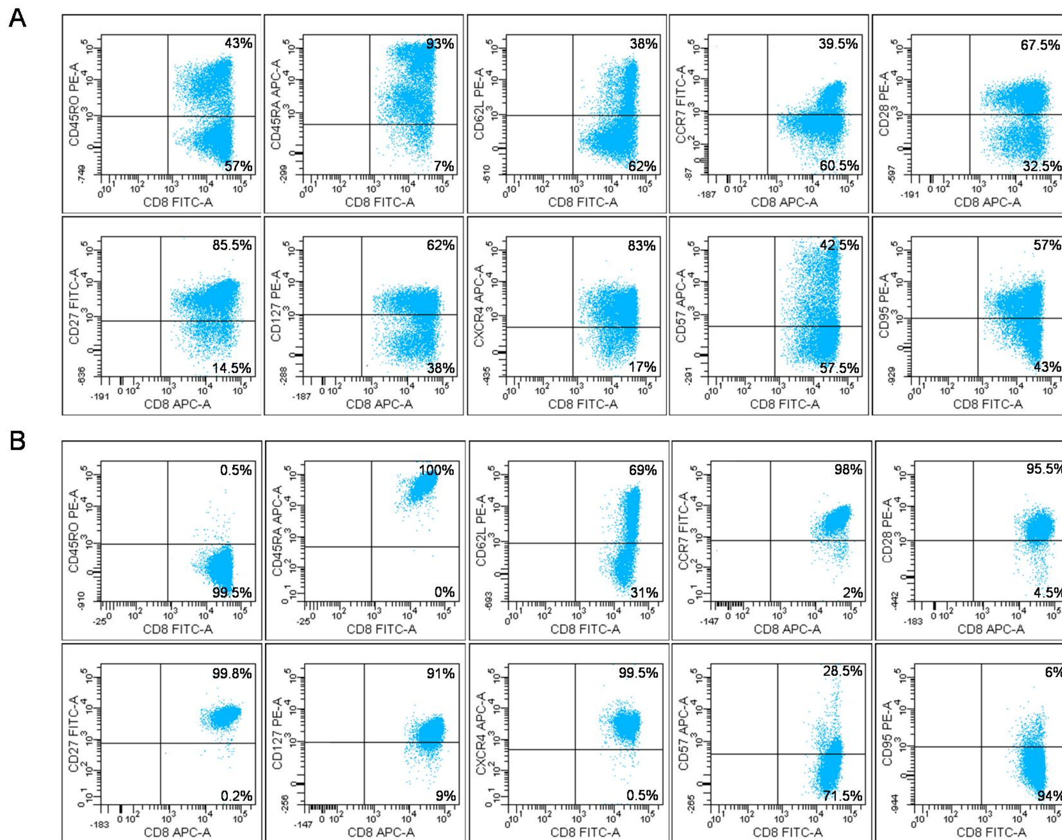

Figure 8: Phenotypic properties of unseparated and naive CD8$^+$ T cells after isolation with MACS-beads analyzed by FACS. Expression of several differentiation markers on the surface of (A) unseparated and (B) naive CD8$^+$ T cells which were used to initiate mini-MLLCs are shown. Lymphocytes were first gated in SSC / FSC channel and out of this population additionally CD3$^+$ CD8$^+$ T cells were gated. Gates were set according to isotype-controls.

In the unseparated CD8$^+$ fraction cells with high, intermediate, and low expression of all stained markers could be detected. This indicated the presence of Tn, Tcm, as well as Tem cells in the isolated population (Figure 8A). In contrast, in the naive subset CD8$^+$ cells with a naive phenotype were highly enriched. T cells expressed the naive markers CD45RA, CCR7, CD28, CD27, CD127, and CXCR4, and were negative or low positive for the memory markers CD45RO, CD57, and CD95. The amount of CD62L positive cells was also increased (Figure 8B). Both, the naive and the unseparated CD8$^+$ subset were used to initiate mini-MLLCs in 96-well plates starting with 10^4 T cells per well. Mini-cultures were weekly stimulated with primary AML blasts from a patient with complete HLA-class I-match to the donor in fresh cytokine-containing T-cell medium. At day 19 or 26, respectively, T cells were screened for AML-reactivity in an IFN-γ split-well ELISpot assay. AML-reactive cultures were further expanded by antigen-specific restimulation and were characterized according to their functional and phenotypic properties (Figure 9).

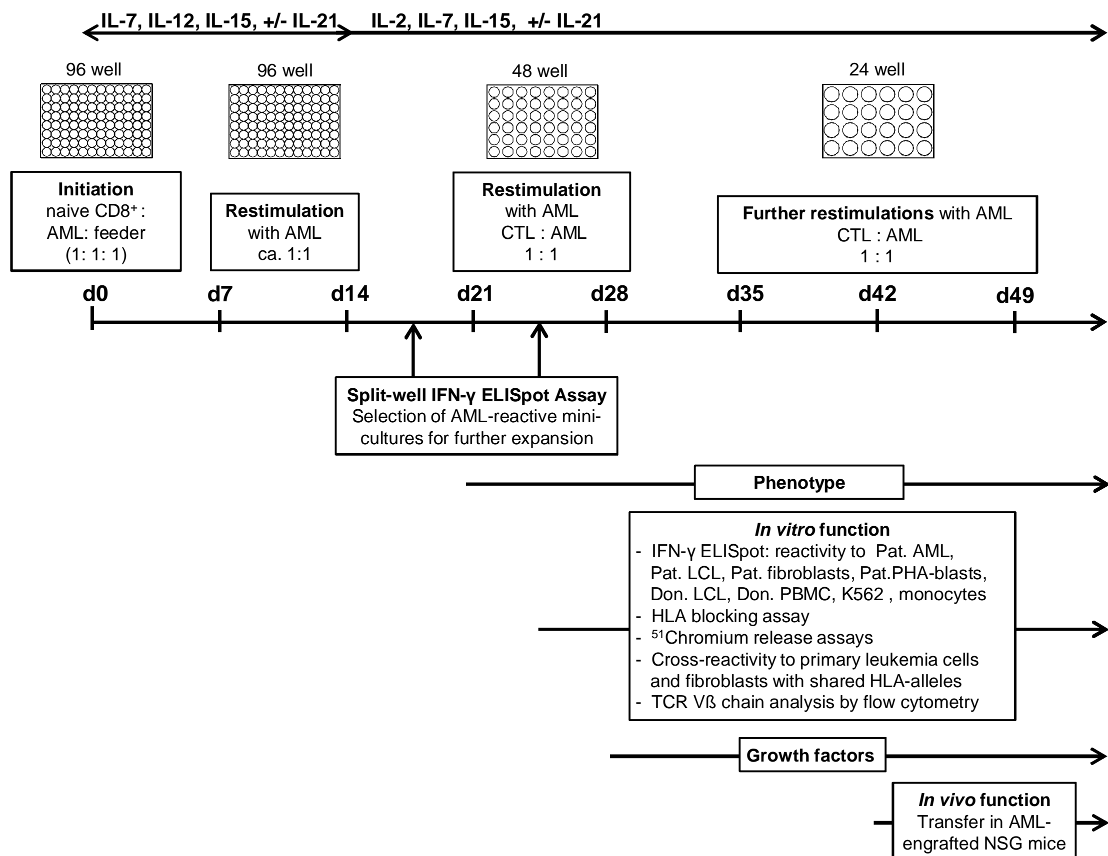

Figure 9: Flow chart of the generation and characterization of CTL clones in mini-MLLCs. Mini-MLLCs were initiated with 10^4 naive $CD8^+$ T cells / well in a 96-well plate and were restimulated weekly with primary AML blasts and a cytokine combination including IL-2 (from day 14 on), IL-7, IL-12 (day 0 to 7), IL-15, and IL-21. AML-reactivity was first tested in split-well IFN-γ ELISpot assays at day 19 or 26. Leukemia-reactive growing cultures were expanded in 48- and 24-well plates. CTLs were further characterized due to phenotypic as well as functional *in vitro* and *in vivo* properties.

In all three AML patient / donor pairs tested significantly higher numbers of leukemia-reactive T cells were observed in cultures initiated with naive $CD8^+$ T cells compared to unseparated $CD8^+$ T cells (p=0.043 by Wilcoxon signed-rank test, Figure 10). Overall, a 1.5-10.0-fold (mean 4.5-fold) increase in AML-reactive cultures initiated with Tn cells was detected compared to cultures from unseparated $CD8^+$ T cells, indicating that the naive subset also was an adequate starting population when T cells were cultured under clonal conditions. Interestingly, the number of reactive CTLs could be further increased by adding IL-21 to culture medium.

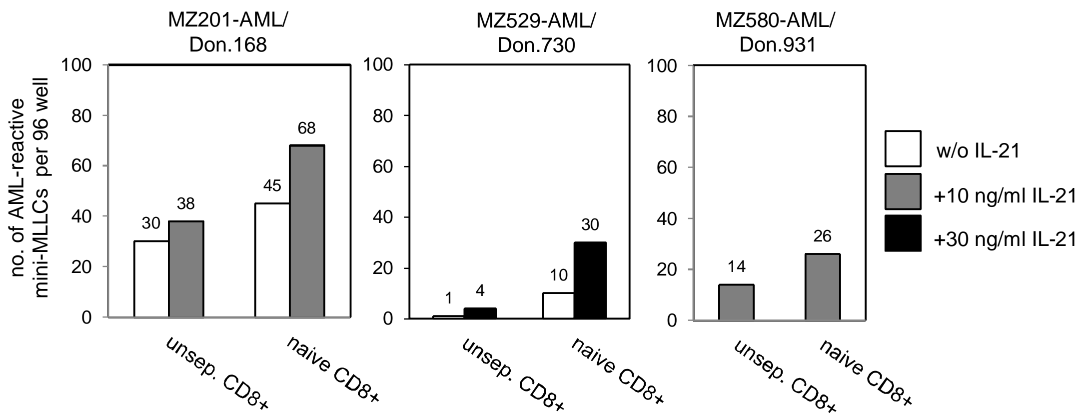

Figure 10: Number of AML-reactive mini-MLLCs generated from the unseparated or naive CD8^{+} T-cell subset, respectively, analyzed by IFN-γ splitwell-ELISpot assays. Number of AML-reactive cultures per 96-well from three different HLA-class I-matched patient / donor pairs after two to three antigen-specific restimulations with primary AML blasts. Allogeneic mini-MLLCs were initiated in presence or absence of 10 to 30 ng / ml IL-21 when enough cell material was available. Wells considered as "positive" for AML recognition had ≥ 5-fold higher spot numbers compared to background level.

4.4 Influence of IL-21 on the generation of AML-reactive CD8^{+} mini-cultures

In order to further improve our protocol regarding a faster and more efficient generation of leukemia-reactive T cells, the common γ-chain cytokine IL-21 was added to the mini-MLLCs because it appears to promote the development of antigen-specific T cells predominantly from the naive subset (Li et al. 2005). To analyze the impact of IL-21 in side-by-side mini-MLLCs, the cytokine was added at 10 ng / ml to 30 ng / ml or was omitted, respectively. In one related and six unrelated patient / donor pairs the AML-reactivity of the cultures was analyzed in IFN-γ split-well ELISpot assays. Again, it could be confirmed that naive T cells were a very reliable source for producing leukemia-reactive CD8^{+} T cells since from all seven donors tested leukemia-reactive T cells could be generated. The addition of IL-21 significantly increased the number of AML-reactive cultures (Figure 11). Overall, this cytokine resulted in 1.1- to 3.3-fold (mean 1.8-fold) higher numbers of AML-reactive CD8^{+} T cells compared to untreated cultures [p=0.042, n=5 (10 ng / mL IL-21); p=0.028, n=7 (30 ng / mL IL-21) by Wilcoxon signed-rank test].

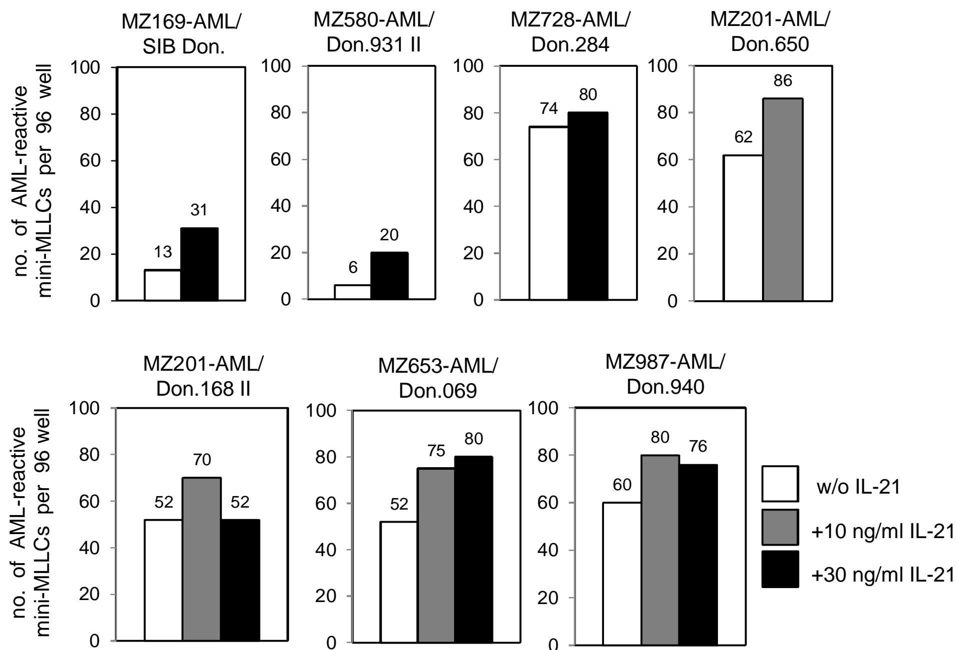

Figure 11: Number of AML-reactive mini-MLLCs generated from the naive CD8$^+$ T-cell subset in presence or absence of IL-21, respectively, analyzed by IFN-γ split-well ELISpot assays. Numbers of AML-reactive cultures per 96-well from seven different HLA-class I-matched patient / donor pairs after two to three antigen-specific restimulations with primary AML blasts are shown. Allogeneic mini-MLLCs were initiated in presence or absence of 10-30 ng / ml IL-21 to determine the influence of the cytokine in early culture times. Wells considered as “positive” for AML recognition had ≥ 5-fold higher spot numbers compared to background level.

In total, 257 mini-cultures deriving from seven patient / donor pairs with AML-reactivity in the IFN-γ split-well ELISpot assay were transferred into 48- / 24-well plates. 150 of these cultures were further expanded due to their HLA-class I-restriction. Interestingly, most of these CD8$^+$ T-cell populations grew out from mini-MLLCs treated with IL-21 (110 of 165, 67.0%) compared to plates without IL-21 (40 of 92, 43.5%). One reason for that could be a better viability of T cells treated with IL-21. To analyze this aspect, 37-38 mini-MLLCs from two different patient / donor pairs started with or without IL-21, respectively, were stained with 7-AAD and Annexin-V at culture day 21 to 28. 7-AAD intercalates in the DNA and is used to stain dead cells because it is excluded by the cell membrane of vital cells. Annexin-V stains a phosphatidylserine expressed on the cell surface of apoptotic cells. In AML-reactive CD8$^+$ T cell populations cultured with IL-21 a decrease of dead and apoptotic cells could be detected (Annexin-V: 7.7% vs. 4.0%, p=0.037; 7-AAD: 6.1% vs. 3.7%, p=0.081 by Mann-Whitney *U* test; Figure 12).

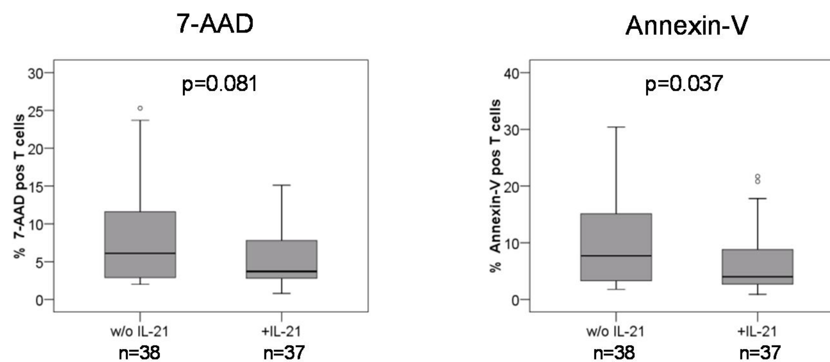

Figure 12: Amount of apoptotic and dead cells in mini-MLLCs generated in presence or absence of 10 ng / ml IL-21 analyzed by FACS. 37-38 mini-MLLCs from two different HLA-class I-matched patient / donor pairs (MZ201-AML / Don.168, MZ653-AML / Don.069) were stained with 7-AAD and Annexin-V at day 21 or 28 of culture. $CD8^+$ T cells were gated and the percentage of 7-AAD and Annexin-V positive cells was measured. P-values were calculated with the Mann-Whitney *U* test using SPSS-15.

This result indicated an advantage of survival in IL-21 treated cultures or suggested that IL-21 supported the stimulation and activation of T cells. To support these data, cell numbers of CTLs generated with or without IL-21, respectively, were determined at day 28, the first time point when cell counting was feasible. Significantly higher cell numbers were detected in cultures started with IL-21 (median $2.6x10^6$, range 0.25-$3.54x10^6$) compared to cultures without the cytokine (median $1.3x10^6$, range 0.30-$3.15x10^6$, p=0.036 by Wilcoxon signed-rank test) which correlated with the described decrease of apoptotic and dead cells in presence of IL-21.

4.5 Functional properties of mini-cultures from the naive subset *in vitro*

Of all AML-reactive mini-MLLCs expanded, 26 well proliferating $CD8^+$ T-cell cultures were selected out of six different AML patient / donor pairs from which sufficient amounts of AML blasts were available and were further characterized regarding their functional properties. First, the release of IFN-γ and TNF-α, two cytokines typically produced by $CD8^+$ T cells upon antigenic stimulation, was verified. All $CD8^+$ T-cell populations showed strong IFN-γ secretion in ELISpot assays after co-incubation with stimulator cells (Table 12). In six AML patient / donor pairs, nine CTLs were exemplarily analyzed for TNF-α production by ELISA three days after stimulation with AML blasts. All T cells produced this cytokine in a range of 8.5 to 598.6 pg / ml (median 71.0 pg / ml).

A second characteristic feature of cytotoxic T cells is the release of effector molecules like perforin and granzyme to lyse their target cells. The lytic activity of the

CTLs was tested in ^{51}Cr-release assays. As exemplarily shown for four CTLs in figure 13, all $CD8^+$ T cells showed strong cytotoxicity against either AML blasts exclusively or against AML blasts and patient-derived LCLs. Donor-derived LCLs serving as control for auto-reactivity and to ensure that the target antigen was not an EBV-derived peptide were not recognized. To exclude NK-cell activity in the $CD8^+$ T-cell populations, K562 cells were also tested. None of the CTLs was reactive to these cells. This reactivity pattern suggested LAAs or mHags as antigens. To analyze the impact of IL-21 on effector functions of $CD8^+$ T cells in long-term culture, CTLs (>day 42) that were generated in presence or absence of IL-21, respectively, were restimulated two weeks in medium supplemented with 10 ng / ml IL-21, 30 ng / ml IL-21, and without the cytokine in side-by-side cultures. Subsequently, specific lysis of target cells was tested. As shown for four representative CTLs in figure 13, no significant differences induced by IL-21 could be observed. Out of 12 T-cell cultures generated with IL-21 (Figure 13 A+B), the pre-incubation with IL-21 induced slightly stronger lysis of target cells in only one case. Also when IL-21 was added to CTLs generated without the cytokine, four out of five CTLs did not show stronger lysis if stimulated with IL-21 in later culture periods (Figure 13 C+D). One CTL showed slightly more lytic activity upon IL-21 treatment (data not shown). Similar results were obtained when CTLs were tested in IFN-γ ELISpot assays as readout system (Figure 13 E). Thus, no enhancing effect of IL-21 on antigen-specific lytic activity and IFN-γ production in long-term culture could be observed.

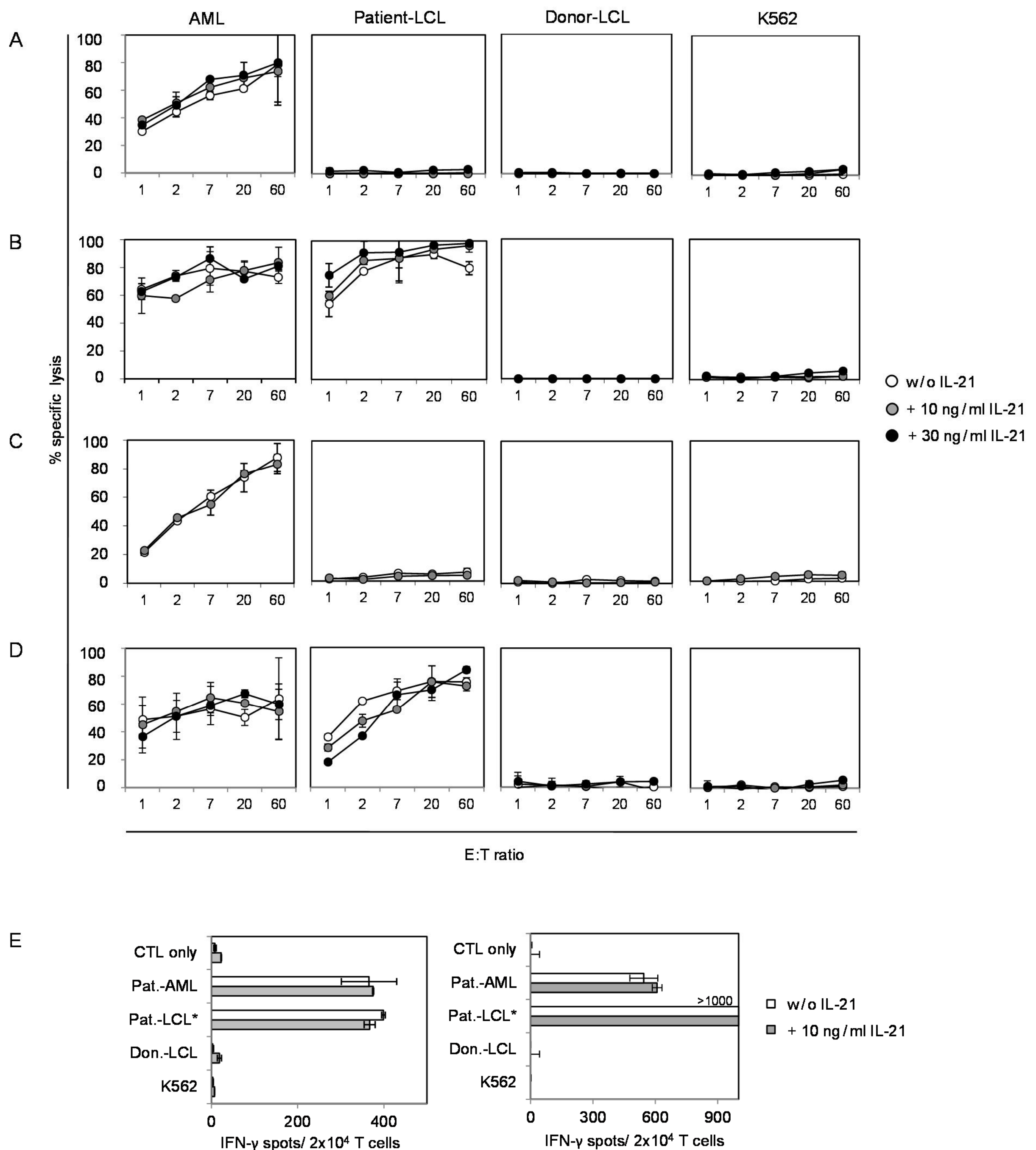

Figure 13: Lytic activity and IFN-γ release of AML-reactive CTLs restimulated for two weeks with AML blasts in presence or absence of IL-21. CTLs were tested against patient's AML and LCL cells, donor's LCLs and K562 in ^{51}Cr-release (A to D) and IFN-γ ELISpot assay (E). (A) CTL 2D8 (MZ201-AML / Don.168) was originally generated with IL-21 and recognized AML blasts exclusively. (B) CTL 7H1 (MZ529-AML / Don.730) was originally generated with IL-21 and recognized AML blasts as well as patient-derived LCL cells. (C) CTL 1E3 (MZ653-AML / Don.069) was originally generated without IL-21 and recognized AML blasts. (D) CTL 2E8 (MZ529-AML / Don.730) was originally generated without IL-21 and recognized AML blasts as well as patient-derived LCL cells. (E) CTLs 5G11 (left panel) and 5H11 (right panel) (MZ580-AML / Don.931) originally generated with IL-21 recognized AML blasts as well as patient-derived LCL cells. Cytokines were not added to the assay medium.
**5x10^3 T cells per well were used instead of 2x10^4 T cells.*

As a third feature, classical cytotoxic CD8$^+$ T cells are restricted in the recognition of their epitope by HLA-class I-molecules. By the use of saturating concentrations of

anti-HLA-class I mAbs, reactivity of all expanded T-cell populations could be blocked regardless of their generation with or without IL-21. Antibodies specific for single or groups of HLA-class I-molecules and leukemic blasts from other patients sharing at least one HLA-class I-molecule with the patient originally used for T-cell generation were further used to analyze the restriction of the mini-cultures in more detail. As potential restriction elements HLA-A01, -A02, -B13, -B15, -B57, -C03, -C06, and -C07 could be identified (Table 12).

Another characteristic property of CTLs is their proliferation after antigenic activation. By weekly stimulations with AML blasts, CTLs could be easily expanded from 10^4 cells at day 0 to numbers exceeding 10^8 within five to ten weeks. Within the first two to three weeks of culture significantly more AML-reactive mini-MLLCs grew out in the presence of IL-21 (Figure 11). However, single CTL populations started without the cytokine (e.g. 2E8 and 1E3) could also be expanded to comparably high cell numbers. To analyze the effect of the cytokine on proliferation during long-term cultures, frozen aliquots of CTLs from day 35 to 70 were restimulated over two to three weeks with AML blasts in side-by-side cultures in presence and absence of IL-21. Cell numbers were determined weekly by trypan blue staining. Of 14 CTLs generated in presence of IL-21, six grew better if restimulated with IL-21 to later time points, four had a lower growth rate, and another four showed no differences in growth. Of five CTLs generated in absence of IL-21, four CTLs first showed a better growth if restimulated with the cytokine but this effect could not be confirmed in control experiments. A clear impact of IL-21 on T-cell expansion could therefore not be detected in long-term culture.

One reason for the use of mini-MLLCs was to generate clonal T-cell populations by starting the cultures with very low cell numbers. The expression of a single TCR Vß chain indicates clonal derivation of CTLs. Flow cytometric staining with all available mAbs against 24 different Vß chains revealed that of 26 CTLs analyzed, 14 CTLs (i.e. 54%) expressed TCRs with a single Vß chain family, suggesting monoclonality. Eight T cells (i.e. 31%) showed expression of two or three Vß chains, indicating oligoclonal derivation. The major Vß chain could not be detected in four CTL cultures, probably due to the lacking availability of mAbs for their TCR. Taken together, more than 80% of the T-cell cultures were mono- or oligoclonal.

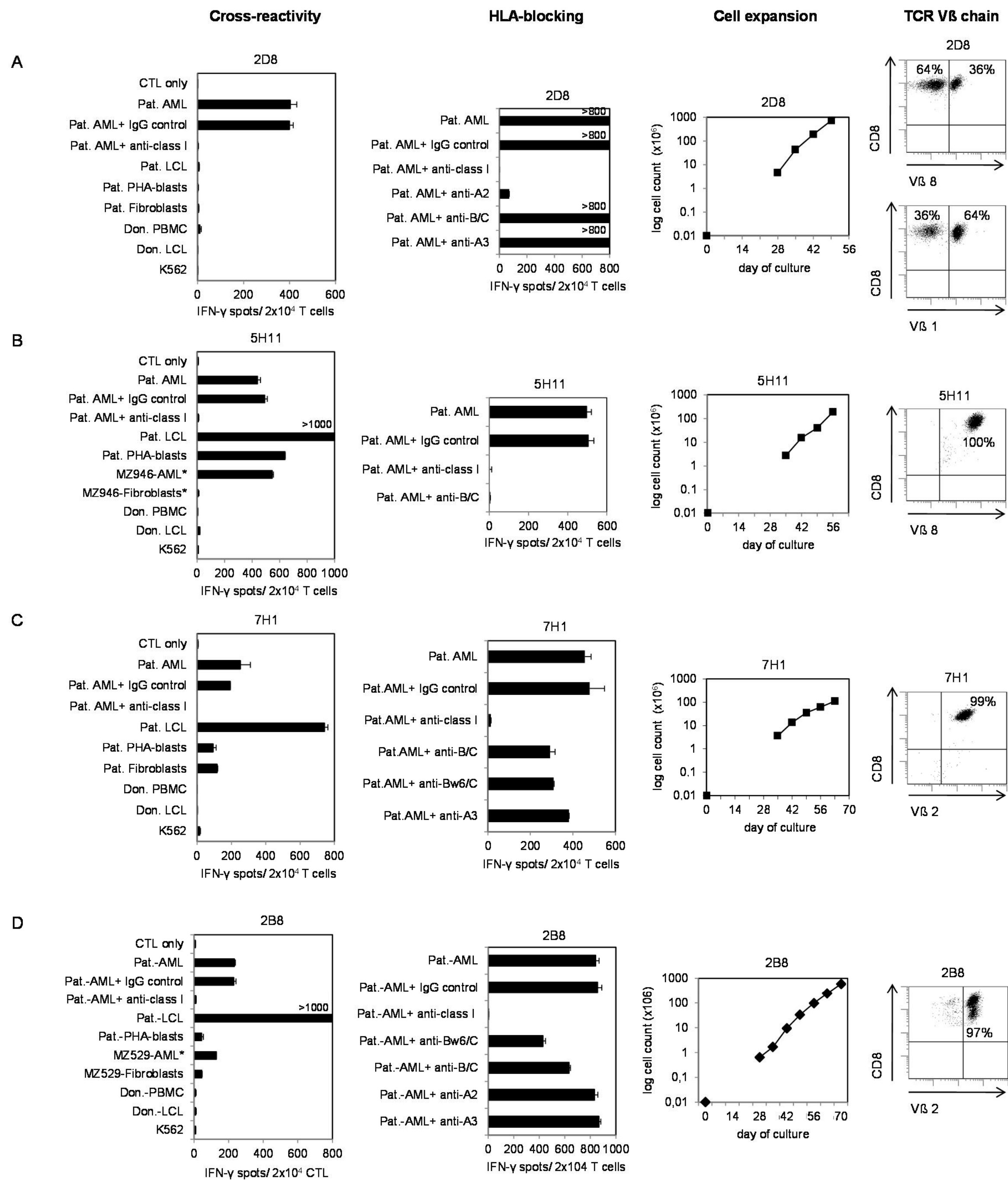

Figure 14: Functional properties of CTL clones generated from the naive CD8^{+} T-cell subset in the presence of IL-21. HLA-class I-restriction, cross-reactivity, *in vitro* expansion, and TCR Vß chains of AML-reactive CTLs are illustrated. Shown are four representative CTL clones from four different AML patient / donor pairs. (A) CTL 2D8 (MZ201-AML / Don.168) recognizing exclusively patient's AML blasts, (B) CTL 5H11 (MZ580-AML / Don.931) reacting to patient's AML blasts as well as patient-derived LCLs, (C) CTL 7H1 (MZ529-AML / Don.730) recognizing patient's hematopoietic as well as non-hematopoietic cells, (D) 2B8 (MZ987-AML / Don.940) reacting to patient's AML blasts and LCLs but not PHA T blasts. Left panel: Cross-reactivity patterns to patient's and donor's hematopoietic cells, stromal fibroblasts, and K562 cells determined by IFN-γ ELISpot assay. Cross-reactivity was defined as significant if values were >10% of AML reactivity. Middle panel: Numbers of expanding cells in mini-MLLCs were determined weekly. Right panel: FACS characterization of the TCR Vß chain. CTLs were defined as monoclonal if > 90% of cells expressed a single TCR Vβ chain.

**CTL 5H11 cross-reacted with AML blasts from patient MZ946. Because bone marrow aspirate from patient MZ580 (whose AML cells were used for stimulation) was not available, stromal fibroblasts isolated from patient MZ946 were used.*

**CTL 2B8 cross-reacted with AML blasts from patient MZ529. Because bone marrow aspirate from patient MZ987 (whose AML cells were used for stimulation) was not available, stromal fibroblasts isolated from patient MZ529 were used.*

4.6 Cross-reactivity of CTL clones *in vitro*

The reactivity of all CTLs described could be blocked by anti-HLA-class I-mAbs, indicating their recognition of patient-derived peptides presented by HLA-class I-molecules. More detailed analyses of recognized cell types could reveal information about a preferential expression of the epitope on hematopoietic cells or leukemic blasts which could be favorable to achieve a GVL effect. The recognition of non-hematopoietic cells suggests that this CTL has the potential of inducing GVHD. Because HLA-A*02:01 the most frequent HLA-class I-molecule in the caucasian population, there are many known T-cell antigens which are presented by this molecule. To analyze the reactivity of the T-cell clones to several of these known antigenic targets, all T cells from HLA-A*02:01 positive patients were incubated with blocking mAbs that inhibit the recognition of HLA-A2 restricted TCRs. The recognition of AML blasts could be blocked in seven T-cell clones from two patient / donor pairs (Table 12) whose reactivity to epitopes encoded by the HLA-A*02:01-binding LAA WT-1 (p126-134), Proteinase-3 (p169-177), PRAME (p100-108), and the HLA-A*02:01-binding mHag HA-1 (p166-174) was further analyzed. Peptide epitopes were loaded on T2 cells and recognition was tested in an IFN-γ ELISpot assay. None of the CTLs reacted to any of the epitopes (data not shown). To get further information about the distribution of the antigens recognized by the CTLs, recognition of patient-derived stromal fibroblasts as non-hematopoietic cells and PHA-activated T-cell blasts (T blasts) as well as donor's PBMCs and, if available, monocytes were tested. Out of 26 T cells analyzed in total, five CTLs recognizing patient's AML blasts, LCLs, and T blasts also showed significant reactivity to patient's stromal fibroblasts (e.g. 7H1, Figure 14C). This recognition pattern indicated that these CTLs may recognize recipient mHags broadly expressed in hematopoietic and non-hematopoietic cells which could be a hint that these CTLs might induce GVHD in the patient. Interestingly, three CTLs recognized AML blasts, LCLs, and stromal fibroblasts but not T blasts from the same patient (e.g. 2B8, Figure 14D), suggesting antigens with low or absent expression in the T-cell lineage. A single CTL reactive to patient's AML blasts but not LCLs and stromal fibroblasts also recognized monocytes to a minor extent, probably consistent with a myeloid-associated antigen overexpressed in AML cells as target. Another CTL only reactive to patient's AML blasts showed recognition of donor PBMCs what could be explained by the presence of monocytes. Two CTLs recognizing patient's AML blasts and LCLs also showed reactivity to patient's T

blasts but not to stromal fibroblasts (e.g. 5H11, Figure 14B), suggesting reactivity to recipient mHags with hematopoiesis-restricted expression. Remarkably, 14 CTLs exclusively showed reactivity to patient's AML blasts and to none of the other targets (e.g. 2D8, Figure 14A). This recognition pattern could indicate LAAs as target structures which may be overexpressed or aberrantly expressed by leukemic blasts. A complete overview of the characteristics of all CTLs is shown in table 12. The presentation of the T-cell antigens in leukemic blasts of other patients was also analyzed to find out if the peptides are specific for only one patient or if they are present in blasts of more patients. Therefore, IFN-γ production of CTLs was tested which were incubated with leukemic blasts from other patients that shared the HLA-class I-molecule with the original patient that is responsible for T-cell recognition. Appropriate patients were selected from the leukemia-bank of the KFO183 containing cells from more than 200 patients. Out of 26 CTLs analyzed from six different AML patient / donor pairs, only four CTLs did not cross-react with leukemic blasts from other patients. The remaining 22 CTLs recognized at least blasts from one other patient. Briefly, 11 CTLs recognized up to 20% of the tested leukemic blast from other patients, 8 CTLs reacted with 21% to 40% and 3 CTLs released IFN-γ to 41% up to more than 60% of leukemic samples. Representative data from one HLA-C07-restricted (1E3 from MZ653-AML / Don.069) and two HLA-A02-restricted T cells (2D8 from MZ201-AML / Don.168 and 2D3 from MZ201-AML / Don.650) cross-reacting with AML blasts from other patients are shown in figure 15.

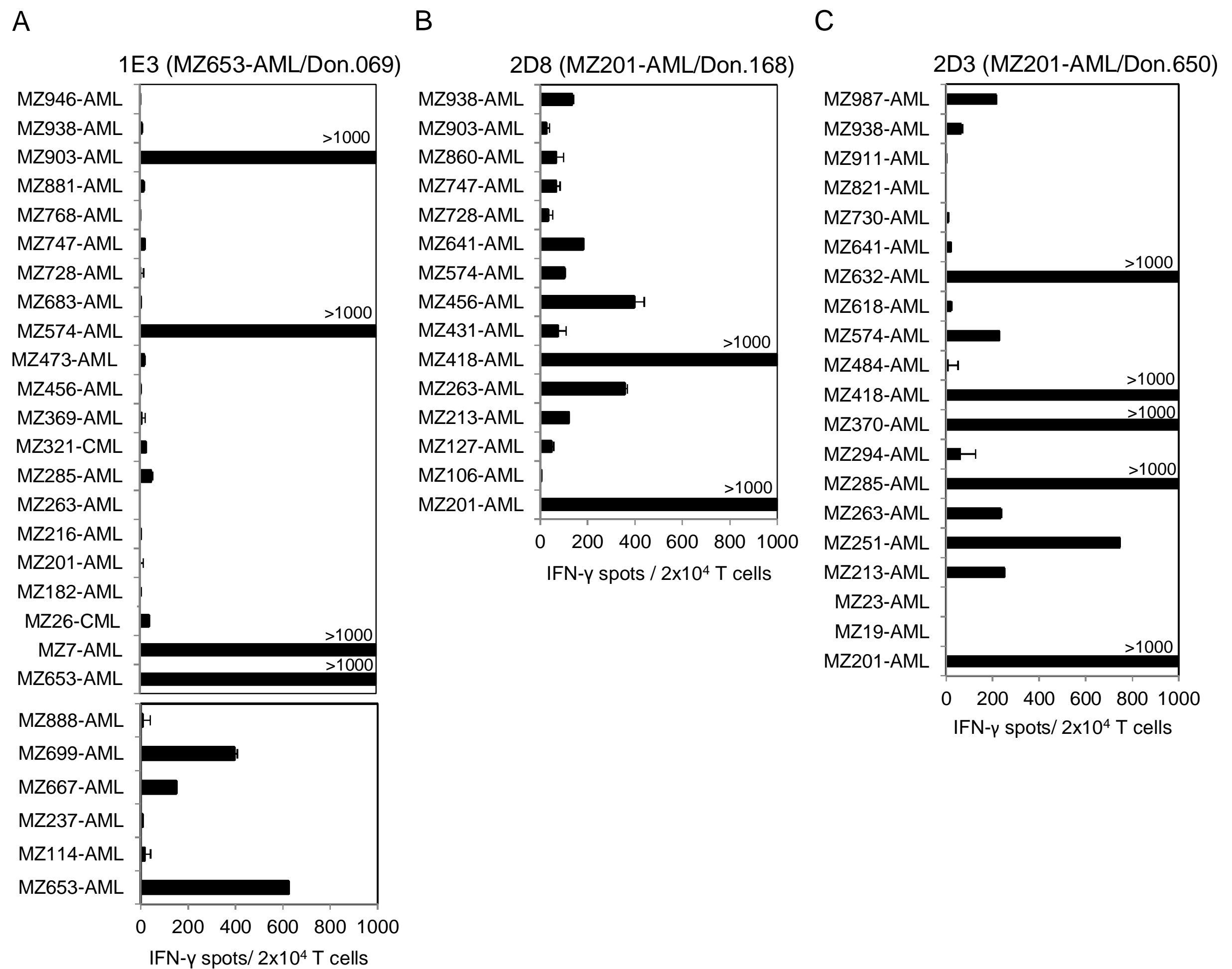

Figure 15: Cross-reactivity of CTLs with AML blasts of other patients who shared HLA-class I-molecules with the original patient used for primary stimulation. Reactivity to AML blasts from other patients was analyzed by IFN-γ ELISpot assays. Exemplarily shown are three CTLs from three different patient / donor pairs. Cross-reactivity was defined as significant if values were >10% of AML reactivity of the original patient.

Table 12:Charaterization of CD8+ AML-reactive CTL clones and oligoclonal lines derived from six patient / donor pairs with complete HLA-class I-match.

Model	CTL	HLA-class I-restriction	Cytolytic activity to patient's cells (% lysis at E/T 7:1)		IFN-γ production to patient's cells (% of AML-reactivity)		IFN-γ production to cells with complete HLA-class I-match (% of AML-reactivity)		IFN-γ production to leukemias with shared HLA-class I-alleles (>100 IFN-γ spots/ 2x10^4 CTL)		T-cell receptor
			AML	LCL	Fibroblasts[b]	T blasts[b]	Don. PBMC[b]	Monocytes[b]	AML+CML[c]	ALL+CLL[c]	Vß chain[d]
MZ201-AML/ Donor 168	2D5	B/C	36%	0.5%	0%	0%	<10%	<10%	0/23	n.t.	Vß 20 (99%)
	2D8	A02	68%	1%	0%	0%	<5%	0%	8/15	n.t.	Vß 1 (64%), Vß 8 (36%)
	2E3	A02/Cw07	51%	6%	0%	0%	<10%	<10%	9/27	n.t.	Vß 13.1 (56%), Vß 8 (33%)
	2E4	B/C	68%	1%	<10%	0%	0%	20%	0/23	n.t.	Vß 13.6 (100%)
	3G9	A01/A02/Cw07	48%	5%	0%	0%	<10%	<10%	18/28	n.t.	Vß 11 (43%)
MZ201-AML/ Donor 650	2C7	A01/A02	44%	1%	<5%	0%	n.t.	n.t.	10/28	n.t.	Vß 12 (48%), Vß 14 (24%), Vß 18 (21%)
	2D3	A02	32%	7%	<5%	0%	n.t.	n.t.	9/19	n.t.	Vß 3 (99%)
	2E7	A02/B/C	31%	8%	0%	0%	n.t.	n.t.	4/30	n.t.	Vß 14 (56%), Vß 2 (39%)
	2G8	A02	57%	8%	0%	0%	n.t.	n.t.	7/19	n.t.	Vß 3 (99%)
MZ529-AML/ Donor 730	2E8[a]	B15	57%	69%	25%	<10%	0%	n.t.	0/4	1/3	Vß 17 (23%), Vß14 (47%), Vß13.2 (30%)
	7E1	B15/Cw03	85%	100%	67%	31%	0%	n.t.	0/8	1/3	Vß 2 (100%)
	7G9	B15	100%	100%	46%	22%	0%	n.t.	0/4	1/3	Vß 11 (100%)
	7H1	B15	86%	92%	46%	37%	0%	n.t.	0/4	1/3	Vß 2 (99%)
MZ580-AML/ Donor 931	5G11	B57/Cw06	32%	80%	<5%	>100%	0%	n.t.	2/9	0/3	Vß 7.2 (88%), Vß 8 (10%)
	5H11	B57/Cw06	32%	89%	0%	>100%	0%	n.t.	2/9	0/3	Vß 8 (100%)
MZ653-AML/ Donor 069	1E3[a]	Cw07	61%	6%	0%	0%	0%	n.t.	5/22	0/2	n.d.
	4B2	B/C	36%	56%	50%	<10%	<5%	n.t.	9/25	2/3	Vß 11 (96%)
	4F3	Cw06/Cw07	66%	26%	13%	10%	<5%	n.t.	6/25	2/3	Vß 4 (72%), Vß17 (9%)
	5B2	B/C	22%	0%	0%	0%	<5%	n.t.	5/25	0/3	Vß 21.3 (92%)
	5C1	B/C	38%	1%	<5%	0%	<5%	n.t.	4/25	2/3	Vß 14 (86%), Vß 16 (10%)
	7A3	B/C	28%	2%	0%	0%	<5%	n.t.	0/6	n.t.	Vß 21.3 (72%), Vß 3 (25%)
	7C12	B/C	18%	0%	<10%	0%	24%	n.t.	0/26	0/3	Vß 8 (100%)
	7H1	Cw07	44%	0%	<5%	0%	0%	n.t.	6/23	1/2	Vß 23 (51%)
	8F11	B13/Cw06	73%	0%	0%	0%	<5%	n.t.	1/4	0/3	Vß 7.2 (94%)
MZ987-AML/ Donor 940	2B8	B15	50%	92%	67%	<10%	0%	n.t.	1/5	0/3	Vß 2 (97%)
	2D10	B15	57%	77%	35%	19%	0%	n.t.	1/5	0/3	Vß 2 (100%)

[a] CTLs generated without IL-21, [b] reactivity to fibroblasts, T blasts, monocytes, or PBMCs was defined as significant if value is >10% of AML-reactivity, [c] x/y means: CTL reacted with x primary leukemias out of y leukemias tested, [d] CTL was defined as monoclonal if >90% of cells expressed a single T-cell receptor Vß chain
AML acute myeloid leukemia, *LCL* EBV-transformed B lymphoblastoid-cell line, *ALL* acute lymphatic leukemia, *CLL* chronic lymphatic leukemia, *CML* chronic myeloid leukemia, *T blasts* PHA-activated T-cell blasts, *n.d.* not defined, *n.t.* not tested

4.7 Phenotypic features of CTL clones *in vitro*

In order to analyze the presence of major T cell surface markers, CTLs were stained for markers of differentiation (CD45RA, CD45RO, and CD57), homing (CCR7, CD62L, and CXCR4), responsiveness to cytokines (CD127), and co-stimulation (CD28 and CD27). The naive T cells of the starting population at day 0 expressed the markers CD45RA, CCR7, CD28, CD27, CD127, and CXCR4, and were negative or low positive for the memory markers CD45RO, CD57, and CD95. Also T cells expressing CD62L were enriched (Figure 8). To follow the course of these the molecules to later culture periods, 49 AML-reactive CTLs from seven patient / donor pairs were analyzed between culture day 21 and 91. CTLs regularly expressed the markers CD45RO, CD45RA, CD27, CD57, CD95, and CXCR4 at intermediate to high levels during the whole observation period. The expression of the other surface molecules analyzed was down regulated with increasing culture time. The chemokine receptor CCR7 was consistently expressed at intermediate to low levels until day 28 to 35. CD62L was regularly present at the cell surface until day 35 to 42 but it could be detected on single CTLs for more than 10 weeks of culture. The IL-7 receptor alpha chain (CD127) and CD28 were expressed until day 49 to 56, respectively. Representative data of a single CTL at day 35 is shown in figure 16.

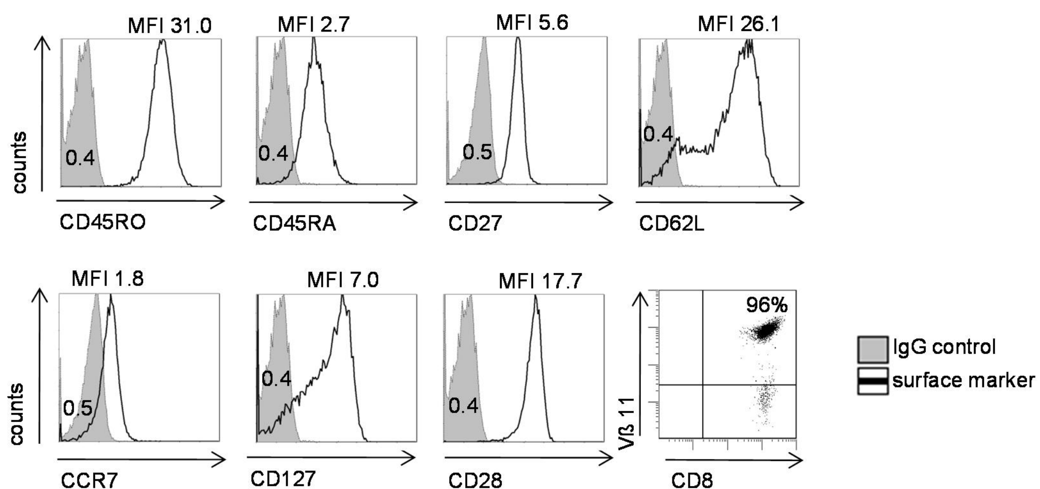

Figure 16: Characterization of differentiation markers on CTL clones generated in presence of IL-21 analyzed by FACS. Shown are the stainings for several T-cell differentiation markers and the respective isotype control as well as the co-staining for CD8 and TCR Vβ 11 of CTL clone 4B2 (MZ653-AML / Don.069) at day 35 of culture.

To analyze the impact of IL-21 on phenotypic features, further comparisons of CTLs which were isolated from three AML patient / donor pairs that were stimulated for three to six weeks with or without the cytokine, respectively, were performed. As shown in figure 17, the expression of CD45RO was significantly reduced (p=0.01 at day 35 and 42 by Mann-Whitney *U* test) on T cells generated in presence of IL-21 with a concomitant higher expression of CD45RA. CD62L showed an increased expression from day 28 to 35 on CTLs treated with IL-21. The chemokine receptor CXCR4 in contrast was less expressed on cells generated with IL-21 (p=0.03 between day 35 and 42 by Mann-Whitney *U* test) but it was nevertheless present on the surface of these cells up to day 70 of culture. In addition, significantly more CD28 (p=0.04 between day 21 and 35 by Mann-Whitney *U* test) and also more CD127 (p=0.03 at day 28 by Mann-Whitney *U* test) was present on the surface of cells cultured with IL-21 (Figure 17). There was no enhancing effect of IL-21 on expression levels of CD27, CCR7, CD57, and CD95 during the entire observation period (data not shown).

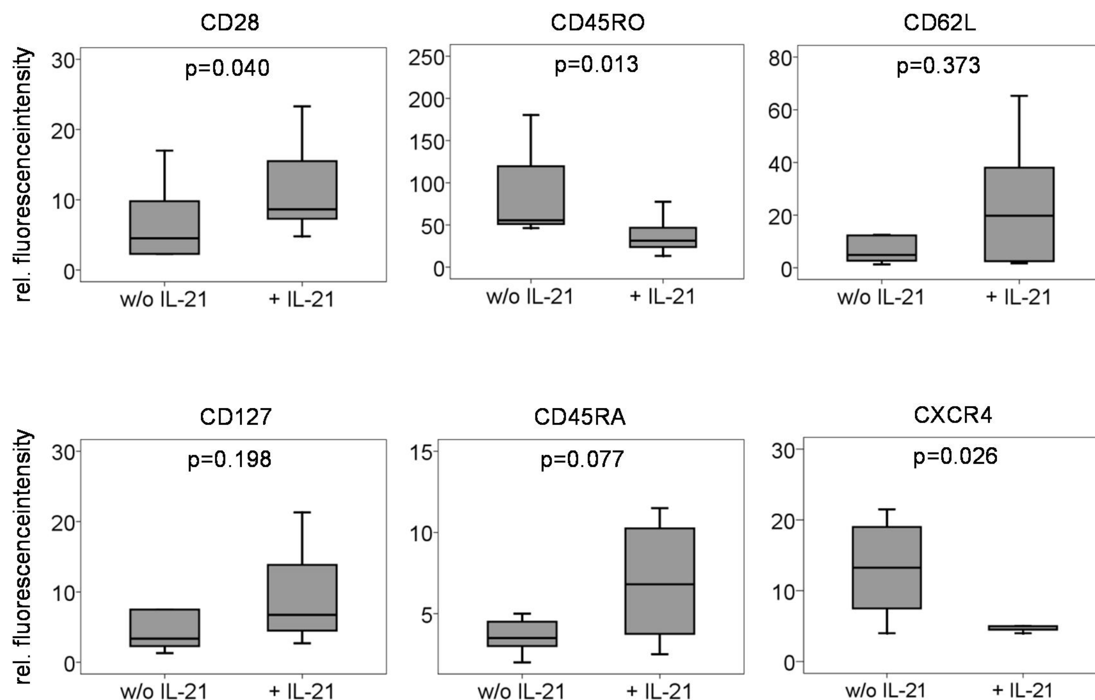

Figure 17: Influence of IL-21 on the expression of differentiation markers on CTL clones analyzed by FACS. Box plots showing the median expression level of surface molecules on 5 to 11 CTLs generated in presence or absence of IL-21, respectively, in three AML patient / donor pairs analyzed at day 35 of culture. The expression level is shown as relative fluorescence intensity calculated from the MFI (median fluorescence intensity) value of the relevant staining divided by the MFI value of the respective IgG isotype control staining. All control stainings had absolute MFI values in the range of 0.2 to 0.8 depending on the used fluorochrome, analyzed with EXPO32TM software. P-values were calculated with the Mann-Whitney U test.

4.8 Functional properties of CTL clones *in vivo*

Specific allogeneic T-cell therapy leading to improved GVL effects after adoptive transfer requires the efficient *in vitro* generation of leukemia-reactive T cells and humanized mouse models to analyze the anti-leukemic potential of transferred cells *in vivo*. Therefore, the group of PD Dr. Udo F. Hartwig (Dep. of Med. III, University Medical Centre Mainz) recently established an immunodeficient NSG mouse model that allows reliable engraftment of human primary AML blasts, particularly those with high-risk FLT3-ITD mutations (Brunk 2011). To investigate *in vivo* functional properties of $CD8^+$ T-cell clones generated from the naive subset in mini-MLLCs, T cells were transferred into AML-engrafted NSG mice. Because there were substantial differences in the engraftment rate of AML blasts from different patients, only CTLs recognizing the reliably engrafting leukemic cells from patient MZ580 and MZ667 were used for adoptive transfer experiments.

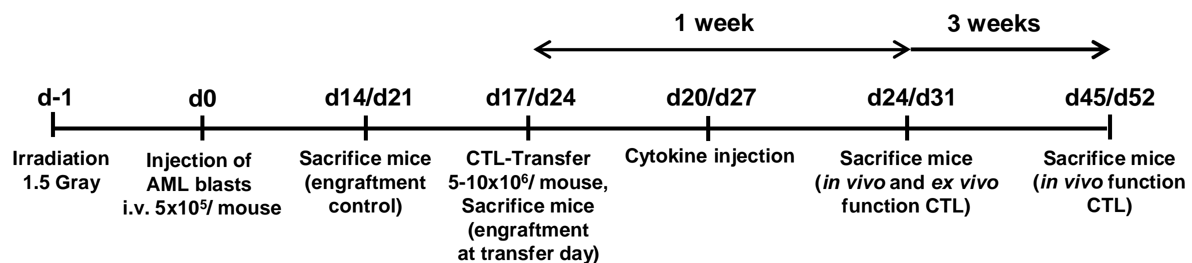

Figure 18: Flow chart of AML-engraftment and CTL transfer into NSG mice to test *in vivo* reactivity of CTLs generated from the naive subset in mini-MLLCs. Mice were engrafted with $5x10^5$ primary AML blasts for 17 days (MZ580-AML) or 24 days (MZ667-AML), respectively. AML-reactive CTL clones were transferred at 5 to $10x10^6$ cells per mouse in parallel with human IL-2, IL-7-Fc, and IL-15. IL-2 and IL-15 were additionally injected three days after transfer. Mice were sacrificed one or four weeks later to analyze the AML infiltration and presence of human T cells.

As illustrated in figure 18, NSG mice (6 to 8 weeks old) were sublethally irradiated (150 cGy) and injected with $5x10^5$ primary AML blasts i.v. into the tail vein. To achieve 1% to 3% human leukemic blasts in the murine bone marrow, resembling minimal residual disease, mice were engrafted for 17 to 24 days depending on AML samples used. Subsequently, 5 to $10x10^6$ AML-reactive and control CTLs cultured for 42 to 56 days were transfused intravenously three days after the last antigen-specific restimulation *in vitro*. Mice were supplemented with human IL-2, IL-7-Fc, and IL-15 in parallel to CTL transfer. IL-2 and IL-15 were additionally injected three days after transfer to improve the milieu for the CTLs. Mice receiving no CTLs but the cytokines served as engraftment-controls. One and four weeks after T-cell transfer, mice were

sacrificed and bone marrow, spleen, as well as peripheral blood were analyzed with regard to the presence of human $CD45^+$ $CD33^+$ AML blasts and $CD45^+$ $CD3^+$ $CD8^+$ T cells. Additionally, in one model *ex vivo* function of transferred human T cells re-isolated from spleens one week after transfer could be analyzed for their capability to recognize AML blasts. As therapeutical model CTL clone 5H11 (day 59) from MZ580-AML / Don. 931 was transferred at $5x10^6$ T cells / mouse into mice that were engrafted with MZ580-AML. T cells expressed 100% TCR Vß8 indicating monoclonality and were restricted by HLA B*57:01 or C*06:02 as determined by HLA-blocking experiments and cross-reactivity tests. 5H11 was reactive to patient's AML blasts, LCLs, and T blasts but not to stromal fibroblasts, indicating a hematopoietic-restricted antigen as target structure. Further, *in vivo* function of CTLs 1E3 (day 52) and 5B2 (day 45) from MZ653-AML / Don.069, cross-reacting with HLA-C*07:01-matched AML blasts from patient MZ667 (Figure 20), were analyzed in mice that were engrafted with MZ667-AML. Both CTLs were transferred at $10x10^6$ T cells / mouse. CTL clone 5B2 expressed 92% TCR Vß21.3 and was restricted by patient's B / C alleles, most probably by HLA-C*07:01. The clonality of 1E3 could not be confirmed because all available anti-Vß-mAbs showed negative results. CTL 1E3 was restricted by C*07:01. Both CTLs were exclusively reactive to patient's AML blasts and not to LCLs, T blasts, and stromal fibroblasts, suggesting AML-associated target structures. More detailed information about the CTLs is summarized in table 12. To ensure that anti-leukemic effects of the AML-reactive CTLs were no unspecific events caused only by the presence of any human T cell, Melan A-reactive (i.e. a melanoma antigen) control CTLs (day 45) generated from the naive subset of Don.689 by stimulation with peptide-loaded APCs were used. These CTLs did not recognize leukemic cells from MZ580-AML and MZ667-AML (Figure 20). At day of transfer, cells were stained with a Melan A-peptide (p26-35) / HLA-tetramer. 90% of the cells showed positive results for tetramer-binding (data not shown). Adoptively transferred CTLs are summarized in table 13.

Table 13: Overview of AML-reactive CTLs and control CTLs generated from the naive subset of healthy donors that were adoptively transferred into AML-engrafted NSG mice.

CTL	Day of culture at transfer	T-cell donor	Stimulator cells used for T-cell generation	Cross-reactivity for engrafting AMLs	Therapeutical model	Purpose of transfer
5H11	59	Donor931	MZ580-AML blasts	-	MZ580-AML	Therapy
1E3	52	Donor069	MZ653-AML blasts	MZ667-AML	MZ667-AML	Therapy
5B2	45		MZ653-AML blasts	MZ667-AML	MZ667-AML	Therapy
Melan A	45	Donor689	Autologous APCs and T2 cells loaded with Melan A-peptide (ELAGIGILTV, p26-35)	-	MZ580-AML+ MZ667-AML	Control

At day of T-cell transfer into the AML-engrafted mice, lytic activity and expression of surface markers of CTLs were analyzed by ^{51}Cr-release assay and FACS staining, respectively. Surface molecules important for homing and co-stimulation of T cells like the chemokine receptor CXCR4, the L-selectin CD62L, the chemokine receptors CCR7 and CD127, as well as the co-stimulatory molecules CD27 and CD28 were stained. CD62L was expressed on CTL 5B2 at transfer day. It was not present on the surface of 1E3 and 5H11 which had been cultured for longer time periods. CCR7 and CD127 were only slightly or not expressed. CD28 was only expressed by 5H11. CXCR4 and CD27 were strongly expressed by all CTLs. All CTLs expressed the isoform CD45RO, showing their antigen-experienced phenotype. Melan A-specific control CTLs showed a comparable expression pattern (Figure 19).

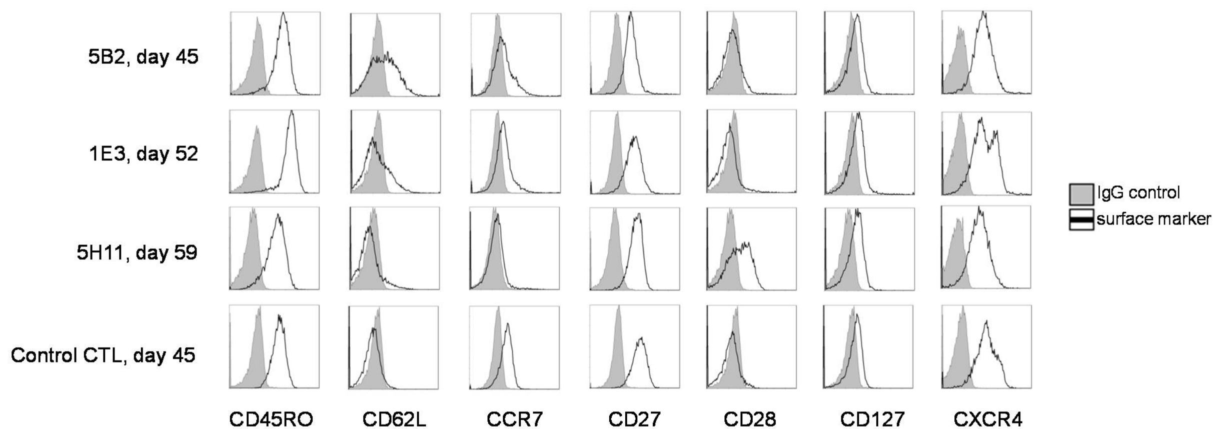

Figure 19: Phenotypic properties of CTLs at day of transfer into AML-engrafted NSG mice analyzed by FACS. CTL 5B2 and 1E3 (MZ653-AML / Don.069), CTL 5H11 (MZ580-AML / Don.931), and Melan A-specific control CTL (Don.689) were analyzed. $CD8^+$ T cells were gated and the stainings for several T-cell homing and co-stimulatory molecules and the respective isotype control are shown.

In a ^{51}Cr-release assay the CTLs 1E3 and 5B2 showed specific lysis to AML blasts from patient MZ667, the blasts that mice were engrafted with. Additionally, blasts from patient MZ653 were recognized which were used in an HLA-class I-matched setting for primary stimulation. LCLs of patient and donor origin were not lysed. CTL 5H11 had lytic activity against patient's AML blasts. Patient's LCLs but not donor's LCLs were also recognized. Control CTLs showed a very specific lysis of Melan A-peptide loaded T2 cells (Figure 20). These data confirmed that *in vitro* functionally active T cells were transferred into the mice.

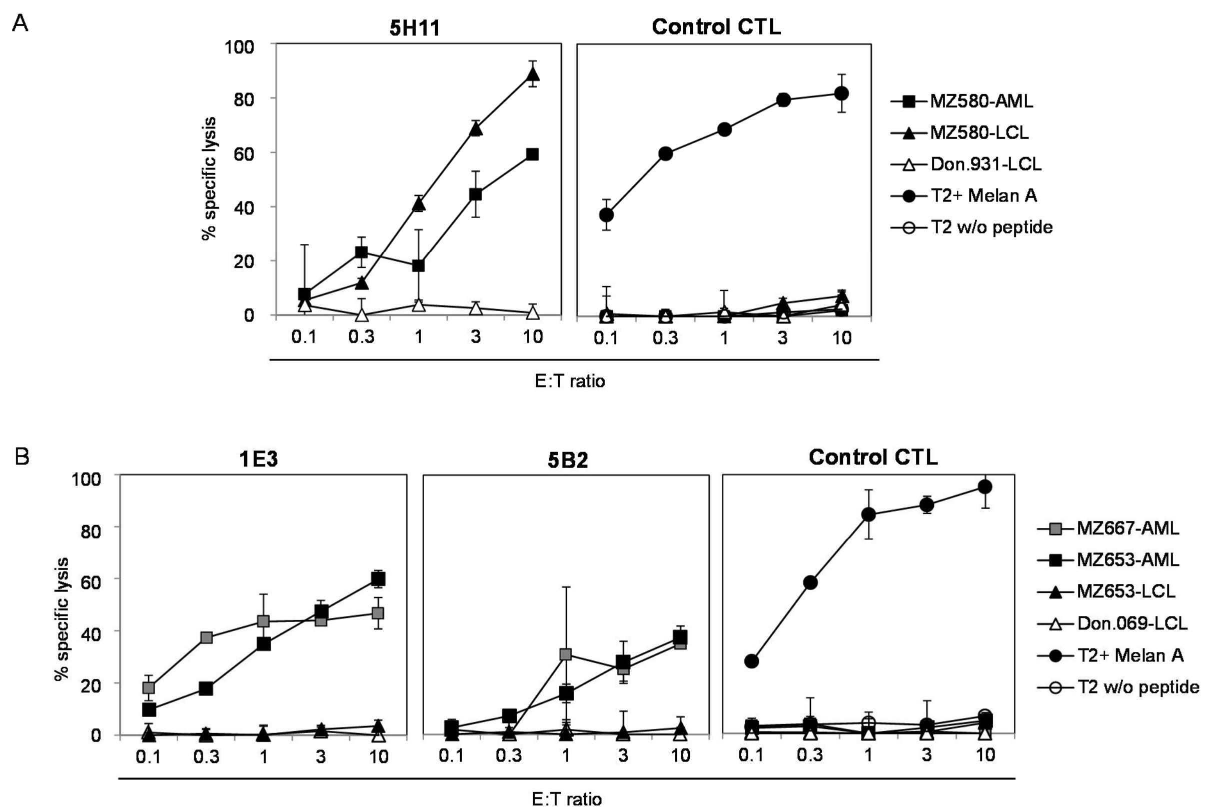

Figure 20: Cytotoxicity of CTLs at day of transfer into AML-engrafted NSG mice analyzed by ^{51}Cr-release assays. (A) Lytic activity of CTL 5H11 (MZ580-AML / Don.931) and Melan A-reactive control CTL (Don.689) at day of transfer in MZ580-AML engrafted mice. (B) Lytic activity of CTL 1E3, 5B2 (MZ653-AML / Don.069), and control CTL (Don.689) at day of transfer in MZ667-AML engrafted mice. Recognition of patient's AML blasts and LCLs as well as donor's LCLs was analyzed. 1E3 and 5B2 were also tested for cross-reactivity of AML blasts from HLA-C*07:01 matched patient MZ667. Lytic activity of control CTL was additionally tested with peptide-loaded T2 cells.

One week after T-cell transfer, the mice were sacrificed and the presence of human CD8^{+} T cells in murine bone marrow, spleen, and peripheral blood was measured. To ensure that no T cells grew out of patient's leukemic blast samples that had been injected to engraft the mice, mice that received no CTLs were additionally analyzed. In the murine bone marrow all human CTLs were detectable one week after transfer [median percentage of T cells: 1E3 0.8% (n=5); 5B2 3.4% (n=5); 5H11 0.1% (n=5);

Control CTL 0.9% (n=10)]. In the spleen even more human CD8+ T cells could be detected [median percentage of T cells: 1E3 2.9% (n=5); 5B2 6.4% (n=5); 5H11 0.4% (n=5); Control CTL 4.1% (n=10)]. Except for 5H11, CTLs could also be recovered from peripheral blood [median percentage of T cells: 1E3 1.0% (n=5); 5B2 6.1% (n=5); Control CTL 0.1% (n=10)]. In organs of the mice which were not transfused with CTLs no human T cells could be detected (Figure 21). Four weeks after T-cell transfer only few cells from CTL clone 5B2 could be recovered in single mice, whereas all other CTLs were not detectable (data not shown). These data strongly suggested that the AML-reactive adoptively transferred CTLs could reach primary and secondary lymphoid organs in the NSG mice and survived at least one week in the animals.

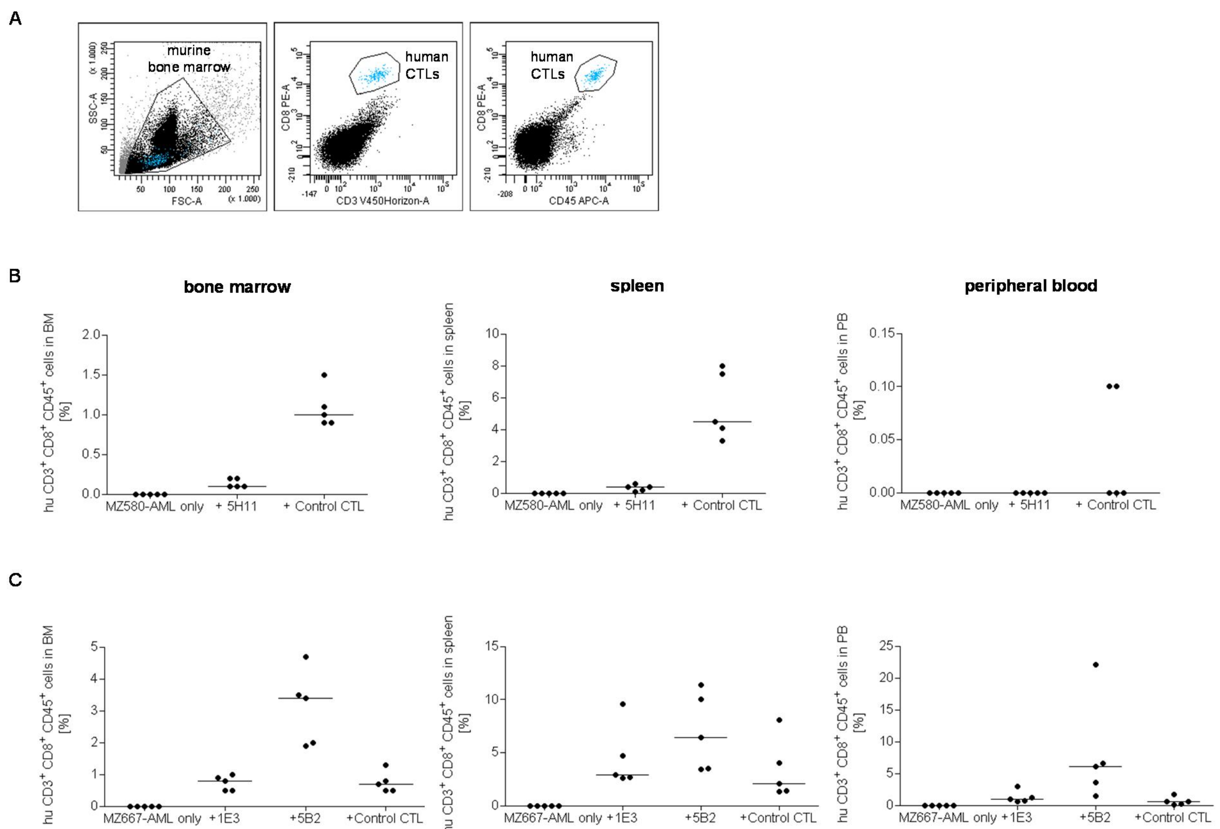

Figure 21: Presence of human CTLs in the murine bone marrow, spleen, and peripheral blood one week after CTL transfer into AML-engrafted NSG mice analyzed by FACS. Mice were engrafted with primary AML blasts until 1% to 3% were present in bone marrow. Subsequently, AML-reactive CTLs were transferred into the engrafted NSG mice. (A) FACS staining to detect human T cells exemplarily shown for bone marrow of one mouse. (B) NSG mice engrafted with MZ580-AML were treated with CTL 5H11 (MZ580-AML / Don.931). (C) NSG mice engrafted with MZ667-AML were injected with CTL 1E3 or 5B2 (MZ653-AML / Don.069). Melan A-reactive CTLs (Don.689) were included as unspecific control cells. One week after transplantation the presence of human CD3+ CD8+ CD45+ T cells in bone marrow, spleen, and peripheral blood was analyzed. The median values (n=5) are shown as bars.

Functional properties of human AML-reactive CTLs that could be recovered from adoptively transferred mice were analyzed. Therefore, CTLs 1E3 and 5B2, re-isolated from spleens one week after transfer, were restimulated twice with AML blasts from patient MZ653 in cytokine containing medium. CTLs were re-tested in ^{51}Cr-release and IFN-γ assays three days after the second *ex vivo* stimulation. Both CTLs were still reactive towards AML blasts to different extents. 1E3 showed persistent lytic activity against AML blasts of patient MZ667 as well as against those of patient MZ653 at a minor level. Moreover, IFN-γ production could be detected. 5B2 had a minor lytic activity but showed significant IFN-γ production upon stimulation with blasts from patient MZ653. AML blasts from patient MZ728 expressing shared HLA-molecules were not recognized by both CTLs indicating specific reactivity. Control CTLs had a very strong and specific lytic activity and cytokine release when stimulated with peptide-loaded T2 cells (Figure 22), demonstrating that all human CTLs were still functional after circulating one week in the mice.

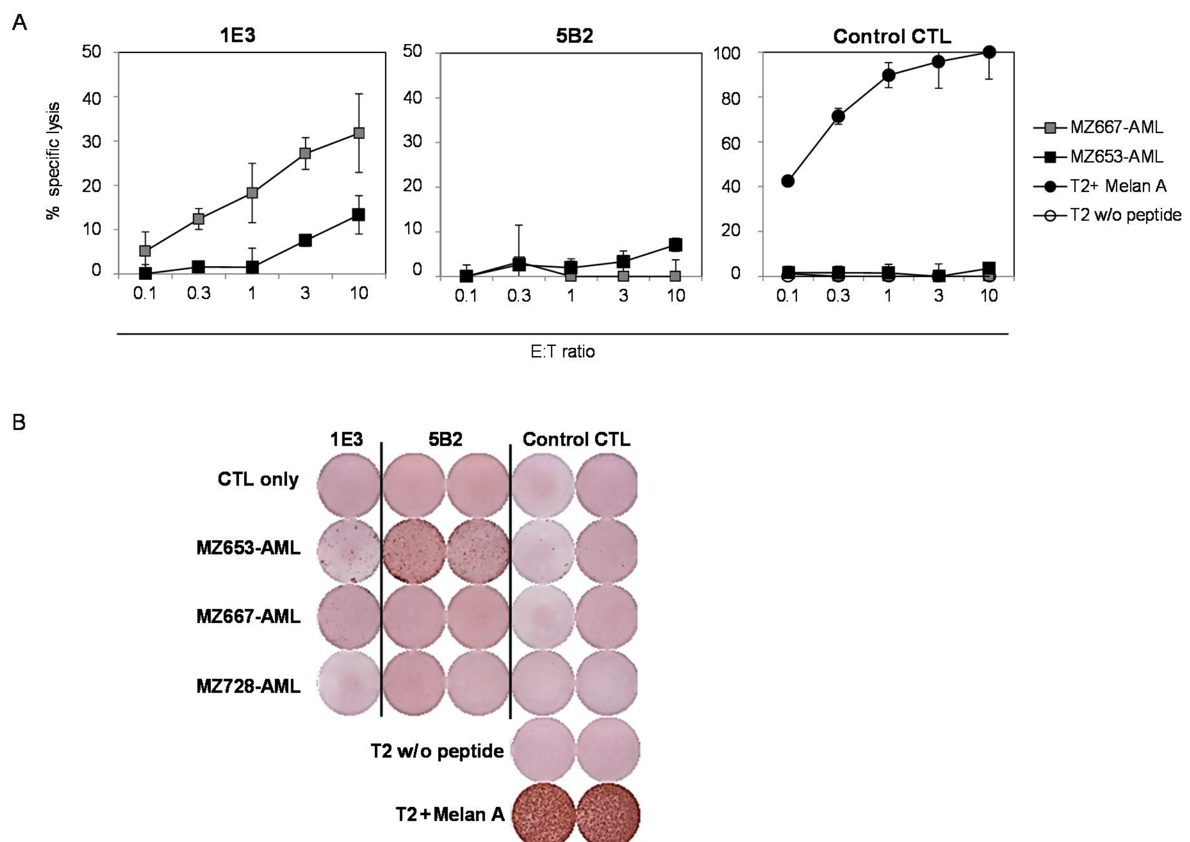

Figure 22: Functional *ex vivo* properties of CTL 1E3 and 5B2 re-isolated from spleens of NSG mice one week after CTL transfer and restimulated twice *in vitro* with AML blasts. Mice were engrafted with primary AML blasts until 1% to 3% were present in bone marrow. Subsequently, AML-reactive CTLs were transferred into the engrafted NSG mice. One week after transplantation the spleens were isolated, pooled from all five mice per group and frozen. Thawed cells were restimulated with MZ653-AML blasts twice. Analysis were performed three days after last antigen-specific restimulation by (A) ^{51}Cr-release at indicated effector-to-target ratios and (B) by IFN-γ ELISpot assay with 1x10^{4} T cells / well of 1E3 and 2x10^{4} T cells / well of 5B2 and control CTL.

Finally, the impact of human AML-reactive CTLs on the AML infiltration in organs of the mice was determined. Mice were sacrificed one and four weeks after T-cell transfer and human CD33$^+$ CD45$^+$ AML blasts were stained in FACS analyzes. The animals showed no signs of a xenogenic GVHD caused by the presence of human CD8$^+$ T cells. In general, a reduced percentage of leukemic blasts in the mice could be detected after a single injection of the AML-reactive CTLs. As illustrated in figure 23, in presence of 5H11 a significant reduction of AML blasts in the murine bone marrow was measurable at day seven after transfer of CTLs [median percentage of AML blasts: AML only 1.4% (n=5); 5H11 0.3% (n=5); Control CTL 1.2% (n=5). p-values: AML only versus 5H11 p=0.016; 5H11 versus control CTL p=0.056; calculated by Mann-Whitney *U* test]. At that time point AML blasts were could not be detected in murine spleen and peripheral blood (median 0% to 0.1%), hence T-cell mediated effects were not measurable in these compartments. In mice that were engrafted with MZ667-AML and treated with CTL 1E3 or 5B2 no significant reduction could be detected after one week (data not shown).

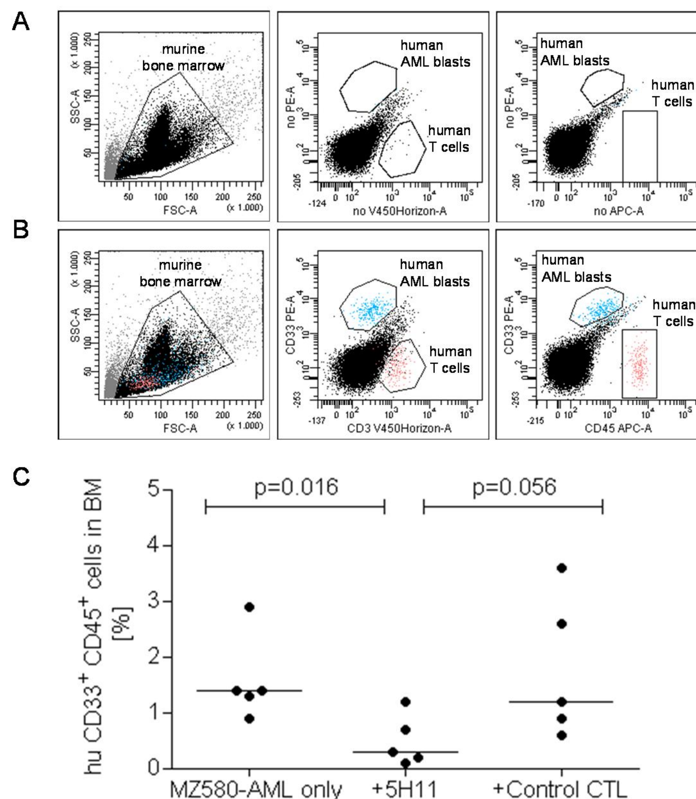

Figure 23: Infiltration of human leukemic blasts in the murine bone marrow of MZ580-AML engrafted NSG mice one week after transfer of AML-reactive CTL clone 5H11. Mice were engrafted with primary AML blasts from patient MZ580. Subsequently, AML-reactive CTL clone 5H11 was transferred into the engrafted NSG mice. One week after transplantation the presence of human CD33$^+$ CD45$^+$ leukemic cells in the murine bone marrow was analyzed. Melan A-reactive control CTLs were included as specificity control. Gating-strategy for the detection of human AML blasts is exemplarily shown for bone marrow cells of one mouse (A) unstained and (B) stained for CD3, CD33, and CD45. (C) Percentage of human leukemic cells in murine bone marrow. The median values (n=5) are shown as bars. Statistical analysis was performed with Mann-Whitney *U* test using SPSS-17.

Interestingly, four weeks after T-cell transfer the anti-leukemic effect in both models was strongly increased. In mice that were engrafted with MZ667-AML, CTL 1E3 and 5B2 decreased the percentage of human leukemia in murine bone marrow significantly compared to mice that received no T cells [median percentage of AML blasts: AML only 73.7% (n=5); 1E3 15.2% (n=5); 5B2 56.7% (n=5). p-values: AML only versus 1E3 p=0.008; AML only versus 5B2 p=0.032; calculated by Mann-Whitney *U* test; Figure 24]. In spleen (Figure 24) and peripheral blood (data not shown) no significant reduction could be observed. Melan-A reactive control CTLs were not included in this experiment because of lacking availability. In a further experiment mice showed an unexpectedly high initial engraftment rate of 10% AML blasts in bone marrow at day of CTL transfer instead of the usually seen 1% to 3%. In this setting the infusion of CTL 1E3 or 5B2 was not able to mediate a significant decrease of the leukemic burden. Even after repetitive administration of CTL 5B2 no effect was measurable, indicating a relevance of the ratio between CTLs and leukemic blasts (data not shown).

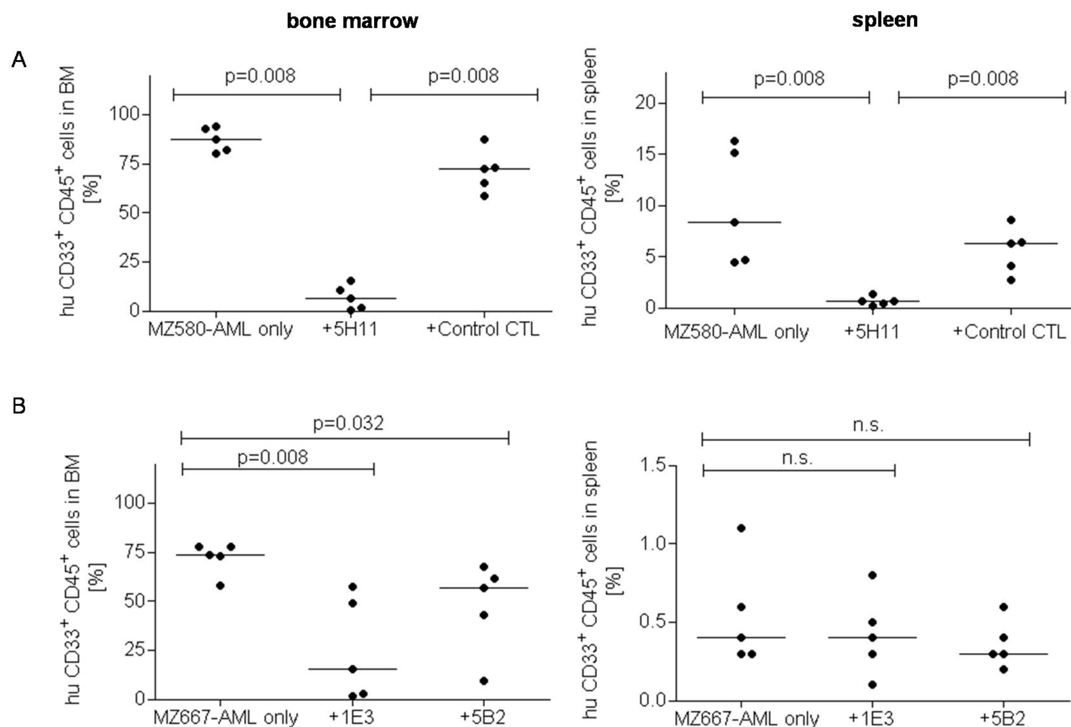

Figure 24: Presence of human leukemic blasts in murine bone marrow and spleens of (A) MZ580-AML and (B) MZ667-AML engrafted NSG mice four weeks after transfer of AML-reactive CTLs. Mice were engrafted with primary AML blasts until 1% to 3% were present in bone marrow. Subsequently, AML-reactive CTLs were transferred into the engrafted NSG mice. (A) NSG mice engrafted with MZ580-AML were treated with CTL 5H11 (MZ580-AML / Don.931). (B) NSG mice engrafted with MZ667-AML were injected with CTL 1E3 or 5B2 (MZ653-AML / Don.069), respectively. Melan A-reactive CTLs (Don.689) were included as unspecific control cells in MZ580-AML engrafted mice. Four weeks after transplantation the presence of human $CD33^+$ $CD45^+$ leukemic cells in murine bone marrow and spleen was analyzed. Shown are median values (n=5) as bars. Statistical analysis was performed with Mann-Whitney *U* test using SPSS-17.

In mice that were engrafted with MZ580-AML, CTL clone 5H11 strongly decreased the percentage of leukemic blasts. In murine bone marrow a decrease from a median of 87.5% human blasts in mice with AML only and 72.4% in mice receiving control CTL to 6.4% in mice that were transferred with 5H11 was achieved (p-values: AML only versus 5H11 p=0.008; 5H11 versus control CTL p=0.008; calculated by Mann-Whitney *U* test). In spleen the leukemic blasts were also reduced significantly after a single injection of 5H11 [median percentage of AML blasts: AML only 8.3% (n=5); 5H11 0.6% (n=5); Control CTL 6.3% (n=5). p-values: AML only versus 5H11 p=0.008; 5H11 versus control CTL p=0.008; calculated by Mann-Whitney *U* test; Figure 24]. The presence of the control CTL caused a minor reduction of AML blasts in both organs as well. In peripheral blood a decrease of blasts could also be observed but only few AML blasts (median percentage 0.3%) were present in mice even without CTL injection (data not shown). These data demonstrated that, even with the barriers caused by the use of a xenogenic model, AML-reactive CTL clones could reach lymphoid organs and showed a significant GVL effect *in vivo*.

5. Discussion

Clinical tumor remissions following adoptive immunotherapy with *in vitro* expanded tumor- and leukemia-reactive T-cell lines and clones are frequently not durable (Huang et al. 2005; Robbins et al. 2004; Warren et al. 2010). Among other reasons these observations can be explained by insufficient persistence and homing of adoptively transferred T cells which have reached a terminal differentiation state during *in vitro* expansion over several weeks. One potential strategy to overcome this limitation is to improve the efficiency of *in vitro* protocols allowing more leukemia-reactive T-cell precursors to grow during the primary stimulation phase. This may help to reduce the number of cell cycles of each single precursor and should translate into improved immune functions upon adoptive transfer *in vivo*. Here, a very reliable and efficient method for the generation of leukemia-reactive CTLs from naive $CD8^+$ T lymphocytes of healthy donors in an HLA-class I-matched setting during a comparably short culture period is introduced. The approach allows the *in vitro* expansion of a large diversity of strongly proliferating HLA-class I-restricted CTLs with an early differentiation phenotype and sustained effector functions towards AML blasts. In a therapeutical mouse model with an established human-derived leukemic hematopoiesis AML-reactive CTL clones could reach lymphoid tissues as well as bone marrow and significantly reduced the leukemic burden *in vivo* after a single transfusion.

5.1 Generation of AML-reactive $CD8^+$ T cells from the naive subset of healthy donors

AML-reactive $CD8^+$ T lymphocytes were generated from HLA-class I-identical healthy donors as T-cell source. In a clinical setting this would have the advantage that T cells from the stem-cell donor can be expanded in advance and CTLs are already available before the HSCT procedure is initiated. The precursor frequency of leukemia-reactive CTLs is very low in healthy individuals (Smit et al. 1998). To improve the protocol for the stimulation with primary AML blasts, different T-cell subsets were screened for their potential to induce anti-leukemic responses. T-cell compartments were separated by flow cytometric sorting. $CD8^+$ T-cell fractions were subsequently stimulated in short-term maxi-MLLCs and AML-reactivity was analyzed during the second and third week of culture. There is an ongoing debate which

combination of differentiation markers characterizes the naive T-cell pool most precisely (Appay et al. 2008). Here, the common definition by isoforms of CD45 was used to isolate the naive pool corresponding to the $CD45RO^-$ subset and also CCR7 was included comprising naive / central memory T cells in the $CCR7^+$ compartment (Sallusto et al. 2004; Sallusto et al. 1999). The generation of AML-reactive T-cell cultures was most efficient when naive / central memory cells were used as responder population in comparison to memory T cells in one sibling and two unrelated patient / donor pairs. CTLs from the naive / central memory subset showed specific lysis and IFN-γ production in response to patient's AML blasts and LCLs which was not obtained to that extent in their counterpart fractions. Although AML-reactive CTLs could be generated from both, the $CD45RO^-$ and the $CCR7^+$ subset, we decided to separate T cells according to their CD45RO expression in following experiments because a MACS Kit (Naive $CD8^+$ T-Cell Isolation Kit, Miltenyi) was available that comprises the depletion of $CD45RO^+$ cells along other markers to purify naive T cells. Thus, the naive $CD45RO^-$ $CD45RA^+$ subset of $CD8^+$ T cells was used as a source to generate AML-reactive CTLs under clonal conditions in mini-MLLCs. With that approach, in eight out of eight HLA-class I-matched patient / donor pairs AML-reactive mini-cultures could reliably be generated. In addition to previous work of our group (Distler et al. 2008), further publications have confirmed that CTL-mediated reactivity to leukemic cells mainly derives from the naive subset of healthy unprimed individuals (Bleakley et al. 2010; Quintarelli et al. 2008). These findings are further supported by HLA-peptide multimer data in *ex vivo* PBMCs showing most precursors of LAA-reactive $CD8^+$ T cells in the naive subset of healthy individuals (Griffioen et al. 2006). In contrast, other groups detected HLA-peptide multimer binding leukemia-reactive $CD8^+$ T cells also in the antigen-experienced memory pool. This was demonstrated for LAA specificities in healthy donors (Rezvani et al. 2003) as well as for mHag specificities in donors with a history of allo-antigen priming *in vivo* (Mommaas et al. 2005; Verdijk et al. 2004).

The set-up of allogeneic MLLCs with naive $CD8^+$ T cells as responders has, besides containing the majority of AML-reactive CTL precursors, another major advantage: It reduces the likelihood that leukemia-reactive CTLs rapidly enter a status of terminal differentiation that is accompanied by functional exhaustion and cell death. The superiority of early differentiated (naive / central memory) $CD8^+$ T-cell subsets in mediating antitumor and antiviral immunity after adoptive transfer has been

impressively demonstrated in murine and primate models (Berger et al. 2008; Hinrichs et al. 2009; Klebanoff et al. 2005). These observations may be explained with less-shortened chromosome telomeres in the naive subset (i.e. less cell division cycles) of less-differentiated T cells that have not yet reached replicative senescence, correlating with persistence after adoptive transfer (Klebanoff et al. 2006; Shen et al. 2007; Zhou et al. 2005). The differentiation phenotype of CTLs generated herein is discussed more detailed in chapter 5.5 and 5.7.

5.2 Generation of AML-reactive CD8$^+$ T cells under clonal conditions

Traditionally, allogeneic MLLC approaches are based on the stimulation of leukemia-reactive CTLs in bulk cultures (Bleakley & Riddell 2004). Herein, AML-responses were first also stimulated in maxi-MLLCs but reactivity decreased rapidly in long-term cultures. Also the proliferation rate was reduced with repeated restimulations. It has been shown previously that CML-reactive CTLs derived from bulk MLLCs lose their leukemia-reactivity during long-term culture compared to CTLs that were generated under limiting-dilution-conditions (Smit et al. 1998). One difficulty may be the very low precursor frequency of antigen-specific T cells in PBMCs of healthy individuals (Alanio et al. 2010; Smit et al. 1998). Another issue with the use of bulk cultures is that they are polyclonal and may also contain T cells with other specificities than for antigens presented on AML blasts which could induce undesired immune effects such as allo-reactivity in patients after transplantation. Therefore, the application of T-cell clones might be advantageous (Bleakley & Riddell 2011). To improve culture conditions for the outgrowth of leukemia-reactive CTLs, our group has established a protocol to generate CTL clones in so called mini-MLLCs (Distler et al. 2008). With a modified approach of mini-MLLCs developed in this study, HLA-class I-restricted T-cell clones with a sustained specificity towards AML blasts could reliably be generated. CTLs kept their specific lytic activity *in vitro* for more than ten weeks and could easily be expanded to cell numbers exceeding $1x10^8$. Importantly, CTL clones that were cultured for 42 to 56 days mediated an anti-leukemic effect in a therapeutical mouse model, indicating the biological significance of these T cells. Our data are in accordance with a recent publication, showing that mHag-reactive CTLs generated from the naive T-cell subset of sibling HSCT donors by stimulation with AML-derived DCs under similar clonal conditions as used herein had cytolytic activity

after expansion and could inhibit AML-engraftment by *in vitro* co-incubation with the leukemic blasts (Bleakley et al. 2010).

5.3 Primary blasts as stimulator cells

As stimulator cells in allogeneic MLLCs leukemic blasts previously isolated from peripheral blood of AML patients at initial diagnosis were used. AML blasts were pre-cultured over night before they were used as stimulator cells in MLLCs. Several important co-stimulatory as well as HLA-class I-molecules were clearly expressed on the cells after incubation, indicating their potential to activate T-cell responses. Maturation of AML blasts with cytokines as performed by other groups (Brouwer et al. 2000; Marijt et al. 2007) was analyzed for leukemic cells used herein but the enhancing effect on the expression of those molecules was not strong enough to regularly follow this procedure that is associated with a loss of cells. The use of primary AML blasts assures the *in vitro* stimulation of donor T cells against hematopoietic mHags as well as LAAs presented by recipient AML cells. In contrast to other groups, non-malignant mononuclear cells (Warren et al. 2010) or dendritic cells of patient origin (Bleakley et al. 2010) as stimulator cells were not used because it would restrict *in vitro* reactivity exclusively to hematopoietic mHags. This difference in stimulation conditions may be an important point since the contribution of both antigen categories (i.e. hematopoietic mHags and LAAs) to the induction of effective GVL responses *in vivo* is still not fully understood (Rezvani & Barrett 2008). AML blasts from patients categorized in different FAB subtypes were used to stimulate donor T cells, including FAB M1, M4, M5, and M5b. In all models AML-reactive mini-cultures could be generated. From these data a general impact of the FAB subtype could not be observed.

5.4 Cytokine usage in MLLCs

Another beneficial component of our protocol appears to be the cytokine combination used during primary stimulation. The common gamma chain cytokines IL-7 and IL-15 are essential for the survival and homeostasis of naive and memory T cells (Berard et al. 2003; Rochman et al. 2009; Schluns et al. 2000; Surh & Sprent 2008). Furthermore, IL-15 has been shown to favor the *in vitro* generation of leukemia-reactive $CD8^+$ central memory T cells (Daudt et al. 2008). IL-12 is necessary as “third signal” for efficient priming of naive $CD8^+$ precursors (Mescher et al. 2006). IL-2,

described to support proliferation and effector phenotype (Cui & Kaech 2010; Jameson & Masopust 2009), was used for T-cell expansion. The cytokine was not added at the initial two stimulations but from day 14 on to avoid outgrowth of regulatory T cells which are supported by IL-2 (Ma et al. 2006). While this favorable cytokine combination was already described in a previous study by our group (Distler et al. 2008) the current addition of IL-21 clearly improved the efficacy in generating leukemia-reactive CTLs.

5.5 Contribution of IL-21 to the generation of AML-reactive CD8$^+$ T cells

The main benefit in our protocol was that the addition of IL-21 significantly increased the number of AML-reactive mini-cultures and that more HLA-class I-restricted CTLs could be expanded out of IL-21-treated cultures. This was demonstrated in eight different AML patient / donor pairs with remarkable consistency. Our observation with leukemia-reactive CTL clones is in accordance with earlier reports showing that IL-21 promotes the priming of naive CD8$^+$ T cells and enhances anti-tumor effects of antigen-specific CTLs (Casey & Mescher 2007; Kaka et al. 2009; Li et al. 2005; Parrish-Novak et al. 2000; Zeng et al. 2005). During the first three to four culture weeks the presence of the cytokine significantly increased T-cell numbers and decreased the percentage of dead and apoptotic cells. These results are consistent with previous data from Li *et al.* showing that the advantageous effect of IL-21 on the production of antigen-specific CTLs can partly be attributed to decreased apoptosis and increased survival of T cells (Li et al. 2005). According to these authors the enhancing effect on the proliferation could be the primary impact of IL-21 in this study. However, even though IL-21 clearly improved the yield of AML-reactive CTLs during the first weeks of MLLCs, it did not increase antigen-dependent proliferation and effector functions during later culture periods consistently for all CTLs tested. There are other reports showing a synergistic positive effect of IL-15 and IL-21 on proliferation and effector functions of tumor-specific CTL at early time points of culture (Huarte et al. 2009; Pouw et al. 2010). The approach herein also included IL-7 (throughout the culture) and IL-2 (from d14 on) which might have enhanced the expansion and effector functions of CTLs to a level where the synergism of IL-15 and IL-21 is hard to detect at later culture periods. Besides different cytokine settings, previous studies also differed in several other important parameters (e.g. CTL bulk

cultures, stimulation with specific peptides, time point of testing) from our work, making direct comparisons of findings very difficult.

Another important beneficial effect of IL-21 was that CTL clones showed a stronger expression of CD28, CD127, CD62L, and CD45RA along with decreased expression of CD45RO, leading to a more "early" phenotype which might promote the survival and homing of T cells after adoptive transfer. These findings are in accordance with previous reports demonstrating improved *in vivo* survival and the expression of central memory markers on antigen-specific T-cell lines primed in the presence of IL-21 (Alves et al. 2005; Ansén et al. 2008; Hinrichs et al. 2008; Kaka et al. 2009; Li et al. 2005; Parmigiani et al. 2011; Wölfl et al. 2011).

The biological relevance of CTLs generated with or without IL-21 was tested by adoptive transfer in AML-engrafted NSG mice. The CTLs 5H11 (MZ653-AML / Don.931) and 5B2 (MZ653-AML / Don.069) were generated in presence of IL-21, while CTL 1E3 (MZ653-AML / Don.069) was stimulated without the cytokine for expansion. All three CTLs showed anti-leukemia effects *in vivo*. From these data an impact of the cytokine could not be concluded, confirming the *in vitro* experiments which showed no influence on effector function in long-term culture.

5.6 T-cell antigens

One aim to enhance a GVL effect and reduce GVHD was to stimulate donor T cells against hematopoietic mHags as well as LAAs presented by recipient AML cells which were not present on non-hematopoietic cells such as fibroblasts. Cross-reactivity data shown herein indicate that both of these proposed CTL specificities are most likely expanded by our approach. The CTLs generated from the naive compartment showed several distinct reactivity patterns towards patient's, donor's, and third-party cells. More than half of the CTLs exclusively recognized patient's AML blasts and no other hematopoietic or non-hematopoietic cells. This recognition pattern could indicate LAAs as T-cell epitopes which may be aberrantly expressed or overexpressed in leukemic blasts. Other CTLs recognizing patient's AML blasts also showed reactivity to other hematopoietic cells of patient origin but not to stromal fibroblasts suggesting reactivity to recipient mHags with hematopoiesis-restricted expression. Other groups have reported that cytotoxic T lymphocytes derived from the naive subset target polymorphic mHags and non-polymorphic LAAs (Bleakley et al. 2010; Quintarelli et al. 2008; Wölfel et al. 2008).

In a clinical approach these two types of T-cell recognition patterns could be favorable to achieve anti-leukemic reactivity with a reduced risk to induce GVHD. However, the risk of an antigen expression in other body tissues than stromal fibroblasts remains, as shown in a phase I study to treat relapsing patients with *in vitro* expanded T-cell clones specific for hematopoiesis-restricted mHags which did not react with dermal fibroblasts. Some patients suffered from pulmonary toxicity because of unexpected expression of the mHags in lung tissue (Warren et al. 2010). One third of isolated CTLs reacted to patient's hematopoietic cells as well as stromal fibroblasts. This suggested recognition of antigens ubiquitously expressed by hematopoietic and non-hematopoietic recipient cells (Feng et al. 2008). If such CTLs would be adoptively transferred into patients, they might induce serious GVH reactivity and therefore should be excluded from any therapeutic approach. However, additional work including the molecular definition of CTL antigens are required before CTLs generated by the described approach appear safe enough to be investigated in pilot clinical trials. The molecular identification of antigens recognized by CTLs generated herein is currently ongoing. The antigenic peptide recognized by CTL 4B2 (MZ653-AML / Don.931) is being identified by two approaches in our group. This project herein focused on the identification of the antigen by a straightforward approach using biochemical purification of HLA-presented peptides from the AML lysate with subsequent separation by HPLC and sequencing by mass spectrometry which is still ongoing. A different project is dealing with the identification of the antigenic peptide by genetic linkage-analysis. The antigen recognized by CTL 2E8, 7G9, and 7H1 (MZ529-AML / Don.730) as well as by 2B8 and 2D10 (MZ987-AML / Don.940) has already been identified by cDNA expression cloning in collaboration with Dr. Catherine Wölfel and Prof. Dr. Thomas Wölfel, Mainz (unpublished data).

Most CTLs reacted not only to leukemic cells from the patient that were used to expand the T cells *in vitro* but also showed cross-reactivity against blasts from other patients sharing the HLA-restriction element with the original patient. Most CTLs reacted with other myeloid leukemias, indicating antigens that were overexpressed, aberrantly expressed, or mutated in myeloid malignant cells. Since patient-derived non-malignant myeloid cells such as monocytes or granulocytes were often not available for testing, also mHags with myeloid expression present in several patients could be target structures. Single AML-reactive CTLs recognized blasts from

lymphatic leukemias indicating antigens expressed in both lineages. H-Y derived antigens could be excluded because all healthy T-cell donors matched to male patients were also male. Some CTLs recognized more than half of all myeloid leukemias tested, showing a widespread distribution of the T-cell epitopes which makes the molecular identification of further antigens very attractive.

5.7 Adoptive transfer of AML-reactive CTLs

After adoptive transfer into patients, leukemia-reactive CTLs should survive and expand *in vivo*, migrate to end organs and exert strong effector functions. It is widely assumed that these important properties are mediated by cytokine receptors (e.g. CD127), homing molecules (e.g. CD62L), chemokine receptors (e.g. CCR7, CXCR4), and co-stimulatory molecules (CD27, CD28) (Hinrichs et al. 2006; Klebanoff et al. 2006; Powell et al. 2005). Herein, we show that several of these “early” (naive / central memory) differentiation markers were expressed by CTLs over several weeks of *in vitro* culture. In contrast to CD62L, we did not observe the regular expression of CCR7 beyond 5 weeks of culture. Furthermore, although the chemokine receptor CXCR4 was found at a lower expression level on IL-21-treated CTLs, all of them expressed this marker. Expression of CXCR4 has been suggested to allow for the migration of T cells into the bone marrow (Mora & von Andrian 2006). Thus CXCR4$^+$ CD62L$^+$ CTLs should be able to home in lymphoid tissues and reach leukemia-infiltrated marrow sites. To test this assumption in an *in vivo* therapeutical model, adoptive transfer experiments of leukemia-reactive CTLs in AML-engrafted immunodeficient NSG mice were performed. This mouse strain carries the severe combined immune deficiency mutation (SCID) and a IL-2 receptor gamma chain knockout which leads to a lack of mature T cells and B cells, functional NK cells, and a deficiency in cytokine signaling (Shultz et al. 2005). These mice showed a reliable engraftment of human stem cells (Ito et al. 2002; Shultz et al. 2005) as well as of AML and ALL cells (Agliano et al. 2008; Sanchez et al. 2009) in experiments of other groups as well. The engraftment procedure used herein was established in another project by the group of PD Dr. Udo Hartwig (Mainz) and has proven to be effective mainly for FLT3-ITD positive AMLs (Brunk 2011). In this study, mice were injected with the reliably engrafting leukemic cells from patient MZ580 or MZ667, respectively, and were incubated until an engraftment rate of 1% to 3% human CD33$^+$ CD45$^+$ AML blasts in murine bone marrow was achieved, resembling minimal residual disease.

The humanized mice were injected intravenously with HLA-class I-restricted CTLs lysing *in vitro* the AML blasts used for engraftment. For MZ580-AML CTL clone 5H11 that was available for transfer experiments was generated from an unrelated HLA-class I-matched healthy donor against the leukemic blasts of this patient. In the case of MZ667-AML no appropriate T-cell donor was available. CTL 1E3 and 5B2, generated in the HLA-matched model MZ653-AML / Don.069 which shares the C*07:01 allele with patient MZ667, cross-reacted with AML blasts from this patient and were therefore used for adoptive transfer experiments. Mice receiving no T cells served as engraftment controls, while the specificity of the GVL effect was controlled by the injection of Melan A-reactive CTLs which were not reactive to both AMLs. One or four weeks after T-cell transfer, mice were sacrificed and the presence of infiltrating human AML blasts and $CD8^+$ T cells in murine bone marrow, spleen, and peripheral blood was analyzed by FACS. In mice from both therapeutical approaches human $CD8^+$ T cells were found in the bone marrow and spleen one week after transplantation. In mice that were engrafted with MZ667-AML T cells were also detected in the peripheral blood and in single mice CTL clone 5B2 was present in very low frequencies even after four weeks. Thus, AML-reactive CTLs were able to migrate to the murine bone marrow and secondary lymphatic tissues. At transfer day only CTL 5B2 and 1E3 expressed low levels of CD62L, whereas CXCR4 was clearly present on the surface of all four CTLs. Furthermore, there was only a very slightly or no expression of CD127 and CCR7. The expression pattern of these molecules seemed to be sufficient for homing to the lymphatic organs even across the species barriers. To support T-cell function and proliferation in the mice, human recombinant IL-2, IL-7-Fc, and IL-15 were injected in parallel to T-cell transfer and additionally three days afterwards. T cells persisted at least for one week in the analyzed murine organs but disappeared afterwards. The same effect was observed in the clinical trial performed by Warren *et al*. including the repetitive injection of patient mHag-reactive CTLs into chemotherapy-treated patients with relapsing B-ALL or MDS. Transplanted CTLs which showed negligible expression of CD62L, CCR7, and CD127 but mostly expressed CXCR4 could home to bone marrow but failed to persist more than three weeks *in vivo*. In two patients the low-dose administration of IL-2 could not promote longer persistence (Warren et al. 2010). A reason for the short persistence could be an insufficient cytokine milieu compared to *in vitro* culture conditions or lacking co-stimulating signals. But since also the unspecific control CTLs persisted comparably

or even at higher numbers than the AML-reactive CTLs in the engrafted mice, a short-termed unspecific effect on proliferation, potentially driven by injected cytokines, could not be excluded. Similar observations have been made previously in ALL engrafted NOD / SCID mice where higher T cells numbers could be recovered from animals treated with unspecific CTLs (Nijmeijer et al. 2002). The administration of the three common γ-chain cytokines might not be enough to establish a serum level which is needed by the T cells for longer survival and proliferation. Probably a repetitive cytokine administration over a longer period of time could improve the survival of the transferred T cells. Also the co-transfer of $CD4^+$ T-helper cells, ideally AML-reactive $CD4^+$ T cells, might have a beneficial effect by the provision of co-stimulatory signals. This point was demonstrated in an infection model where after the adoptive transfer of $CD8^+$ T cells a maintenance of memory cells was dependent on the presence of $CD4^+$ T cells (Sun et al. 2004).

Despite the short-term persistence of the transferred CTLs, an impressive GVL effect in the mice with the established leukemic hematopoiesis was observed herein without signs of xenogenic GVHD. In mice that were engrafted with MZ580-AML the presence of CTL clone 5H11 induced a significant decrease of infiltrating AML blasts in the bone marrow after one week. This effect was even more pronounced in bone marrow, spleen, and peripheral blood of recipient mice analyzed four weeks post transfer. In the second model, both CTLs 1E3 and 5B2 significantly reduced the percentage of leukemic blasts in bone marrow of animals engrafted with MZ667-AML when compared to controls four weeks following T-cell transfer. These are the first data showing that human cytotoxic $CD8^+$ T lymphocytes generated from the naive subset of healthy donors by *in vitro* stimulation with primary AML blasts have a GVL effect *in vivo* in HLA-class I-matched settings in mice with an established leukemic hematopoiesis. The potential of AML-reactive T cells has been demonstrated in mouse models before. Human $CD8^+$ T-cell clones isolated from patients after allogeneic HSCT or generated *in vitro* by stimulation with primary AML blasts or AML-derived DCs, respectively, could inhibit engraftment of AML cells in immunodeficient mice. In contrast to the experiments performed herein, the anti-leukemic effect was demonstrated after *in vitro* co-incubation of AML cells with the T cells prior to injection into the mice (Bonnet et al. 1999; Distler et al. 2008; Bleakley et al. 2010). An *in vivo* GVL effect of peptide-stimulated or mismatched leukemia-reactive CTLs generated from healthy unrelated donors has been shown in NOD / SCID mouse models that

were engrafted with CML or ALL cells, respectively, before the adoptive transfer of T cells (Hambach et al. 2006; Nijmeijer et al. 2002) and also in clinical trials for T-cell therapy of different leukemia subtypes including few AML patients (Falkenburg et al. 1999; Marijt et al. 2007; Warren et al. 2010). The GVL effect shown herein was more potent four weeks post transfer, even though T cells could mostly be detected only one week following transfer. The time points between week one and four could not be analyzed within this project due to limited T-cell numbers as well as treated mice. There is a chance that the T cells persisted for more than one week in the mice and caused a even more severe reduction of leukemic cells two or three weeks post transfer. Possibly, blasts were already growing out again four weeks post transfer after T cells had vanished. This issue should be addressed in a following project.

CTLs reduced the leukemic burden in the mice significantly but were not able to eradicate it. In *ex vivo* analyses from spleens of MZ667-AML engrafted mice that received the CTLs 1E3, 5B2, or control CTLs, the reactivity against leukemic target cells after two restimulations *in vitro* was confirmed. These data demonstrated that persisting CTLs were still functional after circulating one week in the mice. The incomplete eradication of AML blasts in mice therefore could not be attributed to an abrogated functionality of transferred T cells. Although T cells were present in AML infiltrated organs, the interaction of CTLs and AML blasts might not be sufficient due to species specific differences between mice and humans. This also could be a reason for the short-term persistence that was also observed with unspecific control CTLs. Another reason could be an occurring immune escape of AML blasts through a selection pressure mediated by the presence of the CTL clones, probably leading to a loss of cells presenting the antigenic peptide or to HLA-loss variants. In a NOD / SCID mouse model engrafted with CML cells this issue was addressed and refuted by Hambach and colleagues. CML blasts isolated out of engrafted mice which were treated with a peptide-specific CTL clone were equally sensitive to *in vitro* killing by the CTL as leukemic cells that had not been passaged in the mice (Hambach et al. 2006). Another reason could be an insufficient eradiation of leukemic stem cells in the mice. Also the ratio between infused CTLs and leukemic cells could be relevant for the biological significance. In one experiment where the leukemic burden of MZ667-AML was unexpectedly high (i.e.10%) in the murine bone marrow at day of T-cell transfer, the percentage of infiltrating AML blasts could not be reduced significantly in the presence of AML-reactive CTLs. This indicates that

adoptive T-cell therapy might be most effective in the case of minimal residual disease. A repetitive CTL infusion could possibly also increase the GVL effect in this setting. A published clinical trial with DLI treatment of AML patients indicates that mainly patients with favorable cytogenetics, those achieving a hematological remission before DLI, or patients with a lower tumor burden at time of relapse benefit from DLI which supports this assumption (Schmid et al. 2007).

Finally, the *in vitro* culture time of the T cells might have an influence on the effectiveness *in vivo*. CTLs used herein were already cultured for 45 to 59 days before transfer into the engrafted mice. It has been shown previously for AML-reactive T cells recognizing a single HLA-mismatch that the anti-leukemic effect decreases with increasing *in vitro* culture time (Brunk 2011). Therefore, the application of CTLs cultured over a shorter period of time could improve the approach. Previous reports described that T-cell proliferation and survival correlate with the anti-tumor effect after adoptive transfer (Gattinoni et al. 2005; Hinrichs et al. 2009; Klebanoff et al. 2005). CTLs cultured for up to five weeks showed herein a more “early” phenotype with stronger expression of CD62L, CCR7, CD127, CD28, and CD45RA what could be associated with better proliferative capacity and a longer survival due to less terminal differentiation. A future clinical protocol could include the transfer of a pool of several less expanded AML-reactive mini-cultures. A single transfusion also might not be sufficient to achieve remissions, therefore a repetitive administration is expected to induce a beneficial effect in the treatment of minimal residual diseases.

6. Outlook to clinical translation of the established protocol

A clinical protocol for adoptive T-cell transfer at minimal residual disease after HSCT should ensure the eradication of leukemic blasts and ideally leukemic stem cells in patients without destroying healthy tissues. T cells should therefore be able to enter AML infiltrated and lymphoid organs and should ideally establish an immunological memory. To make AML-reactive CTLs already available at time point of HSCT, $CD8^+$ T cells could be generated under good manufacturing practice (GMP) conditions from PBMCs of the healthy HLA-class I-identical donor who donates the stem cells prior to the transplantation. Naive $CD8^+$ T cells could be stimulated in mini-MLLC approaches as described herein with irradiated primary AML blasts in presence IL-2 (from day 14 on), IL-7, IL-12 (day 0 to 7), IL-15, and IL-21. AML-reactivity of the mini-cultures could be analyzed from culture week three on in IFN-γ split-well ELISpot assays. To ensure the expression of the important co-stimulatory molecules CD27 and CD28 as well as the expression of the homing molecule CD62L and the cytokine receptors CXCR4, CCR7, and CD127, T cells should not have longer culture times than 28 to 35 days. At that time point half of the AML-reactive mini-cultures could be frozen for adoptive transfer while the other half could be used to analyze cytolytic activity, phenotypic features, clonality, and growth potential. AML-reactive T-cell cultures for adoptive transfer into patients should satisfy following criteria: First, CTLs should be HLA-class I-restricted in their recognition and should lyse patient's AML blasts or AML blasts and patient-derived LCLs but not to patient's fibroblasts and donor-derived LCLs. This should increase the chances for recognition of hematopoietic-restricted mHags or LAAs by the T cells to minimize the risk of GVHD. Second, AML-reactive CTLs should be of clonal origin to avoid unspecific reactivity and collateral damage such as GVHD. Third, AML-reactive CTLs should express the homing and co-stimulatory molecules mentioned above to increase the chance that T cells enter lymphoid and AML infiltrated organs. Last, AML-reactive $CD8^+$ T-cell cultures should be expanded to a minimum of $1x10^6$ cells per unit within 35 days to ensure feasibility of CTL transfer. Mini-cultures which satisfy all safety criteria could be pooled and used for treatment of AML patients. Although the translation of the entire approach into a GMP protocol is challenging, clinical testing would afterwards demonstrate if such CTLs are capable of mediating sufficient GVL effects in the absence of GVHD in humans.

7. Summary

Available options to treat chemo-refractory acute myeloid leukemia (AML) include immunotherapeutical approaches. The curative effect of this therapy is mediated by immune cells, especially by T cells, of donor origin which recognize antigens presented on the surface of patient's leukemic cells. The induction of this beneficial graft-versus-leukemia effect is frequently associated with complications including graft-versus-host disease which occurs when donor-derived T cells recognize patient's healthy tissues. Therefore, approaches to mediate graft-versus-leukemia responses using selected leukemia-reactive T cells which do not recognize normal tissues could be an option to reduce the risk of inducing graft-versus-host disease.

An *in vitro* protocol was previously established in our group to isolate AML-reactive $CD8^+$ T cells from the $CD62L^+$ subset of healthy individuals by stimulation with primary AML blasts in HLA-class I-identical settings (Distler et al. 2008). Within this project the protocol could substantially be improved by several modifications. First, the naive / central memory $CD8^+$ T-cell subset of healthy donors was found to contain most AML-reactive precursors in the $CD45RO^-$ and the $CCR7^+$ fractions. Because an isolation kit was available that depletes $CD45RO^+$ cells among other markers to purify naive T cells, the resulting $CD45RO^-$ $CD45RA^+$ population was used in following stimulations to initiate T-cell cultures under clonal conditions. With this approach AML-reactive T-cell cultures could successfully be generated in eight different patient / donor pairs. Second, to further improve the stimulation protocol, the common γ-chain cytokine IL-21 was added to the cultures. The addition of IL-21 enabled more precursors to develop into potent leukemia-reactive cytotoxic T lymphocytes (CTLs), probably by its promoting effects on cell survival and antigen-specific proliferation during the first weeks of cultures. High numbers of HLA-class I-restricted CTL clones and oligoclonal lines with lytic abilities to AML blasts *in vitro* for more than ten weeks could be expanded to cell number exceeding $1x10^8$. These CTLs showed expression of central memory markers important for homing and co-stimulation over a longer period of time. Importantly, most CTLs did not react with patient-derived stromal fibroblasts used as non-hematopoietic surrogate targets to show a potential of CTLs to recognize normal tissues. These CTLs were promising candidates for cellular immunotherapy. To analyze the anti-leukemic potential of these CTLs *in vivo*, immunodeficient NSG mice were engrafted with AML blasts and were then adoptively transferred with tree CTLs expanded for 45 to 59 days *in vitro*.

Even after long-term culture these CTLs persisted at least for one week in the mice and were able to mediate a significant reduction of the leukemic burden after adoptive transfer, demonstrating for the first time an impressive GVL effect *in vivo* in a therapeutical AML model. These promising results are a valuable basis for future translation of the approach into a good manufacturing practice (GMP) protocol for clinical application. Clinical testing has to demonstrate if the generation of AML-reactive CTL clones by stimulation with primary AML blasts in a patient-donor specific manner can improve cellular therapy regarding the induction of GVL responses in parallel with reduced graft-versus-host effects. Furthermore, the identification of new T-cell defined antigens can be promoted by CTLs generated with this protocol by their use to screen candidate antigens in currently available identification approaches.

8. References

Agliano A, Martin-Padura I, Mancuso P, Marighetti P, Rabascio C, Pruneri G, Shultz LD, Bertolini F (2008): **Human acute leukemia cells injected in NOD / LtSz-scid / IL-2Rgamma null mice generate a faster and more efficient disease compared to other NOD / scid-related strains**. Int. J. Cancer 123 (9):2222–2227

Alanio C, Lemaitre F, Law HKW, Hasan M, Albert ML (2010): **Enumeration of human antigen-specific naive CD8⁺ T cells reveals conserved precursor frequencies**. Blood 115 (18):3718–3725

Alves NL, Arosa FA, van Lier RAW (2005): **IL-21 sustains CD28 expression on IL-15-activated human naive CD8⁺ T cells**. J. Immunol 175 (2):755–762

Alyea EP, Canning C, Neuberg D, Daley H, Houde H, Giralt S, Champlin R, Atkinson K, Soiffer RJ (2004): **CD8⁺ cell depletion of donor lymphocyte infusions using CD8 monoclonal antibody-coated high-density microparticles (CD8-HDM) after allogeneic hematopoietic stem cell transplantation: a pilot study**. Bone Marrow Transplant 34 (2):123–128

Ansén S, Butler MO, Berezovskaya A, Murray AP, Stevenson K, Nadler LM, Hirano N (2008): **Dissociation of its opposing immunologic effects is critical for the optimization of antitumor CD8⁺ T-cell responses induced by interleukin 21**. Clin. Cancer Res 14 (19):6125–6136

Appay V, van Lier RAW, Sallusto F, Roederer M (2008): **Phenotype and function of human T lymphocyte subsets: consensus and issues**. Cytometry A 73 (11):975–983

Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, Ziegler A (1978): **Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis**. Cell 14 (1):9–20

Barrett AJ (2008): **Understanding and harnessing the graft-versus-leukaemia effect**. Br. J. Haematol 142 (6):877–888

Barrett AJ, Le Blanc K (2010): **Immunotherapy prospects for acute myeloid leukaemia**. Clin. Exp. Immunol 161 (2):223–232

Barrett DM, Zhao Y, Liu X, Jiang S, Carpenito C, Kalos M, Carroll RG, June CH, Grupp SA (2011): **Treatment of advanced leukemia in mice with mRNA engineered T cells**. Hum. Gene Ther 22 (12):1575–1586

Barry M, Bleackley RC (2002): **Cytotoxic T lymphocytes: all roads lead to death**. Nat. Rev. Immunol 2 (6):401–409

Belz GT, Kallies A (2010): **Effector and memory CD8⁺ T cell differentiation: toward a molecular understanding of fate determination**. Curr. Opin. Immunol 22 (3):279–285

Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C (1985): **Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group**. Ann. Intern. Med 103 (4):620–625

Berard M, Brandt K, Bulfone-Paus S, Tough DF (2003): **IL-15 promotes the survival of naive and memory phenotype CD8+ T cells**. J. Immunol 170 (10):5018–5026

Berger AE, Davis JE, Cresswell P (1982): **Monoclonal antibody to HLA-A3**. Hybridoma 1 (2):87–90

Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR (2008): **Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates**. J. Clin. Invest 118 (1):294–305

Berger C, Turtle CJ, Jensen MC, Riddell SR (2009): **Adoptive transfer of virus-specific and tumor-specific T cell immunity**. Curr. Opin. Immunol 21 (2):224–232

Betz BL, Hess JL (2010): **Acute myeloid leukemia diagnosis in the 21st century**. Arch. Pathol. Lab. Med 134 (10):1427–1433

Blattman JN, Greenberg PD (2004): **Cancer immunotherapy: a treatment for the masses**. Science 305 (5681):200–205

Bleakley M, Otterud BE, Richardt JL, Mollerup AD, Hudecek M, Nishida T, Chaney CN, Warren EH, Leppert MF, Riddell SR (2010): **Leukemia-associated minor histocompatibility antigen discovery using T-cell clones isolated by in vitro stimulation of naive CD8+ T cells**. Blood 115 (23):4923–4933

Bleakley M, Riddell SR (2004): **Molecules and mechanisms of the graft-versus-leukaemia effect**. Nat. Rev. Cancer 4 (5):371–380

Bleakley M, Riddell SR (2011): **Exploiting T cells specific for human minor histocompatibility antigens for therapy of leukemia**. Immunol. Cell Biol 89 (3):396–407

Bonnet D, Warren EH, Greenberg PD, Dick JE, Riddell SR (1999): **CD8+ minor histocompatibility antigen-specific cytotoxic T lymphocyte clones eliminate human acute myeloid leukemia stem cells**. Proc. Natl. Acad. Sci. U.S.A 96 (15):8639–8644

Borchers S, Provasi E, Silvani A, Radrizzani M, Benati C, Dammann E, Krons A, Kontsendorn J, Schmidtke J, Kuehnau W, Neuhoff N von, Stadler M, Ciceri F, Bonini C, Ganser A, Hertenstein B, Weissinger EM (2011): **Genetically modified donor leukocyte transfusion and graft-versus-leukemia effect after allogeneic stem cell transplantation**. Hum. Gene Ther 22 (7):829–841

Brouwer RE, van der Heiden P, Schreuder GMT, Mulder A, Datema G, Anholts JDH, Willemze R, Claas FHJ, Falkenburg JHF (2002): **Loss or downregulation of HLA class I expression at the allelic level in acute leukemia is infrequent but**

functionally relevant, and can be restored by interferon. Hum. Immunol 63 (3):200–210

Brouwer RE, Zwinderman KH, Kluin-Nelemans HC, van Luxemburg-Heijs SA, Willemze R, Falkenburg JH (2000): **Expression and induction of costimulatory and adhesion molecules on acute myeloid leukemic cells: implications for adoptive immunotherapy**. Exp. Hematol 28 (2):161–168

Brunk A (2011): **Untersuchungen zu Migration, Homing und Effektor-Funktionen humaner T-Lymphozyten in NOD.Cg-Prkdc scid Il2r gtm1Wjl, SzJ-Mäusen im Rahmen der allogenen hämatopoetischen Stammzelltransplantation**. Dissertation, Johannes-Gutenberg University, Mainz

Buggins AG, Milojkovic D, Arno MJ, Lea NC, Mufti GJ, Thomas NS, Hirst WJ (2001): **Microenvironment produced by acute myeloid leukemia cells prevents T cell activation and proliferation by inhibition of NF-kappaB, c-Myc, and pRb pathways**. J. Immunol 167 (10):6021–6030

Burnett A, Wetzler M, Löwenberg B (2011): **Therapeutic advances in acute myeloid leukemia**. J. Clin. Oncol 29 (5):487–494

Casey KA, Mescher MF (2007): **IL-21 promotes differentiation of naive CD8 T cells to a unique effector phenotype**. J. Immunol 178 (12):7640–7648

Chaplin DD (2010): **Overview of the immune response**. J. Allergy Clin. Immunol 125 (2 Suppl 2):S3-23

Chowdhury FZ, Ramos HJ, Davis LS, Forman J, Farrar JD (2011): **IL-12 selectively programs effector pathways that are stably expressed in human CD8^{+} effector memory T cells in vivo**. Blood 118 (14):3890–3900

Collins RH, Shpilberg O, Drobyski WR, Porter DL, Giralt S, Champlin R, Goodman SA, Wolff SN, Hu W, Verfaillie C, List A, Dalton W, Ognoskie N, Chetrit A, Antin JH, Nemunaitis J (1997): **Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation**. J. Clin. Oncol 15 (2):433–444

Cui W, Kaech SM (2010): **Generation of effector CD8^{+} T cells and their conversion to memory T cells**. Immunol. Rev 236:151–166

Daudt L, Maccario R, Locatelli F, Turin I, Silla L, Montini E, Percivalle E, Giugliani R, Avanzini MA, Moretta A, Montagna D (2008): **Interleukin-15 favors the expansion of central memory CD8^{+} T cells in ex vivo generated, antileukemia human cytotoxic T lymphocyte lines**. J. Immunother 31 (4):385–393

Delmer A, Marie JP, Thevenin D, Cadiou M, Viguie F, Zittoun R (1989): **Multivariate analysis of prognostic factors in acute myeloid leukemia**: value of clonogenic leukemic cell properties. J Clin Oncol 7 (6):738–746

Delves PJ, Roitt IM (2000): **The immune system. First of two parts**. N. Engl. J. Med 343 (1):37–49

den Haan JM, Meadows LM, Wang W, Pool J, Blokland E, Bishop TL, Reinhardus C, Shabanowitz J, Offringa R, Hunt DF, Engelhard VH, Goulmy E (1998): **The minor histocompatibility antigen HA-1**: a diallelic gene with a single amino acid polymorphism. Science 279 (5353):1054–1057

Deol A, Lum LG (2010): **Role of donor lymphocyte infusions in relapsed hematological malignancies after stem cell transplantation revisited**. Cancer Treat. Rev 36 (7):528–538

Deschler B, Lübbert M (2006): **Acute myeloid leukemia: epidemiology and etiology**. Cancer 107 (9):2099–2107

Distler E, Wolfel C, Kohler S, Nonn M, Kaus N, Schnurer E, Meyer RG, Wehler TC, Huber C, Wolfel T, Hartwig UF, Herr W (2008): **Acute myeloid leukemia (AML)-reactive cytotoxic T lymphocyte clones rapidly expanded from $CD8^{+}$ $CD62L^{(high)+}$ T cells of healthy donors prevent AML engraftment in NOD / SCID $IL2R\gamma^{null}$ mice**. Exp Hematol 36 (4):451–463

Dutour A, Marin V, Pizzitola I, Valsesia-Wittmann S, Lee D, Yvon E, Finney H, Lawson A, Brenner M, Biondi A, Biagi E, Rousseau R (2012): **In Vitro and In Vivo Antitumor Effect of Anti-CD33 Chimeric Receptor-Expressing EBV-CTL against CD33 Acute Myeloid Leukemia**. Adv Hematol 2012:683065

Falkenburg JH, Wafelman AR, Joosten P, Smit WM, van Bergen CA, Bongaerts R, Lurvink E, van der Hoorn M, Kluck P, Landegent JE, Kluin-Nelemans HC, Fibbe WE, Willemze R (1999): **Complete remission of accelerated phase chronic myeloid leukemia by treatment with leukemia-reactive cytotoxic T lymphocytes**. Blood 94 (4):1201–1208

Falkenburg JHF, Heslop HE, Barrett AJ (2008): **T cell therapy in allogeneic stem cell transplantation**. Biol. Blood Marrow Transplant 14 (1 Suppl 1):136–141

Fan Z, Zhang Q (2005): **Molecular mechanisms of lymphocyte-mediated cytotoxicity**. Cell. Mol. Immunol 2 (4):259–264

Feng X, Hui KM, Younes HM, Brickner AG (2008): **Targeting minor histocompatibility antigens in graft versus tumor or graft versus leukemia responses**. Trends Immunol 29 (12):624–632

Fink PJ, Hendricks DW (2011): **Post-thymic maturation: young T cells assert their individuality**. Nat. Rev. Immunol 11 (8):544–549

Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, Finkelstein SE, Theoret MR, Rosenberg SA, Restifo NP (2005): **Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred $CD8^{+}$ T cells**. J. Clin. Invest 115 (6):1616–1626

Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C, Wang E, Douek DC, Price DA, June CH, Marincola FM, Roederer M, Restifo NP (2011): **A human memory T cell subset with stem cell-like properties**. Nat. Med 17 (10):1290–1297

Gratwohl A, Hermans J, Apperley J, Arcese W, Bacigalupo A, Bandini G, Di Bartolomeo P, Boogaerts M, Bosi A, Carreras E (1995): **Acute graft-versus-host disease: grade and outcome in patients with chronic myelogenous leukemia. Working Party Chronic Leukemia of the European Group for Blood and Marrow Transplantation**. Blood 86 (2):813–818

Grawunder U, West RB, Lieber MR (1998): **Antigen receptor gene rearrangement**. Curr. Opin. Immunol 10 (2):172–180

Greiner DL, Hesselton RA, Shultz LD (1998): **SCID mouse models of human stem cell engraftment**. Stem Cells 16 (3):166–177

Griffioen M, Kessler JH, Borghi M, van Soest RA, van der Minne CE, Nouta J, van der Burg SH, Medema JP, Schrier PI, Falkenburg JHF, Osanto S, Melief CJM (2006): **Detection and functional analysis of CD8^{+} T cells specific for PRAME: a target for T-cell therapy**. Clin. Cancer Res 12 (10):3130–3136

Hambach L, Nijmeijer BA, Aghai Z, van Schie MLJ, Wauben MHM, Falkenburg JHF, Goulmy E (2006): **Human cytotoxic T lymphocytes specific for a single minor histocompatibility antigen HA-1 are effective against human lymphoblastic leukaemia in NOD / scid mice**. Leukemia 20 (2):371–374

Hayday AC, Pennington DJ (2007): **Key factors in the organized chaos of early T cell development**. Nat. Immunol 8 (2):137–144

Hill BT, Copelan EA (2010): **Acute myeloid leukemia: when to transplant in first complete remission**. Curr Hematol Malig Rep 5 (2):101–108

Hinrichs CS, Borman ZA, Cassard L, Gattinoni L, Spolski R, Yu Z, Sanchez-Perez L, Muranski P, Kern SJ, Logun C, Palmer DC, Ji Y, Reger RN, Leonard WJ, Danner RL, Rosenberg SA, Restifo NP (2009): **Adoptively transferred effector cells derived from naive rather than central memory CD8^{+} T cells mediate superior antitumor immunity**. Proc. Natl. Acad. Sci. U.S.A 106 (41):17469–17474

Hinrichs CS, Gattinoni L, Restifo NP (2006): **Programming CD8^{+} T cells for effective immunotherapy**. Curr. Opin. Immunol 18 (3):363–370

Hinrichs CS, Spolski R, Paulos CM, Gattinoni L, Kerstann KW, Palmer DC, Klebanoff CA, Rosenberg SA, Leonard WJ, Restifo NP (2008): **IL-2 and IL-21 confer opposing differentiation programs to CD8^{+} T cells for adoptive immunotherapy**. Blood 111 (11):5326–5333

Hofmann M, Große-Hovest L, Nübling T, Pyż E, Bamberg ML, Aulwurm S, Bühring H, Schwartz K, Haen SP, Schilbach K, Rammensee H, Salih HR, Jung G (2012): **Generation, selection and preclinical characterization of an Fc-optimized FLT3 antibody for the treatment of myeloid leukemia**. Leukemia official journal of the Leukemia Society of America, Leukemia Research Fund, U.K

Hofmann S, Greiner J (2011): **Adoptive Immunotherapy after Allogeneic Hematopoietic Progenitor Cell Transplantation: New Perspectives for Transfusion Medicine**. Transfus Med Hemother 38 (3):173–182

Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, Rimm AA, Ringdén O, Rozman C, Speck B (1990): **Graft-versus-leukemia reactions after bone marrow transplantation**. Blood 75 (3):555–562

Huang J, Khong HT, Dudley ME, El-Gamil M, Li YF, Rosenberg SA, Robbins PF (2005): **Survival, persistence, and progressive differentiation of adoptively transferred tumor-reactive T cells associated with tumor regression**. J. Immunother 28 (3):258–267

Huarte E, Fisher J, Turk MJ, Mellinger D, Foster C, Wolf B, Meehan KR, Fadul CE, Ernstoff MS (2009): **Ex vivo expansion of tumor specific lymphocytes with IL-15 and IL-21 for adoptive immunotherapy in melanoma**. Cancer Lett 285 (1):80–88

Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, Ueyama Y, Koyanagi Y, Sugamura K, Tsuji K, Heike T, Nakahata T (2002): **NOD / SCID / gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells**. Blood 100 (9):3175–3182

Jameson SC, Masopust D (2009): **Diversity in T cell memory: an embarrassment of riches**. Immunity 31 (6):859–871

Kaka AS, Shaffer DR, Hartmaier R, Hartmeier R, Leen AM, an Lu, Bear A, Rooney CM, Foster AE (2009): **Genetic modification of T cells with IL-21 enhances antigen presentation and generation of central memory tumor-specific cytotoxic T-lymphocytes**. J. Immunother 32 (7):726–736

Kessler JH, Beekman NJ, Bres-Vloemans SA, Verdijk P, van Veelen PA, Kloosterman-Joosten AM, Vissers DC, Bosch GJ ten, Kester MG, Sijts A, Wouter Drijfhout J, Ossendorp F, Offringa R, Melief CJ (2001): **Efficient identification of novel HLA-A(*)0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis.** J Exp Med 193 (1):73–88

Klebanoff CA, Gattinoni L, Restifo NP (2006): **CD8^{+} T-cell memory in tumor immunology and immunotherapy**. Immunol. Rev 211:214–224

Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, Palmer DC, Antony PA, Hwang ST, Rosenberg SA, Waldmann TA, Restifo NP (2005): **Central memory self/tumor-reactive CD8^{+} T cells confer superior antitumor immunity compared with effector memory T cells**. Proc. Natl. Acad. Sci. U.S.A 102 (27):9571–9576

Kloosterboer FM, van Luxemburg-Heijs SAP, van Soest RA, Barbui AM, van Egmond HM, Strijbosch MPW, Kester MGD, Marijt WAF, Goulmy E, Willemze R, Falkenburg JHF (2004): **Direct cloning of leukemia-reactive T cells from**

patients treated with donor lymphocyte infusion shows a relative dominance of hematopoiesis-restricted minor histocompatibility antigen HA-1 and HA-2 specific T cells. Leukemia 18 (4):798–808

Kobayashi N, Takata H, Yokota S, Takiguchi M (2004): **Down-regulation of CXCR4 expression on human CD8+ T cells during peripheral differentiation**. Eur. J. Immunol 34 (12):3370–3378

Kolb H (2008): **Graft-versus-leukemia effects of transplantation and donor lymphocytes**. Blood 112 (12):4371–4383

Kolb HJ, Mittermüller J, Clemm C, Holler E, Ledderose G, Brehm G, Heim M, Wilmanns W (1990): **Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients**. Blood 76 (12):2462–2465

Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, Ljungman P, Ferrant A, Verdonck L, Niederwieser D, van Rhee F, Mittermueller J, Witte T de, Holler E, Ansari H (1995): **Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients**. Blood 86 (5):2041–2050

Krueger A, Fas SC, Baumann S, Krammer PH (2003): **The role of CD95 in the regulation of peripheral T-cell apoptosis**. Immunol. Rev 193:58–69

Lampson LA, Levy R (1980): **Two populations of Ia-like molecules on a human B cell line**. J Immunol 125 (1):293–299

Li Y, Bleakley M, Yee C (2005): **IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response**. J. Immunol 175 (4):2261–2269

Lozzio CB, Lozzio BB (1975): **Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome**. Blood 45 (3):321–334

Ma A, Koka R, Burkett P (2006): **Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis**. Annu. Rev. Immunol 24:657–679

Maeurer MJ, Necker A, Salter RD, Castelli C, Hohn H, Karbach J, Freitag K, Neukirch C, Knuth A, Jager E (2002): **Improved detection of melanoma antigen-specific T cells expressing low or high levels of CD8 by HLA-A2 tetramers presenting a Melan-A/Mart-1 peptide analogue**. Int J Cancer 97 (1):64–71

Marijt E, Wafelman A, van der Hoorn M, van Bergen C, Bongaerts R, van Luxemburg-Heijs S, van den Muijsenberg J, Wolbers JO, van der Werff N, Willemze R, Falkenburg F (2007): **Phase I / II feasibility study evaluating the generation of leukemia-reactive cytotoxic T lymphocyte lines for treatment of patients with relapsed leukemia after allogeneic stem cell transplantation**. Haematologica 92 (1):72–80

Mescher MF, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, Popescu F, Xiao Z (2006): **Signals required for programming effector and memory development by CD8^{+} T cells**. Immunol. Rev 211:81–92

Meyer RG, Britten CM, Wehler D, Bender K, Hess G, Konur A, Hartwig UF, Wehler TC, Ullmann AJ, Gentilini C, Uharek L, Huber C, Kolbe K, Herr W (2007): **Prophylactic transfer of CD8-depleted donor lymphocytes after T-cell-depleted reduced-intensity transplantation**. Blood 109 (1):374–382

Meyer RG, Wagner EM, Konur A, Bender K, Schmitt T, Hemmerling J, Wehler D, Hartwig UF, Roosnek E, Huber C, Kolbe K, Herr W (2010): **Donor CD4 T cells convert mixed to full donor T-cell chimerism and replenish the CD52-positive T-cell pool after alemtuzumab-based T-cell-depleted allo-transplantation**. Bone Marrow Transplant 45 (4):668–674

Michl M (2005): **Basics Hämatologie.** 1st ed. Basics. Elsevier Urban & Fischer, München

Molldrem J, Dermime S, Parker K, Jiang YZ, Mavroudis D, Hensel N, Fukushima P, Barrett AJ (1996): **Targeted T-cell therapy for human leukemia**: cytotoxic T lymphocytes specific for a peptide derived from proteinase 3 preferentially lyse human myeloid leukemia cells. Blood 88 (7):2450–2457

Mommaas B, Stegehuis-Kamp JA, van Halteren AG, Kester M, Enczmann J, Wernet P, Kögler G, Mutis T, Brand A, Goulmy E (2005): **Cord blood comprises antigen-experienced T cells specific for maternal minor histocompatibility antigen HA-1**. Blood 105 (4):1823–1827

Mora JR, Andrian UH von (2006): **T-cell homing specificity and plasticity: new concepts and future challenges**. Trends Immunol 27 (5):235–243

Mulford D (2008): **Antibody therapy for acute myeloid leukemia**. Semin. Hematol 45 (2):104–109

Neefjes J, Jongsma MLM, Paul P, Bakke O (2011): **Towards a systems understanding of MHC class I and MHC class II antigen presentation**. Nat. Rev. Immunol 11 (12):823–836

Nijmeijer BA, Willemze R, Falkenburg JHF (2002): **An animal model for human cellular immunotherapy: specific eradication of human acute lymphoblastic leukemia by cytotoxic T lymphocytes in NOD / scid mice**. Blood 100 (2):654–660

Nonn M, Herr W, Khan S, Todorova M, Link I, Thies J, Distler E, Kaltwasser M, Hoffmann J, Huber C, Hartwig UF (2008): **Selective depletion of alloreactive T lymphocytes using patient-derived nonhematopoietic stimulator cells in allograft engineering**. Transplantation 86 (10):1427–1435

Obar JJ, Lefrançois L (2010): **Memory CD8^{+} T cell differentiation**. Ann. N. Y. Acad. Sci 1183:251–266

Oka Y, Elisseeva OA, Tsuboi A, Ogawa H, Tamaki H, Li H, Oji Y, Kim EH, Soma T, Asada M, Ueda K, Maruya E, Saji H, Kishimoto T, Udaka K, Sugiyama H (2000): **Human cytotoxic T-lymphocyte responses specific for peptides of the wild-type Wilms' tumor gene (WT1) product**. Immunogenetics 51 (2):99–107

O'Reilly RJ, Dao T, Koehne G, Scheinberg D, Doubrovina E (2010): **Adoptive transfer of unselected or leukemia-reactive T-cells in the treatment of relapse following allogeneic hematopoietic cell transplantation**. Semin. Immunol 22 (3):162–172

Parham P, Bodmer WF (1978): **Monoclonal antibody to a human histocompatibility alloantigen, HLA-A2**. Nature 276 (5686):397–399

Parkin J, Cohen B (2001): **An overview of the immune system**. Lancet 357 (9270):1777–1789

Parmar S, Fernandez-Vina M, Lima M de (2011): **Novel transplant strategies for generating graft-versus-leukemia effect in acute myeloid leukemia**. Curr. Opin. Hematol 18 (2):98–104

Parmigiani A, Pallin MF, Schmidtmayerova H, Lichtenheld MG, Pahwa S (2011): **Interleukin-21 and cellular activation concurrently induce potent cytotoxic function and promote antiviral activity in human CD8 T cells**. Hum. Immunol 72 (2):115–123

Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, Johnston J, Madden K, Xu W, West J, Schrader S, Burkhead S, Heipel M, Brandt C, Kuijper JL, Kramer J, Conklin D, Presnell SR, Berry J, Shiota F, Bort S, Hambly K, Mudri S, Clegg C, Moore M, Grant FJ, Lofton-Day C, Gilbert T, Rayond F, Ching A, Yao L, Smith D, Webster P, Whitmore T, Maurer M, Kaushansky K, Holly RD, Foster D (2000): **Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function**. Nature 408 (6808):57–63

Porter DL, Levine BL, Kalos M, Bagg A, June CH (2011): **Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia**. N. Engl. J. Med 365 (8):725–733

Pouw N, Treffers-Westerlaken E, Kraan J, Wittink F, Hagen T ten, Verweij J, Debets R (2010): **Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells**. Cancer Immunol. Immunother 59 (6):921–931

Powell DJ, Dudley ME, Robbins PF, Rosenberg SA (2005): **Transition of late-stage effector T cells to CD27^{+} CD28^{+} tumor-reactive effector memory T cells in humans after adoptive cell transfer therapy**. Blood 105 (1):241–250

Quintarelli C, Dotti G, Angelis B de, Hoyos V, Mims M, Luciano L, Heslop HE, Rooney CM, Pane F, Savoldo B (2008): **Cytotoxic T lymphocytes directed to the preferentially expressed antigen of melanoma (PRAME) target chronic myeloid leukemia**. Blood 112 (5):1876–1885

Radka SF, Kostyu DD, Amos DB (1982): **A monoclonal antibody directed against the HLA-Bw6 epitope**. J Immunol 128 (6):2804–2806

Rebai N, Malissen B (1983): **Structural and genetic analyses of HLA class I molecules using monoclonal xenoantibodies**. Tissue Antigens 22 (2):107–117

Rezvani K, Barrett AJ (2008): **Characterizing and optimizing immune responses to leukaemia antigens after allogeneic stem cell transplantation**. Best Pract Res Clin Haematol 21 (3):437–453

Rezvani K, Grube M, Brenchley JM, Sconocchia G, Fujiwara H, Price DA, Gostick E, Yamada K, Melenhorst J, Childs R, Hensel N, Douek DC, Barrett AJ (2003): **Functional leukemia-associated antigen-specific memory CD8$^+$ T cells exist in healthy individuals and in patients with chronic myelogenous leukemia before and after stem cell transplantation**. Blood 102 (8):2892–2900

Ringdén O, Karlsson H, Olsson R, Omazic B, Uhlin M (2009): **The allogeneic graft-versus-cancer effect**. Br. J. Haematol 147 (5):614–633

Ringdén O, Labopin M, Gorin NC, Schmitz N, Schaefer UW, Prentice HG, Bergmann L, Jouet JP, Mandelli F, Blaise D, Fouillard L, Frassoni F (2000): **Is there a graft-versus-leukaemia effect in the absence of graft-versus-host disease in patients undergoing bone marrow transplantation for acute leukaemia?** Br. J. Haematol 111 (4):1130–1137

Robbins PF, Dudley ME, Wunderlich J, El-Gamil M, Li YF, Zhou J, Huang J, Powell DJ, Rosenberg SA (2004): **Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy**. J. Immunol 173 (12):7125–7130

Rochman Y, Spolski R, Leonard WJ (2009): **New insights into the regulation of T cells by gamma(c) family cytokines**. Nat. Rev. Immunol 9 (7):480–490

Russell JH, Ley TJ (2002): **Lymphocyte-mediated cytotoxicity**. Annu. Rev. Immunol 20:323–370

Sallusto F, Geginat J, Lanzavecchia A (2004): **Central memory and effector memory T cell subsets: function, generation, and maintenance**. Annu. Rev. Immunol 22:745–763

Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A (1999): **Two subsets of memory T lymphocytes with distinct homing potentials and effector functions**. Nature 401 (6754):708–712

Salter RD, Cresswell P (1986): **Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid**. Embo J 5 (5):943–949

Sanchez PV, Perry RL, Sarry JE, Perl AE, Murphy K, Swider CR, Bagg A, Choi JK, Biegel JA, Danet-Desnoyers G, Carroll M (2009): **A robust xenotransplantation model for acute myeloid leukemia**. Leukemia 23 (11):2109–2117

Schatz DG, Ji Y (2011): **Recombination centres and the orchestration of V(D)J recombination**. Nat. Rev. Immunol 11 (4):251–263

Schluns KS, Kieper WC, Jameson SC, Lefrançois L (2000): **Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo**. Nat. Immunol 1 (5):426–432

Schmid C, Labopin M, Nagler A, Bornhäuser M, Finke J, Fassas A, Volin L, Gürman G, Maertens J, Bordigoni P, Holler E, Ehninger G, Polge E, Gorin N, Kolb H, Rocha V (2007): **Donor lymphocyte infusion in the treatment of first hematological relapse after allogeneic stem-cell transplantation in adults with acute myeloid leukemia: a retrospective risk factors analysis and comparison with other strategies by the EBMT Acute Leukemia Working Party**. J. Clin. Oncol 25 (31):4938–4945

Schmitt M, Casalegno-Garduño R, Xu X, Schmitt A (2009): **Peptide vaccines for patients with acute myeloid leukemia**. Expert Rev Vaccines 8 (10):1415–1425

Shen X, Zhou J, Hathcock KS, Robbins P, Powell DJ, Rosenberg SA, Hodes RJ (2007): **Persistence of tumor infiltrating lymphocytes in adoptive immunotherapy correlates with telomere length**. J. Immunother 30 (1):123–129

Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Kotb M, Gillies SD, King M, Mangada J, Greiner DL, Handgretinger R (2005): **Human lymphoid and myeloid cell development in NOD / LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells**. J. Immunol 174 (10):6477–6489

Smit WM, Rijnbeek M, van Bergen CA, Willemze R, Falkenburg JH (1998): **Generation of leukemia-reactive cytotoxic T lymphocytes from HLA-identical donors of patients with chronic myeloid leukemia using modifications of a limiting dilution assay**. Bone Marrow Transplant 21 (6):553–560

Smith KD, Lutz CT (1996): **Peptide-dependent expression of HLA-B7 on antigen processing-deficient T2 cells**. J Immunol 156 (10):3755–3764

Smits ELJ, Lee C, Hardwick N, Brooks S, van Tendeloo VFI, Orchard K, Guinn B (2011): **Clinical evaluation of cellular immunotherapy in acute myeloid leukaemia**. Cancer Immunol. Immunother 60 (6):757–769

Soiffer RJ, Alyea EP, Hochberg E, Wu C, Canning C, Parikh B, Zahrieh D, Webb I, Antin J, Ritz J (2002): **Randomized trial of $CD8^+$ T-cell depletion in the prevention of graft-versus-host disease associated with donor lymphocyte infusion**. Biol. Blood Marrow Transplant 8 (11):625–632

Spits H (2002): **Development of alphabeta T cells in the human thymus**. Nat. Rev. Immunol 2 (10):760–772

Sun JC, Williams MA, Bevan MJ (2004): **$CD4^+$ T cells are required for the maintenance, not programming, of memory $CD8^+$ T cells after acute infection**. Nat. Immunol 5 (9):927–933

Surh CD, Sprent J (2008): **Homeostasis of naive and memory T cells**. Immunity 29 (6):848–862

Turtle CJ, Swanson HM, Fujii N, Estey EH, Riddell SR (2009): **A distinct subset of self-renewing human memory CD8⁺ T cells survives cytotoxic chemotherapy**. Immunity 31 (5):834–844

Vago L, Perna SK, Zanussi M, Mazzi B, Barlassina C, Stanghellini MTL, Perrelli NF, Cosentino C, Torri F, Angius A, Forno B, Casucci M, Bernardi M, Peccatori J, Corti C, Bondanza A, Ferrari M, Rossini S, Roncarolo MG, Bordignon C, Bonini C, Ciceri F, Fleischhauer K (2009): **Loss of mismatched HLA in leukemia after stem-cell transplantation**. N. Engl. J. Med 361 (5):478–488

Vardiman JW, Harris NL, Brunning RD (2002): **The World Health Organization (WHO) classification of the myeloid neoplasms**. Blood 100 (7):2292–2302

Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, Harris NL, Le Beau MM, Hellström-Lindberg E, Tefferi A, Bloomfield CD (2009): **The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes**. Blood 114 (5):937–951

Verdijk RM, Kloosterman A, Pool J, van de Keur M, Naipal AMIH, van Halteren AGS, Brand A, Mutis T, Goulmy E (2004): **Pregnancy induces minor histocompatibility antigen-specific cytotoxic T cells: implications for stem cell transplantation and immunotherapy**. Blood 103 (5):1961–1964

Vincent K, Roy D, Perreault C (2011): **Next-generation leukemia immunotherapy**. Blood 118 (11):2951–2959

Warren EH, Fujii N, Akatsuka Y, Chaney CN, Mito JK, Loeb KR, Gooley TA, Brown ML, Koo KKW, Rosinski KV, Ogawa S, Matsubara A, Appelbaum FR, Riddell SR (2010): **Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens**. Blood 115 (19):3869–3878

Whiteway A, Corbett T, Anderson R, Macdonald I, Prentice HG (2003): **Expression of co-stimulatory molecules on acute myeloid leukaemia blasts may effect duration of first remission**. Br. J. Haematol 120 (3):442–451

Williams MA, Bevan MJ (2007): **Effector and memory CTL differentiation**. Annu. Rev. Immunol 25:171–192

Wölfel C, Lennerz V, Lindemann E, Hess G, Derigs HG, Huber C, Herr W, Wölfel T (2008): **Dissection and molecular analysis of alloreactive CD8⁺ T cell responses in allogeneic haematopoietic stem cell transplantation**. Cancer Immunol. Immunother 57 (6):849–857

Wölfl M, Merker K, Morbach H, van Gool SW, Eyrich M, Greenberg PD, Schlegel PG (2011): **Primed tumor-reactive multifunctional CD62L⁺ human CD8⁺ T cells for immunotherapy**. Cancer Immunol. Immunother 60 (2):173–186

Xue S, Gao L, Thomas S, Hart DP, Xue JZ, Gillmore R, Voss R, Morris E, Stauss HJ (2010): **Development of a Wilms' tumor antigen-specific T-cell receptor for clinical trials: engineered patient's T cells can eliminate autologous leukemia blasts in NOD / SCID mice**. Haematologica 95 (1):126–134

Young NT, Mulder A, Cerundolo V, Claas FH, Welsh KI (1998): **Expression of HLA class I antigens in transporter associated with antigen processing (TAP)-deficient mutant cell lines**. Tissue Antigens 52 (4):368–373

Zeng R, Spolski R, Finkelstein SE, Oh S, Kovanen PE, Hinrichs CS, Pise-Masison CA, Radonovich MF, Brady JN, Restifo NP, Berzofsky JA, Leonard WJ (2005): **Synergy of IL-21 and IL-15 in regulating CD8^{+} T cell expansion and function**. J. Exp. Med 201 (1):139–148

Zhou J, Shen X, Huang J, Hodes RJ, Rosenberg SA, Robbins PF (2005): **Telomere length of transferred lymphocytes correlates with in vivo persistence and tumor regression in melanoma patients receiving cell transfer therapy**. J. Immunol 175 (10):7046–7052

Ziegler A, Laudien D, Heinrichs H, Muller C, Uchanska-Ziegler B, Wernet P (1981): **K562 cells express human major histocompatibility antigens**. Immunogenetics 13 (4):359–365

9. Abbreviations

ALL	Acute lymphatic leukemia
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
CAR	Chimeric-antigen receptor
CD	Cluster of differentiation
CLL	Chronic lymphatic leukemia
CML	Chronic myeloid leukemia
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DLI	Donor lymphocyte infusion
DNA	Deoxyribonucleic acid
Don.	Donor
EBV	Epstein-Barr virus
ER	Endoplasmatic reticulum
FAB	French-American-British
GVL	Graft-versus-leukemia
GVHD	Graft-versus-host disease
IFN	Interferon
IL	Interleukin
i.v.	Intra veniously
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
HSCT	Hematopoietic stem cell transplantation
LAA	Leukemia-associated antigen
LCL	Epstein-Barr virus-transformed B lymphoblastoid cell line
MFI	Median fluorescence intensity
mAb	Monoclonal antibody
Mfr.	Manufacturer
mHag	Minor histocompatibility antigen
MHC	Major histocompatibility complex
MLLC	Mixed-lymphocyte / leukemia culture
NK	Natural killer
NSG	NOD / SCID / IL2Rγc^{null}
PHA	Phytohaemagglutinin
PBMC	Peripheral blood mononuclear cell
PBSCT	Peripheral blood stem cell transplantation
RTE	Recent thymic emigrants
SIB	Sibling
SNP	Single-nucleotide polymorphism
TAP	Transporter associated with antigen presentation
TCR	T-cell receptor
Tcm	Central memory T cell
Teff	Effector T cell
Tem	Effector memory T cell
Tm	Memory T cell
Tn	Naive T cell
TNF	Tumor necrosis factor
Tscm	Memory stem T cells
WBC	White blood cell count
WHO	World Health Organization

10. Appendix

10.1 Abstracts of publications derived from this thesis

Cancer Immunol Immunother. 2011 Feb;60(2):235-48. Epub 2010 Nov 3.

IL-21-treated naive CD45RA$^+$ CD8$^+$ T cells represent a reliable source for producing leukemia-reactive cytotoxic T lymphocytes with high proliferative potential and early differentiation phenotype.

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Abstract

Clinical tumor remissions after adoptive T-cell therapy are frequently not durable due to limited survival and homing of transfused tumor-reactive T cells, what can be mainly attributed to the long-term culture necessary for *in vitro* expansion. Here, we introduce an approach allowing the reliable *in vitro* generation of leukemia-reactive cytotoxic T lymphocytes (CTLs) from naive CD8$^+$ T cells of healthy donors, leading to high cell numbers within a relatively short culture period. The protocol includes the stimulation of purified CD45RA$^+$ CD8$^+$ T cells with primary acute myeloid leukemia blasts of patient origin in HLA-class I-matched allogeneic mixed lymphocyte-leukemia cultures. The procedure allowed the isolation of a large diversity of HLA-A/-B/-C-restricted leukemia-reactive CTL clones and oligoclonal lines. CTLs showed reactivity to either leukemia blasts exclusively, or to leukemia blasts as well as patient-derived B lymphoblastoid-cell lines (LCLs). In contrast, LCLs of donor origin were not lysed. This reactivity pattern suggested that CTLs recognized leukemia-associated antigens or hematopoietic minor histocompatibility antigens. Consistent with this hypothesis, most CTLs did not react with patient-derived fibroblasts. The efficiency of the protocol could be further increased by addition of interleukin-21 during primary *in vitro* stimulation. Most importantly, leukemia-reactive CTLs retained the expression of early T-cell differentiation markers CD27, CD28, CD62L and CD127 for several weeks during culture. The effective *in vitro* expansion of leukemia-reactive CD8$^+$ CTLs from naive CD45RA$^+$ precursors of healthy donors can accelerate the molecular definition of candidate leukemia antigens and might be of potential use for the development of adoptive CTL therapy in leukemia.

Impact factor (2011): 3.7

Haematologica. 2011 Jul;96(7):1024-32. Epub 2011 Apr 12.

Alloreactive and leukemia-reactive T cells are preferentially derived from naive precursors in healthy donors: implications for immunotherapy with memory T cells.

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Abstract

BACKGROUND: HLA mismatch antigens are major targets of alloreactive T cells in HLA-incompatible stem-cell transplantation, which can trigger severe graft-versus-host disease and reduce survival in transplant recipients. Our objective was to identify T-cell subsets with reduced *in vitro* reactivity to allogeneic HLA antigens.
DESIGN AND METHODS: We sorted CD4 and CD8 T-cell subsets from peripheral blood by flow cytometry according to their expression of naive and memory markers CD45RA, CD45RO, CD62L, and CCR7. Subsets were defined by a single marker to facilitate future establishment of a clinical-grade procedure for reducing alloreactive T-cell precursors and graft-versus-host disease. T cells were stimulated in mixed lymphocyte reactions against HLA-deficient K562 cells transfected with single HLA-A/-B/-C/-DR/-DQ mismatch alleles. Alloreactivity was measured by interferon-γ spot production and cell proliferation.
RESULTS: We observed that allogeneic HLA-reactivity was preferentially derived from subsets enriched for naive T cells rather than memory T cells in healthy donors, irrespective of the HLA mismatch allele. This separation was most efficient if CD45RA (versus other markers) was used for sorting. The numbers of allogeneic HLA-reactive effector cells were in median 7.2-fold and 16.6-fold lower in CD45RA(neg) memory CD8 and CD4 T cells than in entire CD8 and CD4 T cells, respectively. In contrast, proliferation of memory T cells in response to allogeneic HLA was more variably reduced (CD8) or equivalent (CD4) when compared to that of naive T cells. We also demonstrated in HLA-matched donor-patient pairs that leukemia-reactive CD8 cytotoxic T-lymphocytes were mainly derived from subsets enriched for naive T cells compared to memory T cells.
CONCLUSIONS: Memory T-cell subsets of most healthy individuals showed decreased allogeneic HLA-reactivity, but lacked significant anti-leukemia responses *in vitro*. The clinical use of memory or naive-depleted T cells might be beneficial for HLA-mismatched patients at high risk of graft-versus-host disease and low risk of leukemia relapse. Preferred allografts are those which contain leukemia-reactive memory T cells. Alternatively, replenishment with leukemia-reactive T cells isolated from naive subsets is desirable.

Impact factor (2010): 6.5