In vitro generation and characterization of acute myeloid leukemia-reactive $CD8^+$ cytotoxic T-lymphocyte clones from healthy donors

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1. ABSTRACT

Donor-derived $CD8^+$ cytotoxic T lymphocytes (CTLs) eliminating host leukemic cells mediate curative graft-versus-leukemia (GVL) reactions after allogeneic hematopoietic stem cell transplantation (HSCT). The leukemia-reactive CTLs recognize hematopoiesis-restricted or broadly expressed minor histocompatibility and leukemia-associated peptide antigens that are presented by human leukocyte antigen (HLA) class I molecules on recipient cells. The development of allogeneic CTL therapy in acute myeloid leukemia (AML) is hampered by the poor efficiency of current techniques for generating leukemia-reactive CTLs from unprimed healthy donors *in vitro*. In this work, a novel allogeneic mini-mixed lymphocyte/leukemia culture (mini-MLLC) approach was established by stimulating $CD8^+$ T cells isolated from peripheral blood of healthy donors at comparably low numbers (i.e. 10^4/well) with HLA class I-matched primary AML blasts in 96-well microtiter plates. Before culture, $CD8^+$ T cells were immunomagnetically separated into $CD62L^{(high)+}$ and $CD62L^{(low)+/neg}$ subsets enriched for naive/central memory and effector memory cells, respectively. The application of 96-well microtiter plates aimed at creating multiple different responder-stimulator cell compositions in order to provide for the growth of leukemia-reactive CTLs optimized culture conditions by chance. The culture medium was supplemented with interleukin (IL)-7, IL-12, and IL-15. On day 14, IL-12 was replaced by IL-2.

In eight different related and unrelated donor/AML pairs with complete HLA class I match, numerous CTL populations were isolated that specifically lysed myeloid leukemias in association with various HLA-A, -B, or -C alleles. These CTLs recognized neither lymphoblastoid B cell lines of donor and patient origin nor primary B cell leukemias expressing the corresponding HLA restriction element. CTLs expressed T cell receptors of single V-beta chain families, indicating their clonality. The vast majority of CTL clones were obtained from mini-MLLCs initiated with $CD8^+$ $CD62L^{(high)+}$ cells. Using antigen-specific stimulation, multiple CTL populations were amplified to 10^8-10^{10} cells within six to eight weeks.

The capability of mini-MLLC derived AML-reactive CTL clones to inhibit the engraftment of human primary AML blasts was investigated in the immunodeficient nonobese diabetic/severe combined immune deficient IL-2 receptor common γ-chain deficient (NOD/SCID IL2Rγ^{null}) mouse model. The leukemic engraftment in NOD/SCID IL2Rγ^{null} was specifically prevented if inoculated AML blasts had been pre-incubated *in vitro* with AML-reactive CTLs, but not with anti-melanoma control CTLs.

These results demonstrate that myeloid leukemia-specific CTL clones capable of preventing AML engraftment in mice can be rapidly isolated from $CD8^+$ $CD62L^{(high)+}$ T cells of healthy donors *in vitro*. The efficient generation and expansion of these CTLs by the newly established mini-MLLC approach opens the door for several potential applications. First, CTLs can be used within T cell-driven antigen identification strategies to extend the panel of molecularly defined AML antigens that are recognizable by T cells of healthy donors. Second, because these CTLs can be isolated from the stem cell donor by mini-MLLC prior to transplantation, they could be infused into AML patients as a part of the stem cell allograft, or early after transplantation when the leukemia burden is low. The capability of these T cells to expand and function *in vivo* might require the simultaneous administration of AML-reactive $CD4^+$ T cells generated by a similar *in vitro* strategy or, less complex, the co-transfer of CD8-depleted donor lymphocytes. To prepare clinical testing, the mini-MLLC approach should now be translated into a protocol that is compatible with good manufacturing practice guidelines.

2. INTRODUCTION

2.1 Acute Myeloid Leukemia (AML)

2.1.1 General characteristics and subgroups of leukemias

Leukemias are cancers of the white blood cells (leukemia, Greek "white blood"). Risk factors are pre-leukemic blood disorders, ionizing radiation, certain chemicals and viruses, anti-cancer chemotherapy, as well as genetic predispositions. Although leukemias account for only 3% of all tumors, they constitute the leading cause of death due to cancer in children and persons under 39 years of age (Deschler/Lübbert, 2006). Approximately ten thousand of patients are newly diagnosed with leukemia each year in Germany, whereas the incidences of the different leukemic types are age-dependent. Five thousand patients die each year from leukemic diseases in Germany (www.krebsgesellschaft.de).

In general, leukemias are caused by a malignant transformation of one or different subtypes of leukocytes, leading to an uncontrolled proliferation. As a consequence, the malignant leukocyte type spreads out in the bone marrow and passes over into the peripheral blood. Those leukocytes are immature and therefore not functional. At the same time, the normal hematopoiesis is displaced, leading to a lack of healthy blood cells. Symptoms are fatigue, paleness, shortness of breath, weight loss, as well as an increased susceptibility for infections and bleeding due to a lack of mature leukocytes and platelets. Diagnosis is accomplished by a differential blood count; an exact characterization of the leukemic subtype requires the analysis of leukemic cells from the bone marrow.

Leukemias are subdivided according to their clinical course (acute or chronic) and their origin from the hematopoietic lineages (myeloid or lymphoid). Acute leukemias have a rapid course, leading without therapy to death within weeks to months. They are characterized by the massive proliferation of leukemic cells, which are deposited in different organs (liver, spleen, central nervous system). Caused by missing normal blood cells, the above mentioned symptoms occur. Acute lymphatic leukemias (ALL) count for 80% of childhood leukemias, whereas the acute myeloid/myelogenous leukemia (AML) occurs predominantly in adults. Chronic leukemias have a slow course with longer stable phases. Chronic lymphatic leukemia (CLL) occurs rarely before the age of 50 years, whereas chronic myeloid leukemia (CML) occurs at earlier ages. Typical for CML is the blast crisis, corresponding to a transition into acute leukemia (www.krebsgesellschaft.de; Deschler/Lübbert, 2006).

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of myeloid progenitor cells. Occurrence of AML shows two peaks in early childhood and later adulthood. The disease has an incidence of 3.7 per 100,000 persons and an age-dependent mortality of 2.7 to nearly 18 per 100,000 persons (Deschler/Lübbert, 2006). The malignant cell type in AML is the myeloblast, which is in normal hematopoiesis an immature precursor of myeloid white blood cells. A normal myeloblast will gradually develop into a mature white blood cell. In AML, two major types of genetic events have been described that are highly interdependent and crucial for leukemic transformation: Alterations in myeloid transcription factors that regulate hematopoietic differentiation, and activating mutation of signal transduction intermediates. A single myeloblast accumulating such genetic changes will be arrested in its immature state and is prevented from differentiation. The combination of such a differentiation arrest together with mutations affecting genes that control proliferation, results in an uncontrolled growth of immature cell clones, leading to the clinical entity of AML (Steffen et al, 2005). Additionally, the differentiation of normal blasts into mature progeny cells is inhibited by AML cells, presumably mediated by various chemokines produced by AML cells (Youn et al, 2000).

2.1.2 Diagnosis of AML

AML is diagnosed when myeloid blasts comprise more than 20-30% of the cells in the bone marrow. Several types of myeloid blasts are recognized (see *2.1.3 Classification*); it is also possible that more than one type of blasts occurs in individual patients. Distinctive red-staining peroxidase-positive structures called *Auer rods*, which represent abnormal granules, are present in many cases and are particularly numerous in AML associated with the translocation t(15;17) (acute promyelocytic leukemia). The presence of Auer rods is taken to be definitive evidence of myeloid differentiation. Histochemical staining for peroxidase, specific esterase, and nonspecific esterase, and immunostainings for myeloid specific antigens are important in defining the type of myeloid differentiation that the leukemic blast exhibit. The number of leukemic cells in the peripheral blood is highly variable. White blood cell counts (WBC) can be more than 100,000 per microliter, but are under 10,000 per microliter in about 50% of the patients (www.robbinspathology.com).

2.1.3 Classification of AML

The two most commonly used classification systems for AML are the French-American-British Cooperative Group (FAB) system (Bennet et al, 1985) and the newer system of the

World Health Organization (WHO) (Vardiman et al, 2002). AML is typically categorized with the revised FAB classification, which divides AML into 8 subtypes, M0 to M7, based on the degree of maturity and the lineage of the leukemic blast (table 1). In this system, AML is confirmed when the marrow contains more than 30% blasts.

FAB subtype		**Incidence (% of AML)**
M0	undifferentiated AML	2-3%
M1	myeloblastic, without maturation	20%
M2	myeloblastic, with maturation	30-40%
M3	promyelocytic, or acute promyelocytic leukemia (APL)	5-10%
M4	myelomonocytic	15-20%
M4eo	myelomonocytic, together with bone marrow eosinophilia	
M5	monoblastic leukemia (M5a) or monocytic leukemia (M5b)	10%
M6	erythrocytic, or erythroleukemia	5%
M7	megakaryoblastic	1%

Table 1. AML sybtypes according to the French-American-British Cooperative Group (FAB) classification (modified and adapted from: www.robbinspathology.com)

The WHO classification of AML attempts to be more clinical useful, it comprises more prognostic information and has lowered the blast minimum criterion to 20% (Estey/Döhner, 2006). The WHO schema categorizes AML into the following 5 subtypes (Vardiman et al, 2002):

- **AML with characteristic genetic abnormalities** (includes translocations between chromosome 8 and 21 [t(8;21)], inversions in chromosomes 16 [inv(16)], or translocations between chromosome 15 and 17 [t(15;17)]; generally high rate of remission and a better prognosis compared to other AML types)
- **AML with multilineage dysplasia** (includes patients with a prior myelodysplastic syndrome (MDS) or myeloproliferative disease (MPD) that transforms into AML; most often in elderly patients; often worse prognosis)
- **AML and MDS, therapy-related** (includes patients who have had prior chemotherapy and/or radiation and subsequently develop AML or MDS; these leukemias may be characterized by specific chromosomal abnormalities; often worse prognosis)

- **AML not otherwise characterized** (subtypes of AML that do not fall into the above categories)
- **Acute leukemias of ambiguous lineage** (mixed phenotype or biphenotypic acute leukemia; leukemic cells cannot be classified as either myeloid or lymphoid cells, or both types of cells are present)

2.1.4 Prognosis of AML

Acute myeloid leukemia is a potentially curable disease. The chance for cure of a specific patient with AML depends on a number of prognostic factors of which cytogenetics is the most important one (Estey, 2001). Certain cytogenetic abnormalities are associated with very good outcomes (i.e. the balanced chromosomal translocations t(15;17) in promyelocytic leukemia (FAB M3), the t(8;21) translocation, and the chromosome 16 inversion). About half of all AML patients have normal cytogenetics and fall into an intermediate risk group. A number of other cytogenetic abnormalities, for example chromosomal deletions, are associated with a poor prognosis and a high risk of relapse (Estey, 2001). Secondary AML, which arises from a pre-existing myelodysplastic syndrome (MDS) or myeloproliferative disorder (MPD), as well as treatment-related AML occurring after chemotherapy for another previous malignancy (10-15% of AML patients; Estey/Döhner, 2006), have a worse prognosis. These two entities are commonly associated with deletions or monosomies involving chromosomes 5 and 7 and usually lack chromosomal translocations (www.robbinspathology.com).

Mutations in the FLT3 (fibroblast-macrophage stimulating factor receptor (FMS)-like tyrosine kinase receptor 3) gene, encoding for a class III receptor tyrosine kinase (RTK), are the most frequent genetic alterations reported in AML. FLT3 plays a role in the proliferation and differentiation of early hematopoietic progenitor cells and is expressed by up to 90% of AMLs (Steffen et al, 2005). Internal tandem duplications (ITDs) within the juxtamembrane domain occur in 15-35% of patients with AML and lead to the leukemic transformation by a constitutive phosphorylation and therefore uncontrolled activation of the tyrosine kinase (Kiyoi et al, 2002). Several studies have confirmed that FLT3/ITDs are strongly associated with leukocytosis and a poor prognosis (Kiyoi et al, 2005; Steffen et al, 2005), and are also found in putative leukemic stem cells (Levis et al, 2005). Further FLT3 mutations observed in AML patients are point mutations within the kinase domain (Yamamoto et al, 2001; Kindler et al, 2005). Activating mutations of the receptor tyrosine kinase c-KIT (CD117, receptor for stem cell factor) in AML have also clinical significance.

Clinical studies try to block the RTK activity by specific tyrosine kinase inhibitors. Beside those genetic alterations, epigenetic lesions, for example promoter silencing by hypermethylation, are increasingly investigated concerning the pathogenesis of AML (Deschler/Lübbert, 2006).

2.1.5 Treatment of AML

Primary treatment of AML consists of chemotherapy, divided into two phases: Induction and consolidation therapy. Induction chemotherapy shall achieve a complete remission by reducing the amount of leukemic cells to an undetectable level (<5% leukemic cells in the bone marrow). The durability of remission depends on the prognostic features of the original leukemia. The goal of consolidation (post remission) chemotherapy is to eliminate any residual undetectable disease. Otherwise, almost all patients will eventually relapse (Estey/Döhner, 2006). For patients with high risk of relapse, or when relapse occurred despite chemotherapy, the only proven potentially curative therapy is allogeneic hematopoietic stem cell transplantation (HSCT; Copelan, 2006), leading to a graft-versus-leukemia immune effect. In particular, infusions of T lymphocytes from the original donor (DLI, donor lymphocyte infusion) can produce remissions following relapse after HSCT (Kolb et al, 1995). Complications are cytomegalovirus (CMV) and Epstein Barr-Virus (EBV) reactivations, leading to a treatment-related mortality of 10-25% (Kolitz, 2006).

2.2 Immunological background

2.2.1. The Immune System

The human immune system is a complex system of lymphoid organs, cells, humoral factors, and cytokines that interact in order to protect the organism from infection with pathogens, as it is able to discriminate between "self" and "non-self", and also from the development of malignancies ("tumor immune surveillance", see *chapter 2.3.1*).
Immunity is divided into two parts, termed innate and adaptive (acquired, specific) immune responses. Innate immunity includes physical, chemical and microbiological barriers, but also cellular and molecular factors (neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins) that provide immediate host defence. Adaptive immunity consists of highly specific responses of T and B lymphocytes. Whereas the innate immune response is rapid, but sometimes leads to the damage of healthy tissue due to its unspecificity, the adaptive immune response is very specific but needs days or weeks to develop. Since the adaptive immunity has a memory function, subsequent exposure to the same antigen leads to, not immediate, but more rapid and vigorous responses. For an efficient elimination of pathogens, innate and adaptive immunity usually work together. Inappropriate function of the immune system may result either in severe infections, or in allergy and autoimmune diseases (Delves/Roitt, 2000; Parkin/Cohen, 2001).

2.2.2 Innate Immunity

The first innate reaction occurring after infection is the recruitment of *neutrophils* to the site of infection, a process that can lead to tissue inflammation when happening inappropriately strong. Activated *macrophages* release cytokines that stimulate myeloid precursor division in the bone marrow, leading to neutrophil leukocytosis. Recruited neutrophils kill pathogens by phagocytosis. This process is even more effective if the pathogenic organism is first opsonised with specific antibodies or complement proteins.
The *complement system* consists of at least 20 serum glycoproteins that are activated in a cascade-like amplification sequence. There exist three different pathways of complement activation that all converge with the activation of the central C3 component, leading to a final common pathway and the formation of a transmembrane pore. Osmotic lysis leads to death of the foreign organism. Beside the complement proteins, other soluble factors

involved in innate immunity are so-called *acute-phase proteins* that enhance resistance to infection and promote the repair of tissue damage.

Infections with parasites (particularly nematodes) induce the production of antigen-specific immunoglobulin (Ig) E that coat the pathogenic organism. IgE molecules are bound by *eosinophils*. Those immune cells are not phagocytic, but have large granules containing cytotoxic molecules that kill the parasite when released. Eosinophils are also involved in the pathology of allergic reactions.

Basophils and *mast cells* are immune cells that occur in the blood or in mucosa and connective tissue, respectively, at relative low number compared to other leucocytes. Both cell types possess receptors for IgE molecules. When those receptors are cross-linked by antigen-binding to IgE, basophils and mast cells are triggered to secrete inflammatory mediators such as histamine, prostaglandins, and leukotrienes.

Natural killer (NK) cells are morphologically similar to lymphocytes, but do not express a specific antigen receptor. NK cells recognize their targets cells in two different ways. First, they possess Fc receptors (FcR) that bind immunoglobulins. These receptors link NK cells to IgG-coated target cell, which are subsequently killed by antibody-dependent cellular cytotoxicity (ADCC). The second system of target recognition by NK cells relies on so-called killer-activating receptors and killer-inhibitory receptors (KIRs). The killer-activating receptors recognize several different ubiquitously expressed surface molecules, whereas KIRs recognize major histocompatibility complex (MHC) class I molecules, which are usually also expressed on normal cells, but may be downregulated in case of microbial infection or malignant transformation. If the killer-activating receptors are engaged with their ligands, NK cells are signalled to kill their target. by perforin and granzyme mechanism, inducing apotosis of the target cell. This signal is normally overridden by an inhibitory signal from KIRs that have recognized MHC class I molecules on the target cell. Activated NK cells stimulate the maturation of dendritic cells (DCs), and facilitate therewith adaptive immune responses to infections and tumors. Thus, NK cells link innate and adaptive immunity (Degli-Espoti/Smyth, 2005).

Although the innate immunity is not antigen-specific, it is able to discriminate between foreign and self molecules. This is accomplished by so-called *pattern-recognition receptors (PRRs),* for example Toll-like receptors (TLRs), expressed by phagocytic cells. Pattern-recognition receptors recognize molecular structures termed *pathogen-associated molecular patterns (PAMPs*) present on microbes, but not on host cells (e.g. cell wall components of bacteria and yeast, for example lipopolysaccharide (LPS)). Since pattern-recognition receptors recognize a broad range of microbial structures and present the

processed products to antigen-specific T cells, the innate immunity is closely linked to adaptive responses. The different mechanisms of innate immunity to fight infections – phagocytosis, opsonization, and complement-mediated lysis – need the exposure of innate immune cells to the surface of pathogens. Innate immunity is therefore only able to eradicate *extracellular* microbes, mostly bacteria. *Intracellular* pathogens, such as viruses and mycobacteria, can not be detected by cells of the innate immune system (Delves/Roitt, 2000; Parkin/Cohen, 2001).

2.2.3 Specific Immunity

2.2.3.1 T and B cell receptor

Specific (adaptive, acquired) immunity is characterized by targeted effector responses accomplished by the use of antigen-specific receptors on T and B lymphocytes. The antigen is presented to and recognized by the antigen-specific T or B cell. This process leads to cell priming, activation, and differentiation, usually occurring within lymphoid tissues. Activated T cells leave the lymphoid tissue and home to the disease site to fulfil their effector functions. Activated B cells (plasma cells) release antibody into blood and tissue fluid.

T and B lymphocytes develop from progenitor cells within the bone marrow. B cells complete their development within the bone marrow, whereas T cells migrate at an early stage as thymocytes to the thymus. The formation of antigen-specific B and T cell receptor occurs early in the development, and is accomplished for both lymphocyte types by a similar mechanism of random rearrangement and splicing together of multiple DNA segments, coding for the antigen-binding region of the receptor (complementarity-determining regions, CDRs). Four segments of genes are involved in those gene arrangements, which are located on different chromosomes and are called variable (V), diversity (D), joining (J), and constant (C) regions. Clonal diversity, leading to a repertoire of over 10^8 T cell receptors (TCRs) and 10^{10} antibody specificities (Arstila et al, 1999), is achieved by several mechanisms: (1) Free combination of the final VDJ region out of a multiplicity of genes for the single regions (V, 25-100 genes; D, ~25 genes; J, ~50 genes). (2) Inaccurate splicing and base pair frameshifts leading to different amino acids (junctional diversity). (3) Insertion of additional nucleotides by the enzyme desoxyribonucleotidyltransferase. The repertoire of B cell receptors is even more increased as further immunoglobulin gene arrangements occur during B cell division after antigen stimulation (somatic hypermutation).

The T cell receptor occurs in two forms. The most common is a heterodimer built up out of an α and a β chain, each consisting of a constant and a variable domain (αβ-T cells). αβ-T cells are mainly responsible for major histocompatibility complex (MHC)-restricted antigen-specific immune responses. A minority of T cells express γ and δ chains. Those γδ-T cells represent only about 5% of lymphocytes in the blood, but appear in much larger numbers in epithelial tissues such as skin and intestinal tract. They recognize protein as well as nonprotein antigens and are not MHC-restricted. γδ-T cells are thought to be involved in mucosal immunity, but their function is not finally elucidated. Another subset of TCR $\alpha\beta^+$ cells are the so-called natural killer T cells (NKT cells), that display like many γδ-T cells relatively invariant TCRs, and are positively selected by the MHC-related molecule CD1d. The commitment of bone marrow precursors to the T cell lineage as well as the differentiation of both αβ- and γδ-T cells seems to be particularly dependent on Notch signaling pathways, but the precise mechanisms are not yet fully characterized (Hayday/Pennington, 2007).

B cell fate requires the expression of the "master regulator" Pax5. In B cells, the receptor gene product is a membrane-bound IgM, initially expressed alone, later together with IgG. In early B cell development, this molecule acts as antigen receptor with the ability to induce cell signaling. The bound antigen can be internalized, processed and being re-expressed for antigen presentation to T cells. As soon as B cells are activated, they develop into plasma cells secreting the soluble antibody.

B and T cell receptors recognize antigens by completely different mechanisms. Whereas antibodies recognize the conformational structure of an epitope without the need of processing, the TCR binds linear peptides out of 8 to 9 aminoacids that have been produced by intracellular processing of antigen-presenting cells. The so-called *complementarity-determining region (CDR*), consisting of three subregions, CDR1, CDR2, and CDR3, is the surface of the variable region of an antibody or a TCR that binds to the antigen (Abbas/Lichtman, 2003).

2.2.3.2 Development of αβ-T cells in the thymus

Early T cell precursors (thymocytes) travel from the bone marrow to the thymus and complete their maturation by undergoing a series of selection procedures. These maturation processes occur in a precise order of somatic recombination of TCR genes, the expression of the TCR, CD3, and CD4 and CD8 coreceptors. The most immature thymocytes do not express TCR, or CD4 and CD8. Those double-negative T cells ($CD4^-$ $CD8^-$) are found in the subcapsular sinus and outer cortical region of the thymus. From

here, thymocytes migrate into and through the cortex and first express αβ-TCR, as well as CD4 and CD8 coreceptors (double-positive T cells, $CD4^+$ $CD8^+$). For antigen recognition, foreign peptides need to be presented to T cells by self MHC molecules. The TCR does not only recognize the antigenic peptide alone, but the complex of the epitope in association with a self MHC molecule. Positive selection processes in the thymical cortex sort for T cells that are capable of recognizing self MHC molecules, but only with low affinity in order to exclude autoreactivity. Due to the random nature of TCR gene arrangement, many of the immature T cells are useless because they do not recognize self MHC molecules at all, and die by apoptosis. This mechanism ensures that surviving T cells are self MHC-restricted. Thus, T cells are positively selected if they express a TCR that interacts with the MHC molecules expressed on cortical epithelial cells. In that case, the signal for spontaneous apoptosis that is otherwise triggered naturally in developing T cells is switched off. Negative selection, largely mediated by bone marrow-derived dendritic cells and macrophages in the cortico-medullary junction, induces apoptosis in lymphocytes that express TCRs with a high affinity for a complex of self peptide plus self MHC in order to prevent autoimmunity, a process called *clonal deletion*.

The selection processes in the thymus are accompanied by loss of one of the coreceptors, CD4 and CD8, resulting in single-positive CD4 or CD8 T cells that recognize peptide presented by only MHC class II or class I molecules, respectively. This phenotypic maturation is accompanied by functional maturation. Mature single-positive T lymphocytes leave the thymus and populate peripheral lymphoid tissues.

The process of inducing *central tolerance* through clonal deletion is not absolute, because not all self proteins are expressed in the thymus. However, lymph node stromal cells were found to contribute to self-tolerance by their endogenous expression of a broad range of peripheral tissue antigens. In that context, the autoimmune regulator AIRE seems to be important, since defects in the gene encoding AIRE result in multi-organ autoimmunity (Zehn/Bevan, 2007). The immune system has developed further mechanisms of *peripheral tolerance*, leading to the elimination of self-reactive T cells outside the thymus. These mechanisms are mainly based on the lack of costimulatory signals to T cells that encounter self-antigens, leading to anergy (unresponsiveness) or apoptosis (Delves/Roitt, 2000; Parkin/Cohen, 2001; Abbas/Lichtman, 2003). The induction of both central and peripheral tolerance to peripheral tissue antigens involves dendritic cells that carry and cross-present antigens acquired in nonlymphoid tissues, and nonhematopoietic cells (medullary epithelial cells, lymph node stromal cells) that express a broad range of organ-specific genes (Zehn/Bevan, 2007). Recent studies suggest that there exist also peripheral,

extrathymic maturation events. It seems possible that the thymus not only exports mature, positively selected T cells, but also immature progenitors that thereafter mature *in situ* (Hayday/Pennington, 2007).

2.2.3.3 Antigen processing and presentation

The presentation of protein antigens to T cells in context with MHC molecules requires intracellular processing pathways, which are different for $CD4^+$ and $CD8^+$ T cells. Exogenous antigens, derived from extracellular pathogens, are presented by MHC class II molecules to $CD4^+$ T cells, whereas endogenous antigens, such as viral or tumor proteins, are presented by MHC class I molecules to $CD8^+$ cytotoxic T cells. Alternatively, exogenous proteins can be taken up by antigen-presenting cells (APCs; e.g. DCs, B cells, macrophages) through endocytosis and being cross-presented by MHC class I to $CD8^+$ cytotoxic T cells. Since all nucleated cells express MHC class I, any such cell that is infected or produces abnormal tumor antigens, is able to present those antigens to $CD8^+$ T cells for cytolytic attack. In contrast, MHC class II molecules are only expressed by cells specialized for antigen presentation.

MHC class I pathway. Ubiquitinylated endogenous proteins – viral, tumor, bacterial or defective cellular proteins – are first degraded into smaller fragments by the proteasome, which is a large multienzyme complex in the cytosol with several proteolytic acitivities. The proteasome cleavage defines the C-terminal end of the potentially immunogenic peptides. Subsequently, cytosolic aminopeptidases (e.g. TPPII, tripeptidylpeptidase II; Kloetzel, 2004) cleave N-terminal residues, and the resulting peptides are transported into the endoplasmatic reticulum (ER) by the transporter in antigen processing (TAP). The N-terminus is further trimmed by aminopeptidases within the ER (e.g. the IFN-γ-inducible aminopeptidase ERAPI; Saric et al, 2002) to a peptide length of eight to nine amino acid residues. Those peptides are then loaded onto MHC class I molecules by the peptide loading complex. Before assembly of the MHC peptide loading complex inside the ER, MHC class I heavy chain has been stabilized by calnexin. Binding of β2-microglobulin to the heavy chain leads to an exchange of calnexin by calreticulin. Then, formation of the MHC class I peptide loading complex consisting of TAP, tapasin, MHC class I heterodimer, calreticulin and Erp57 takes place. After peptide loading, the MHC class I peptide complex dissociates from the loading complex and is transported through the Golgi apparatus to the cell surface (figure 1). Antigenic peptides presented at the cell surface by MHC class I molecules can now be recognized by $CD8^+$ cytotoxic T cells (Abbas/Lichtman, 2003; Kloetzel, 2004).

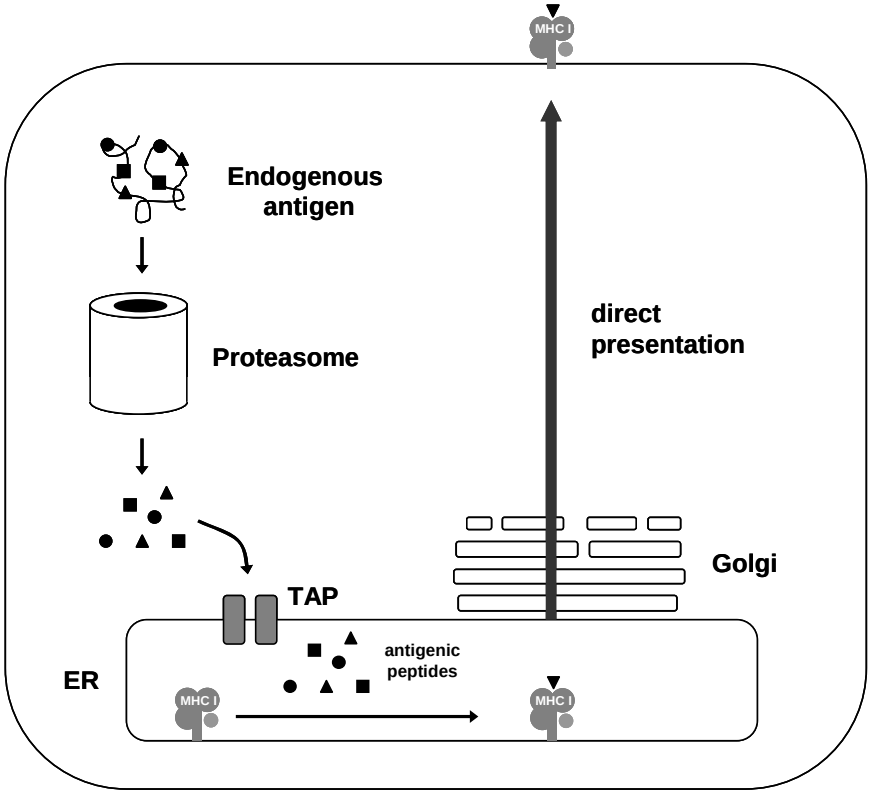

Figure 1: MHC class I presentation pathway (adapted and modified from: Heath et al, 2004)

MHC class II pathway. $CD4^+$ T cells recognize exogenous protein antigens derived from extracellular pathogens in association with MHC class II molecule. Uptake of exogenous proteins by APCs is accomplished by phagocytosis or endocytosis, or in the case of B cells by internalization of surface immunoglobulin-bound proteins. Protein antigens are enclosed in endosomes, which subsequently fuse with MHC class II-containing acidic lysosomes, leading to protein degradation. The assembly of the MHC class II molecule takes place in the endoplasmatic reticulum. The α- and β-chain form a complex with the invariant chain, which prevents the MHC molecule from binding to a peptide inside the ER. Calnexin holds the MHC molecule and the invariant chain assembled together. For export of the MHC complex from the ER, calnexin is released, and the MHC II/invariant chain complex is transported via the Golgi apparatus to specialized lysosomal compartment which fuse with the protein-containing endosomes to endolysosomes. There, the invariant chain is cleaved by proteases, leaving a small fragment (CLIP, class II associated invariant peptide) bound to the MHC II molecule. For antigen binding, the CLIP fragment is replaced by the peptide; this process is supported by a HLA-DM molecule that acts as a

molecular chaperone and hinders the aggregation of MHC class II molecules. Antigen peptides are now presented on the cell surface by MHC class II molecules and can be recognized by antigen-specific $CD4^+$ T cells.

Cross-presentation. It is also possible that exogenous material is presented by MHC class I molecules instead of MHC II. This mechanism, termed cross-presentation or cross-priming, serves to present material of infected cells, after being transported to uninfected professional APCs (DCs), in association with MHC class I molecules to $CD8^+$ cytotoxic T cells (Heath et al, 2004).

Epitope spreading. Once the immune system is stimulated by an immunogenic epitope, it is possible that additional epitopes on the antigen are involved in the subsequent response as a result of general upregulation of antigen processing and presentation. This phenomenon is referred to as *epitope spreading*, and may even occur between different antigens (intermolecular spreading). Epitope spreading is clinical relevant in some autoimmune diseases, such as *lupus erythematosus* (Delves/Roitt, 2000).

2.2.3.4 T lymphocyte activation

Naive T - i.e., they have not yet encountered their specific antigen - populate the secondary lymphoid tissues of the lymph nodes, spleen, tonsils, and mucosas. The lymphoid tissues provide the requirements to increase the chance of lymphocytes to meet their specific antigen. Adhesion molecules and chemokines allow the cells to move through the tissues in search for antigen. Antigen-presenting cells produce cytokines necessary for lymphocyte maintenance. The primary immune response – the proliferation of naive lymphocytes during their first antigen encounter – leads to the clonal expansion of antigen-specific T cells, generating both effector and memory cells. After a subsequent encounter with the same antigen, memory cells enable a superior secondary immune response (Delves/Roitt, 2000; Parkin/Cohen, 2001).

When a T cell has encountered its antigen that was brought to the lymph node by the lymphatics or within APCs, activation occurs over the next 2-3 days. However, for full T cell activation, the engagement of the T cell receptor and CD4 or CD8 coreceptors by MHC-peptide complexes is not sufficient. A second signal, termed costimulation, is provided by the engagement of costimulatory molecules, such as B7 molecules (CD80=B7.1, CD86=B7.2), CD40, and CD58, that are expressed on antigen-presenting cells and bind CD28, CD40L (CD154), and CD2 on the T cell, respectively (figure 2). Activated dendritic cells are the most potent stimulators for naive T cells, because they express large amounts of B7 and CD40. Another receptor on T cells for costimulation is CD45, a tyrosine

phosphatase with a critical role in the activation of both T and B cells. The corresponding costimulatory molecules that bind CD45 are not fully defined (Delves/Roitt, 2000).

Crosslinking of the TCR/CD3 complex after antigen binding causes phosphorylation of tyrosines within the cytoplasmic tail of CD3. This leads to the activation of intracellular signal transduction pathways and activation of cytokine genes responsible for T cell proliferation. If the costimulatory molecules are not simultaneously engaged, different signal cascades are activated leading to T cell anergy and apoptosis (Delves/Roitt, 2000; Parkin/Cohen, 2001).

There are also coinhibitory regulators of T cell activation, which include the attenuators of TCR signaling CTLA-4 (cytotoxic T-lymphocyte-associated protein 4, CD152) and PD-1 (programmed death-1), and the B and T lymphocyte attenuator BTLA (CD272; Carreno/Collins, 2002; Watanabe et al, 2003; Greenwald et al, 2005).

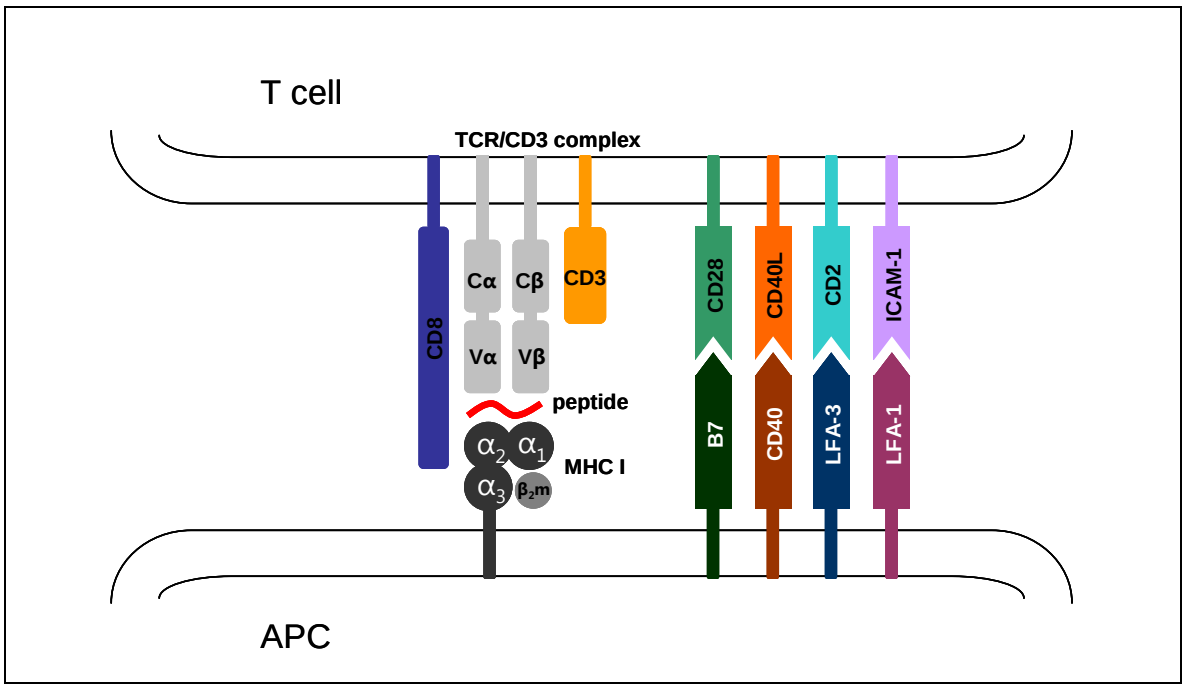

Figure 2: Immunological Synapse. Molecular interactions upon antigen presentation and recognition between an antigen-presenting cell expressing MHC class I, and a $CD8^+$ T cell. ICAM, intercellular adhesion molecule; LFA, leucocyte function associated antigen

2.2.3.5 Effector mechanisms of T lymphocytes

Two major types of effector cells are known; $CD4^+$ T cells are mainly cytokine-secreting helper T cells (Th), whereas $CD8^+$ T cells are mainly cytolytic killer cells (CTLs, cytotoxic T lymphocytes). $CD4^+$ helper T cells were classically categorized into two distinct subsets according to the cytokine patterns they produce. Th1 cells induce a cell-mediated

inflammatory response and may also contribute to the pathogenesis of autoimmune disease. They predominantly produce interferon-γ (IFN-γ), which activates macrophages to kill intracellular bacteria and induces cytotoxicity of NK cells. IFN-γ-stimulated macrophages (and other APCs) secrete IL-12, which further increases the production of IFN-γ by Th1 cells. The second major cytokine of Th1 cells is interleukin-2 (IL-2), which together with IFN-γ induces T cell proliferation of $CD8^+$ cells. IL-2 and IFN-γ have also an autocrine effect on $CD4^+$ T cells. The cytokines produced by Th2 cells are IL-4, IL-5, IL-6, and IL-10 that stimulate antibody production by activated B cells (plasma cells). Th2 responses may be associated with allergic diseases, since IL-4 secretion leads to isotype switching to IgE. The cytokines produces by Th cells are also important in the downregulation of immune responses. For example, the secretion of IFN-γ by Th1 cells inhibits Th2 cells, and the secretion of IL-10 by Th2 cells reciprocally inhibits Th1 cells (Parkin/Cohen, 2001; Delves/Roitt, 2000). An uncontrolled effector T cell response may lead to autoimmune diseases and allergies. Beside helper T cells, there have also been described $CD4^+$ regulatory T cells (T_{regs}) which are able to control effector responses. CD4 T_{regs} are best characterized in mice and can be subdivided into naturally occurring and inducible T_{regs}. They are characterized by the coexpression of CD25 (IL2Rα) and the transcription factor FOXP3 (forkhead box P3); further markers are lymphocyte activation gene-3 (LAG-3, CD223; Huang et al, 2004) and glucocorticoid-induced TNFR family-related gene (GITR). Another subset of $CD4^+$ T cells that has been described are so-called Th17 cells, which produce IL-17, are highly proinflammatory and induce severe autoimmunity. The differentiation of murine Th17 cells from naive precursors has been shown to require IL-6 and TGF-β, whereas IL-23 is needed to expand previously differentiated Th17 cells (Bettelli et al, 2007). The human Th17 subset has until now not been fully characterized (Acosta-Rodriguez et al, 2007).

$CD8^+$ CTLs act directly cytotoxic on their target cells. The principal mechanism of CTL-mediated cytolysis is the delivery of cytotoxic granule proteins to their target cell. After CTL recognition of MHC I-associated peptides, the cytoskeleton of the CTL reorganizes, in order to concentrate the cytoplasmic granules of the CTL in the area near the contact to the target cell. The granule membrane subsequently fuses with the plasma membrane, resulting in exocytosis of the granule contents onto the surface of the target cell. The two most important granule proteins for CTL cytotoxicity are perforin and granzymes. Perforin is a pore-forming protein, which is present as a monomer in the granules of CTLs (and also of NK cells). It undergoes polymerization after being released from the granules and coming in contact with high extracellular calcium concentrations. This polymerization

takes place in the lipid bilayer of the target cell plasma membrane and forms an aquaeos channel. Through these channels, granzymes (granule enzymes) are being passed. Granzyme B, which is the most important of those serine proteases, proteolytically cleaves and thereby activates caspases within the target cell. Activation of caspases and further downstream molecules induce DNA fragmentation and apoptosis of the target cell. Another effect of perforin pores in the target cell membrane is an increased influx of water and ions, inducing osmotic swelling. Additionally, high calcium concentration may trigger apoptosis. Another mechanism of CTL killing is mediated by binding of Fas ligand (FasL) on the CTL to the "death receptor" Fas, which is expressed on many target cell types. The Fas/FasL interaction also induces apoptosis of the target cell by activation of caspases. Cytokines produced by $CD8^+$ T cells upon antigen encounter include IFN-γ, TNF-α, and lymphotoxin, which function to activate macrophages and induce inflammation Some $CD4^+$ T cells are also capable of target cell killing, but as $CD4^+$ T cells lack perforin and granzymes, FasL plays in that case the main role in cytotoxicity (Abbas/Lichtman, 2003).

Beside $CD8^+$ cytotoxic effector T cells, it has also been postulated earlier that there exist $CD8^+$ T cells that have a suppressor function in downregulating T cell responses. This observation was confirmed by a recent publication, which describes a human subset of $CD8^+$ T cells, which expressed LAG-3, CD25 and FOXP3. These $CD8^+$ T_{regs} seemed to be induced *in vivo* by pathogenic stimulation, since there were only found in primed, but not in naive donors. The suppression of T cell responses was suggested to be mediated through the secretion of CCL4 interfering with TCR signaling (Joosten et al, 2007).

Further studies in murine models have found a population of regulatory $CD8^+$ T cells that were generated by exposure of $CD8^+$ T cells to IL-4 and IL-12. In contrast to $CD4^+$ regulatory T cells, $CD8^+$ T_{regs} were additionally stimulated by glucocorticoids. The described $CD8^+$ suppressor T cells secreted IL-10 and IFN-γ, and inhibited Th1, Th2 as well as CTL antigen-specific effector responses (Noble et al, 2006; Waller, 2006).

2.2.3.6 Differentiation of $CD8^+$ T cells

To induce an optimal protective immunity, the adaptive immune system maintains a constant number of naive T cells from which long-lived memory T cells arise. During an immune response, the number of antigen-specific T cells greatly increases to carry out effector functions before contracting and generating memory T cells.

Naive T cells home to lymph nodes in search for their antigen. Homing occurs in high endothelial venules (HEV), which express molecules for the constitutive recruitment of lymphocytes. The chemokine receptor CCR7 and the cell adhesion molecule CD62L

(L-selectin, binds to sialyl-Lewis^X-like sugars) are both involved in the migration of naive T cells to lymph nodes and Peyer's patches. Upon antigenic stimulation, naive T cells proliferate by clonal expansion and differentiate into effector cells, which express receptors that enable them to migrate to sites of inflammation. Although most effector cells are short-lived, a few antigen-experienced cells survive and differentiate into memory cells that provide immunity against a second antigen challenge. Naive and memory T cells were initially distinguished by the cell surface expression of different isoforms of a transmembrane phosphatase, CD45RA and CD45R0, respectively.

Memory cells can be subdivided according to their function and migratory ability. The so-called effector memory T cells (T_{EM}) migrate to peripheral tissues and are involved in immediate protection, whereas central memory T cells (T_{CM}) are important for secondary responses and long-term protection. T_{CM} express a repertoire of homing molecules similar to that of naive T cells (CD62L, CCR7) and migrate preferentially to lymphoid organs. Upon secondary stimulation, T_{CM} become effector cells (Sallusto et al, 1999; von Andrian/ Mackay, 2000; Lanzavecchia/Sallusto, 2005; Klebanoff et al, 2006). The differentiation model proposed by Sallusto and Lanzavecchia assumes that these two memory subsets result from the selective survival of effector cells and intermediates that are generated during the primary response. It is proposed that, depending on the strength of the antigenic stimulation, the avidity of the TCR, and the cytokine stimulation, activated T cells progressively differentiate through early, intermediate and late effector stages that can be phenotypically distinguished. Differentiating T cells acquire tissue homing receptors and effector functions and become able to respond to homeostatic cytokines, while they lose lymph node homing receptors and proliferative capacity. Intermediates in CD8 T cell differentiation have also been shown to downregulate CD27 and CD28 expression (Lanzavecchia/Sallusto, 2005). Precursors of long-lived memory $CD8^+$ T cells have been demonstrated to up-regulate anti-apoptotic molecules such as Bcl_{XL} and surface expression of IL-15R and IL-7Ralpha (CD127), which allows their homeostatic maintenance in the absence of further antigen challenge (Kaech et al, 2003b). Maintenance of CD8 memory cells is depending on IL-15 mediating proliferation, and IL-7 which is important for survival.

CD4 help. Memory $CD8^+$ T cells require two properties to provide long-term protection in response to infections or immunization. First, they must be able to increase their numbers rapidly and exert potent effector functions when the antigen is re-encountered. Second, they need the ability for self-renewal in order to persist for long periods, a state termed homeostatic proliferation. The quality of $CD8^+$ memory T cells depends on help from $CD4^+$

T cells. Several studies demonstrated that although memory $CD8^+$ T cells are generated in the absence of CD4 help, their responses to a second antigen encounter are weaker (impaired proliferation and cytolytic capacity, decreased IL-2 and IFN-γ production) compared to $CD8^+$ memory T cells that were generated in the presence of $CD4^+$ T cells. CD4 help is provided to $CD8^+$ T cells directly by cytokine secretion, as well as indirectly by activating APCs and B cells via CD40L/CD40 stimulation, leading to an efficient priming of naive $CD8^+$ T cells. Additionally, $CD4^+$ T cells can communicate directly with $CD8^+$ T cells through their CD40L, since activated $CD8^+$ T cells also express CD40 (Kaech/Ahmed, 2003a).

$CD8^+$ memory stem cells. Although the progressive pathway of CD8 differentiation has been widely accepted (Wherry et al, 2003), there are also some controversial findings about the linear relationship between T_{CM} and T_{EM}. Memory cells have several qualities that are typically associated with stem cells, such as the capability of self-renewal (Lanzavecchia/Sallusto, 2005). Recently, in a mouse model of graft-versus-host disease (GVHD), a subset of host-reactive CD8 memory cells was discovered that sustained GVHD and was able to self-replicate and differentiate into both central memory and terminally differentiated effector memory cells. These so-called memory stem cells expressed low levels of CD44 and high levels of CD62L like naive $CD8^+$ T cells, but in contrast, they expressed high levels of Sca-1 (stem cell antigen-1), which is marker on self-renewing cells (Zhang et al, 2005; Yu/Anasetti, 2005).

Homeostatic proliferation of naive T cells. During a lymphopenic state (e.g. in newborns, during infection, after chemotherapy or irradiation), naive T cells are maintained by homeostatic proliferation restoring lymphocyte numbers in the absence of effector responses. In contrast to antigen-specific proliferation, homeostatic proliferation of naive T cells is polyclonal, depends on large amounts of IL-7, and requires distinct costimulatory signals, such as CD24 (Li et al, 2004).

T cell exhaustion. During chronic antigenic stimulation, such as in the case of progressive tumors or chronic viral infections, responding $CD8^+$ T cells can become impaired in their effector functions, including cytokine production, cytolysis and proliferation. This phenomenon, termed "exhaustion" correlates with the magnitude of antigen burden and is associated with an upregulated expression of the inhibitory proteins PD-1 and CTLA-4 which both bind to receptors of the B7 family. CTLA-4 can bind B7-1 (CD80) and B7-2 (CD86), whereas PD-1 binds to B7-H1 (PD-L1) and B7-DC (PD-L2; Blattman/Greenberg, 2006; Sharpe et al, 2007). In a mouse model of chronic LCMV (lymphocytic choriomeningitis virus) infection, it could recently be demonstrated that blockade of PD-1,

but not of CTLA-4 alone or in combination with PD-1, can rescue $CD8^+$ T cells from exhaustion (Barber et al, 2006). This observation can be explained by the different functions of PD-1 and CTLA-4 in the regulation of T cell responses. The major role of CTLA-4 is to prevent unwanted T cell responses by blocking early costimulatory signals in lymphoid tissues, whereas PD-1 acts predominantly in peripheral tissues to control T cell responses. The effect of PD-1 can be overridden by IL-2-mediated signals, suggesting that the PD-1 pathway may be more important during chronic stimulation, when Il-2 as well as costimulatory signals become limited. Thus, PD-1 blockade might be a new approach for enhancing T cell responses in settings of therapeutic vaccination or adoptive immunotherapy of chronic infections or malignancies (Blattman/Greenberg, 2006).

Transcriptional regulation. The transcriptional regulation of $CD8^+$ T cell differentiation is up to now not fully understood. The activation of transcriptional regulators after TCR signaling must induce genes important in directing proliferation, acquisition of effector functions, and survival. Two T cell-specific transcription factors, T-bet and eomesodermin, have been found to be essential inducers of target genes important for the acquisition of CD8 effector functions and for the responsiveness to cytokines promoting the survival of long-lived memory cells (Pearce et al, 2003; Sullivan et al, 2003; Glimcher et al, 2004; Intlekofer et al, 2005). The E protein family of transcription factors and their natural inhibitors, the "inhibitor of DNA binding" (Id) proteins are known to regulate many aspects of hematopoiesis, including commitment to the lymphoid lineages (Murre, 2005). In a transgenic mouse model of *Listeria monocytogenes* infection, it could be shown that the transcriptional regulator Id2 is essential in regulating the survival of effector $CD8^+$ T cells and influences their differentiation to memory cells (Cannarile et al, 2006).

2.3 Immunotherapy of malignancies

2.3.1 Immunity to tumors

The concept of *tumor immune surveillance* assumes that the host immune system is capable to recognize and eliminate tumor cells (Burnet, 1970). Evidence for this view comes from several findings. Immunocompromised individuals show an increased cancer incidence (in part due to virally-induced tumors), whereas occasional spontaneous regressions occur in immunocompetent hosts. Tumor immunity could be demonstrated using mouse models with defined immunological defects. Furthermore, the accumulation of immune cells at tumor sites – e.g. tumor-infiltrating lymphocytes (TILs) – correlates with an improved prognosis, and anti-tumor immune responses can directly be detected in the patient. However, although many tumors are potentially immunogenic, they have often developed strategies that promote escape from tumor surveillance (Blattman/Greenberg, 2004). In that context, less is known about immune escape mechanisms of hematological malignancies compared to solid tumor. Downregulation of HLA expression, leading to an insufficient presentation of tumor-associated antigens (TAAs), occurs in many types of solid tumors (van Hall et al, 2006), and is also reported for some cases of leukemia (Elkins et al, 1984; Dermime et al, 1997; Brouwer et al, 2002; Demanet et al, 2004). Poor immunogenicity may also be due to the loss of antigen expression ("antigen loss variants"), or the reduced expression of costimulatory molecules (Costello et al, 1998; Brouwer et al, 2000b; Brouwer et al, 2001). Additionally, solid tumors have developed diverse strategies to escape the immune system by establishing tolerizing conditions in the tumor microenvironment which provides the requirements for neoplastic processes. Those immune escape mechanisms include low levels of molecules promoting immune responses and DC maturation (e.g. granulocyte/macrophage-colony stimulating factor (GM-SCF), IL-12, IL-18, IFN-γ, IL-4) in the tumor microenvironment, and at the same time an enhanced expression of molecules that act immunosuppressive or promote tumor growth (IL-6, IL-10, transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), idoleamine-2,3-deoxygenase (IDO), nitric-oxide synthase-2 (NOS2), cyclooxygenase-2 (COX2); Muller/Scherle, 2006). Insufficient T cell priming in the microenvironment of solid tumors may also rely on "structural defects", like high intestinal pressure, defective blood vessels, fibrosis or lacking lymph vessel. It is furthermore speculated that the stroma of tumor might prevent the efficient release of TAAs at early stages. Antigen-presenting cells might not have sufficient stimulatory capacity, and the

amount of effector T cells or cytokines might be to low. This in turn might be due to the lack of an "acute phase" in the course of tumorigenesis, since priming of $CD8^+$ T cells is a rapid and early process which is crucial for effective expansion of antigen-specific T cells, as shown for acute infections (Zou, 2005). Regulatory T cells (T_{regs}), induced by IL-10 and TGF-β in the tumor microenvironment are suggested to suppress effector T cell responses to tumor by different mechanisms. Tumor-associated T_{regs} might induce expression of B7-H4 on APCs. B7-H4 is a co-inhibitory molecule of the B7 family, which might bind to its (unknown) receptor on effector T cells, leading to cell cycle arrest. T_{regs} expressing PD-1, which is a ligand for B7-H1 (=PDL-1) acting costimulatory as well as coinhibitory, induce an increased expression of IL-12 in myeloid DC when binding by PD-1 to B7-H1 on myeloid DCs (Zou, 2005).

Since the immune system not only protects the host from tumor development, but also exerts a selection pressure on tumor cells promoting the survival of escape variants, the "cancer immunoediting" concept was proposed (Dunn et al, 2002). Cancer immunoediting is considered to result from three phases: (1) The eliminiation phase, which encompasses the original concept of immunosurveillance, (2) the equilibrium phase, in which the host immune system exerts selective pressure on tumor cells that have survived the elimination process, leading to less immunogenic variants, and (3) the escape phase, where surviving tumor cells that have acquired insensitivity to immunological detection begin their uncontrolled expansion (Dunn et al, 2002).

2.3.2 Approaches for cancer immunotherapy

Immunotherapeutic strategies against malignancies can possibly target the different branches of the immune system, i.e. the innate, the humoral, and the cellular immunity. Activation of innate responses can be enhanced by the administration of adjuvants (such as the TLR ligands LPS and CpG), ligands for costimulatory molecules (e.g. CD40L on DCs), or drugs that directly trigger innate immune cells. Cytokines may be used to stimulate innate as well as adaptive response (IL-2, IFN-α, IFN-γ). Attempts of humoral therapy aiming at the activation of B cells have proven until now not successful. Reasons were limited antibody responses, unspecificity, as well as potential autoreactivity.

The passive transfer of monoclonal antibodies (mAbs) is today frequently applied in therapy; one of the most commonly used mAbs is Rituximab, which binds to CD20. Rituximab (MabThera™) is successfully applied in the treatment of B cell lymphomas by the mechanism of antibody-dependent cellular cytotoxicity (ADCC). The anti-CD52 mAb

Alemtuzumab (MabCampath™) is approved for treatment of CLL; it is furthermore used for lymphodepletion prior to stem cell transplantation. Other mAbs act independent from effector cells by blocking receptors essential for cell signaling pathways that affect survival and growth of tumor cells. In clinical use are mAbs against the Her-2/Neu receptor (Trastuzumab, Herceptin™) on breast cancer cells, and against the epidermal growth factor receptor (EGFR) on epithelial tumors (Cetuximab, Erbitux™). Further applications of mAbs in immunotherapy include the linkage to radioisotopes or toxins (e.g. anti-CD33 mAb Gemtuzumab (Mylotarc™) coupled to calicheamicine, approved for AML treatment). Bispecific mAbs are recombinantly engineered to simultaneously bind tumor cells and activate receptors (e.g. CD3, FcR) on immune effector cells. Occurring problems in the treatment with mAbs are the relative lack of specificity, leading to toxicity towards normal tissues, and immunogenicity against murine portions of the antibodies (Blattman/Greenberg, 2004).

Therapeutic vaccination against malignancies is an active immunotherapy approach that aims at stimulating autologous cellular immune responses by the delivery of optimal TAA vaccines. Vaccination strategies that are currently under study have different formats. Activated DCs as APCs may be loaded with peptides or transfected with DNA or RNA encoding the respective TAA. Other modes of antigen delivery are modified inactivated tumor cells and recombinant viral or bacterial vectors (adenoviruses, *Listeria, Salmonella*) that are engineered to express the antigen as well as TLR ligands as adjuvants (e.g. CpG oligonucleotides). Cancer vaccines so far have shown only limited success, since most vaccinated patients developed only weak T cell responses with minor clinical benefit (Rosenberg, 2004; Puré et al, 2005; Blattman/ Greenberg, 2004). An alternative strategy proposed to enhance autologous T cell responses is blocking of negative regulators of T cell activations, such as CTLA-4 and PD-1 (Blattman/Greenberg, 2006; Barber et al, 2005), or to selectively deplete regulatory T cells (Blattman/Greenberg, 2004).

Adoptive transfer of allogeneic T cells as a passive immunotherapy (see *chapter 2.3.6*) has proven to be more successful until now compared to active immunotherapeutic approaches that try to stimulate autologous responses by vaccination (Blattman/Greenberg, 2004; Puré et al, 2005).

2.3.3 Hematopoietic stem cell transplantation (HSCT) as immunotherapy

Hematopoietic stem cell transplantation (HSCT) was initially developed in order to prevent bone marrow toxicity caused by intensive chemoradiotherapy regimens against

hematological malignancies. By transplanting pluripotent hematopoietic stem cells obtained from bone marrow or peripheral blood, it became possible to apply much higher doses of chemo- and/or radiotherapy. It was later recognized that in allogeneic HSCT, donor immune cells, which are transplanted with the stem cell graft, or arise from it, exert a potent graft-versus-malignancy (tumor, leukemia) effect. These findings made allogeneic HSCT to an immunological approach for cancer therapy (Appelbaum, 2001; Bleakley/Riddell, 2004). New transplantation protocols take advantage of the allogeneic graft-versus-leukemia effect for tumor eradication, and use non-myeloablative conditioning regimens, consisting of more specific immunosuppressive chemotherapy and low dose chemotherapy or total-body irradiation sufficient to allow engraftment of stem and immune cells (Appelbaum, 2001).

2.3.4 Immune reactions in allogeneic HSCT: Graft-versus-leukemia (GVL) effect and graft-versus-host disease (GVHD)

Allogeneic HSCT is accompanied by reciprocal immune reactions between donor and recipient. A major role in these reactions plays the human leukocyte antigen (HLA) system, which is the human analogue to the major histocompatibility complex (MHC). This multigene complex is located on chromosome 6 and spans more than 4 megabases, including more than 200 highly polymorphic genes (Klein/Sato, 2000). The most influent genes for allogeneic HSCT are the HLA class I genes HLA-A, -B, and-C, and the class II genes DRB1, DQB1, and DPB1. The HLA molecules present peptide epitopes to T cells and are themselves designated as major histocompatibility antigens. T cells that are confronted with non-identical HLA molecules react with a vigorous allo-response. Regarding one individual, the immune system has developed during its normal maturation tolerance against self proteins. In contrast, in the setting of transplantation, the occurrence of polymorphisms in endogenous proteins gives rise to so-called minor histocompatibility antigens (mHAgs), resulting in the immunological non-identity between the HLA-matched stem cell donor and the recipient. The consequences are reciprocal immune reactions between donor and recipient. Donor T cells in the stem cell graft can react against recipient HLA-peptide complexes on normal host tissues, leading to graft-versus-host disease (GVHD) in the skin, the gastrointestinal tract, and/or the liver. Less frequent is the reaction of residual host T cells against donor stem cells, resulting in graft rejection. The highest risk for GVHD and graft rejection occurs in the setting of HLA-mismatched

transplantation. But also after HLA-matched stem cell transplantation GVHD occurs frequently if no T cell depletion of the graft was applied. The reason for the higher incidence of GVHD in transplant settings with unrelated donors is seen in unrecognized incompatibilities in major histocompatibility antigens or in greater heterogeneity in minor histocompatibility antigens. Until 1998, HLA typing could only be performed using serologic methods that were not able to identify all differences on the allelic level. Recent studies analyzing the HLA class I and II type on allelic level by genomic DNA sequencing detected allele-level mismatches in over 30% of donor-patient pairs that were serologically matched (Appelbaum, 2001). Beside those undesired effects of graft rejection and GVHD, the immunological non-identity between stem cell donor and recipient leads also to the beneficial graft-versus-leukemia (GVL) effect that is responsible for tumor eradication (Bleakley/Riddell, 2004; Appelbaum, 2001). The potency of the GVL effect was impressively demonstrated in the treatment of patients with post-transplant relapse applying T cell infusions from the original stem cell donor (donor lymphocyte infusion, DLI). DLI was used as a successful treatment inducing durable remissions, especially in patients with CML and in some case of acute leukemia (Kolb et al, 1995; Mackinnon et al, 1995; Collins et al, 1997).

Beside $CD8^+$ and $CD4^+$ T cells, it has also been shown for donor natural killer (NK) cells to mediate a GVL effect. The activity of NK cells is modulated by HLA class I antigens interacting with inhibitory and activating killer immunoglobulin-like receptors expressed on NK cells. A GVL effect of donor NK cells against AML was observed after T cell-depleted haplo-identical stem cell transplantation in patients that do not express the HLA class I molecules that are required to engage NK killer inhibitory receptors (KIRs) on NK cells (Parham/McQueen, 2003).

2.3.5 Separation of GVL from GVHD: Potential target antigens for specific allogeneic T cell therapy

In HLA-matched transplantation settings, T cell responses to minor histocompatibility antigens are responsible for the beneficial GVL reactivity, but also cause in most cases GVHD. It was found that recipients of allogeneic (genetically different) transplants have a lower risk of leukemic relapse than recipients of syngeneic transplants (genetically identical twins), or recipients of T cell-depleted allogeneic stem cell transplants (Horowitz et al, 1990). The GVL effect seems to be strongest in the subset of allo-transplant recipients with GVHD (acute or chronic), but the risk for relapse in patients without GVHD

is also lowered compared to T cell-depleted or syngeneic stem cell transplants. These observations show that the GVL effect is closely linked to the development of GVHD. One method to prevent GVHD is T cell depletion of the donor stem cell graft. Several clinical studies have demonstrated that T cell depletion reduces the incidence and severity of GVHD remarkably (Ho/Soiffer, 2001). Disadvantageously, T cell depletion is also associated with a higher occurrence of severe infections (because T cell immunity after transplantation is dependent on donor T cells transplanted with the graft), a higher incidence of graft rejection (probably due to missing donor T cells eradicating residual host immune cells), and an increased risk of leukemia recurrence (Appelbaum, 2001). The application of prophylactic transfer of CD8-depleted DLIs has recently been demonstrated as a promising approach to accelerate immune reconstitution and to lower the risk for GVHD after reduced-intensity HSCT for hematological malignancies (Meyer et al, 2007).

The increased relapse rate observed after T cell-depleted transplantation highlights the significance of T cell-mediated immune responses for the eradication of the disease. A treatment with CTLs that show a relative specificity for leukemic cells may circumvent GVHD. There are two categories of antigens that could serve as potential targets for adoptive T cell therapy of hematological malignancies: Polymorphic minor histocompatibility antigens (mHAgs), and antigens that are associated with the malignant phenotype. The fact that the GVL effect can occur in the absence of GVHD (Horowitz et al, 1990) indicates that there is a subset of mHAgs that is expressed by leukemic cells, but not by cells of the non-hematopoietic tissues that would be otherwise affected by GVHD. Thus, the identification of mHAgs that are differentially expressed by hematopoietic and non-hematopoietic tissue is one strategy to separate GVL from GVHD. An alternative strategy is to identify antigens that are associated with the malignant phenotype, and are suitable to effectively separate anti-malignancy reactivity from GVHD.

2.3.6 Adoptive T cell therapy

Additionally to the well-established use of DLIs for leukemic relapse, adoptive T cell transfer aims at augmenting the GVL effect after allogeneic HSCT by the infusion of donor T cells, which are specific for leukemia antigens (Dudley/Rosenberg, 2003). This approach requires the isolation and expansion of antigen-specific T cells i*n vitro*, and the evidence that such T cells persist and function *in vivo* after transfer.

The concept of adoptive cell transfer with allogeneic T cells has been successfully applied to restore antiviral immunity after HSCT using donor T cells against EBV- and CMV-specific

antigens without causing GVHD, as well as to treat EBV-related lymphoma (Riddell et al, 1992; Walter et al, 1995; Rooney et al, 1998). In hematologic malignancies, it was demonstrated that in a CML patient with relapse after allogeneic HSCT and resistance to DLI, the treatment with *ex vivo*-generated leukemia-reactive CTLs achieved complete remission (Falkenburg et al, 1999). In autologous settings, further support comes from clinical responses that have been observed in melanoma patients after the administration of autologous melanoma-reactive tumor-infiltrating lymphocytes (Dudley et al, 2002; Yee et al, 2002). Other approaches that did not lead to convincing clinical benefits involved the re-infusion of expanded autologous lymphocytes isolated from patients after vaccination with their autologous tumor cells, or stimulation of peripheral blood lymphocytes (PBLs) from melanoma patients with peptide-pulsed dendritic cells (Dudley/Rosenberg, 2003).

Current approaches for adoptive T cell therapies of hematological malignancies focus mainly on the isolation of leukemia-reactive CTLs from chimeric patients after HSCT (Den Haan et al, 1995; Wang et al, 1995; Dolstra et al, 1999; Vogt et al, 2000). Other publications demonstrate the *in vitro* generation of leukemia-reactive lymphocytes independent from the setting of HSCT (Faber et al, 1992; Montagna et al, 2003). But until now those protocols did not yield sufficient T cell expansion, or could not demonstrate a sustaining anti-leukemia reactivity *in vitro* (Bleakley/Riddell, 2004).

Successful transfer of allogeneic T cells additionally requires the assessment of the *in vivo* persistence of transferred lymphocytes, applying PCR analysis for TCRs, flow cytometric tetramer staining, and functional T cell assays. Furthermore, it is essential that T cells not only survive, but are even able to home to sites of residual disease (Dudley/Rosenberg, 2003; Speiser/Romero, 2005).

2.3.7 Minor histocompatibility antigens

Minor histocompatibility antigens (mHAgs) are immunogenic peptides derived from endogenous proteins in recipient cells that differ to donor proteins due to genetic polymorphisms. In murine adoptive transfer models it was demonstrated that adoptively transferred $CD8^+$ cytotoxic T lymphocytes specific for a single minor histocompatibility antigen are capable of eradicating leukemia without causing GVHD (Fontaine et al, 2001; Dazzi et al, 2001), revealing the potency of mHAgs as targets for GVL responses. Furthermore, *in vivo* activated $CD8^+$ and $CD4^+$ T cells that recognize mHAgs can be isolated during graft rejection and GVHD (Warren et al, 1998a). Minor histocompatibility antigens seem to be expressed not only on leukemic blast, but also on early leukemic

progenitors. It could be demonstrated that $CD8^+$ mHAg-specific cytotoxic T cells are capable of inhibiting the growth of leukemic colonies (Falkenburg et al, 1991), as well as preventing the engraftment of AML in NOD/SCID mice (Bonnet et al, 1999).

Name	MHC restriction	Peptide sequence	Distribution	Function / Mechanism
Malignancy-associated minor H antigens				
HA-1	HLA-A*0201 HLA-B60	VLHDDLLEA KECVLHDDL	hematopoietic restricted, solid tumors	Rho-like GTPase-activating protein
HA-2	HLA-A*0201	YIGEVLVSV	hematopoietic restricted	unconventional myosin 1G
HA-3	HLA-A01	VTEPGYAQY	ubiquitous	Guanine nucleotide exchange factors for RhoA
HA-8	HLA-A*0201	RTLDKVLEV	ubiquitous	Pumilio-family member
HB-1	HLA-B44	EEKRGSLHVW	B-ALL + EBV-B-LCL	unknown
BCL2A1	HLA-A*2402 HLA-B*4403	DYLQYVLQI KEFEDDIINW	hematopoietic restricted, selected solid tumors	BCL-2 family member, anti-apoptotic
Normal minor H antigens				
SMCY	HLA-A*0201 HLA-B*0702	FIDSYICQV SIDSYICQV	ubiquitous	Hypothetical transcription factor
UTY	HLA-B08 HLA-B60	LPHNHTDL RESEESVSL	ubiquitous	Spermatogenesis, RPF-containing protein
DFFRY	HLA-A*0101	IVDCLTEMY	ubiquitous	Spermatogenesis, ubiquitin C-terminal hydrolase
DBY	HLA-DQ5	HIENFSDIDMGE	ubiquitous	Spermatogenesis, RNA helicase
RPS4Y	HLA-DRB3	VIKVNDTVQI	ubiquitous	Protein synthesis
Unclassified minor H antigens				
UGT2B17	HLA-A*2902 HLA-B*4403	AELLNIPFLY	ubiquitous	Homozygous gene deletion
LRH-1	HLA-B07	TPNQRQNVC	hematopoietic restricted	Deletion SNP causing a frameshift
PANE1	HLA-A03	RVWDLPGVLK	B-lymphoid cells	NS cSNP causing a stop codon
ECGF-1	HLA-B07	RPHAIRRPLAL	hematopoietic restricted	NS cSNP in an alternatively translated peptide
SP110	HLA-A03	SLPRGTSTPK	hematopoietic restricted	NS cSNP resulting in alternate splicing
TMSB4Y	HLA-A*3303	EVLLRPGLHFR	ubiquitous	Unconventional ORF

Table 2. Currently known minor histocompatibility antigens

Abbreviations: B-ALL, B-cell acute lymphoblastic leukemia; EBV-BLCL, Epstein-Barr virus-transformed B cells; NS, nonsynonymous; cSNP, coding single nucleotide polymorphism; ORF, open reading frame (adapted and modified from: Hambach/Goulmy, 2005; Spierings et al, 2004; Bleakley/Riddell, 2004; Falkenburg et al, 2003; Mullally/Ritz, 2007; mHAg database at: www.lumc.nl).

The differential tissue expression of genes encoding mHAgs is suggested to be a basis for separating the GVL effect from GVHD. Donor T cells specific for mHAgs that are broadly expressed by both hematopoietic and epithelial cells cause GVHD. If the same mHAg is also expressed by leukemic cells, these specific donor T cells could also induce a GVL effect. Donor T cells that are specific for mHAgs selectively expressed by hematopoietic cells might induce a GVL response without causing GVHD (Bleakley/Riddell, 2004). Currently known minor histocompatibility antigens are summarized in table 2. Further 38 mHAgs have been characterized after isolation of $CD8^+$ mHAg-specific T cell clones from recipients of allogeneic HSCT, which seem ubiquitously or hematopoietically restricted (Warren et al, 1998a,b; Riddell et al, 2002).

Minor histocompatibility antigens that are only expressed by hematopoietic cells are for example HA-1, HA-2, HB-1, and BCL2A1. HA-1 and HA-2, which are both presented by HLA-A02, were shown to be targets of a GVL effect after allogeneic HSCT and DLI. Remissions and lower incidences of leukemic relapse were observed to coincide with the expansion of HA-1 and HA-2 specific $CD8^+$ T cells. Those T cells were isolated from the patient after allogeneic HSCT or DLI, and showed *in vitro* lysis of recipient leukemic cells and inhibition of leukemic cell colony formation (Marjit et al, 2003).

Minor histocompatibility antigens with a broad tissue expression include HA-8 and UGT2B17, which were observed to be associated with the occurrence of GVHD after mHAg-mismatched allogeneic HSCT (Bleakley/Riddell, 2004). Several Y-chromosome genes show significant levels of polymorphism with their X-chromosome homologues, and are responsible for GVHD reactions in males who received a stem cell transplant from a female donor. The risk for GVHD might be determined by the level of expression of the mHAg on epithelial cells. SMCY is highly expressed in epithelia, and mHAg mismatches between female donor and male recipient lead to severe GVHD. In contrast, the level of UTY expression in non-hematopoietic cells seems to be insufficient to induce GVHD. On the other hand, UTY is highly expressed in leukemia cells, and the potency of UTY-specific CTLs to induce a GVL effect was demonstrated by the inhibition of leukemia engraftment in NOD/SCID mice (Bonnet et al, 1999; Bleakley/Riddell, 2004). The relevance of mHAg-mediated GVL effects is furthermore demonstrated by the observation that male recipients of stem cell transplants of a female donor have a decreased risk of leukemic relapse, compared to other donor-recipient gender combinations (Bleakley/Riddell, 2004).

2.3.8 Leukemia-associated antigens

Antigens associated with the malignant phenotype can be classified in the following categories (Appelbaum, 2001):

- mutational antigens (e.g. Bcr/Abl in CML, PML/RARα in acute promyelocytic leukemia), should be tumor-specific
- viral antigens (e.g. human papilloma virus (HPV) in cervical cancer); should be tumor-specific
- tissue-specific antigens (e.g. prostate-specific antigen in prostate cancer)
- germ-cell antigens (e.g. the melanoma-associated antigen family, normally expressed in adults only in the testis)
- overexpressed self-proteins (may be recognized based on increased levels of presentation)

A reproducible generation of $CD8^+$ cytotoxic T cells against mutational antigens such as Bcr/Abl that recognize leukemic progenitor cells was until now not possible (Appelbaum, 2001). Alternative targets for T cell-mediated immunotherapy are normal, nonpolymorphic proteins that are overexpressed in leukemic cells and leukemia progenitor cells and are efficiently processed and presented by different common HLA class I alleles. Non-polymorphic proteins, such as Wilms' tumor antigen 1 (WT-1), proteinase 3, survivin, telomerase reverse transcriptase, CYPB1 and immature laminin receptor are being expressed by leukemic cells at much higher level than by normal cells (Bleakley/Riddell, 2004). The best studied AML-associated antigens in this category are proteinase 3 and WT-1.

Proteinase 3 is a serine proteinase with an expression restricted to the promyelocytic stage of myeloid differentiation. In the primary granules of myeloid leukemia cells, particularly in CML, expression seems to be at two to five times the amount found in normal myeloid cells and might be important in the maintenance of the leukemic phenotype. Molldrem et al demonstrated the generation of $CD8^+$ CTLs specific for proteinase 3, by *in vitro* stimulation experiments with PR1, a HLA-A2-binding nonamer peptide derived from proteinase 3 (Molldrem et al, 1996; Molldrem et al, 1997). PR1 has also been shown to induce a specific immune response in HLA-A*0201 positive patients with CML or AML (Molldrem et al, 1999). However, CTLs with this specificity could not be found in the peripheral blood of healthy donors or untreated CML patients, but only in CML patients after allogeneic HSCT or IFN-α treatment (Molldrem et al, 2000).

WT-1 is a zink-finger transcription factor that was originally described as a tumor-suppressor gene in childhood Wilms' tumor. WT-1 is over-expressed by most leukemias, including AML, with a 10 to 100 fold higher expression in leukemic cells compared to normal $CD34^+$ cells. Higher expression levels were found to be associated with worse prognosis (Inoue et al, 1997; Bergmann et al, 1997). Specific $CD8^+$ T cell responses against WT-1 derived peptides could be detected in HLA-A24 and HLA-A*0201 positive patients with AML (Oka et al, 2004). One study demonstrated that WT-1 specific $CD8^+$ CTLs were able to lyse only leukemic blasts, but not normal $CD34^+$ cells. Furthermore, in colony forming assays those CTLs inhibited specifically the growth of leukemic colonies, but not that of normal myeloid colonies. Thus, the results of this study suggest that WT-1 specific $CD8^+$ CTLs are able to distinguish between the different protein expression levels (Gao et al, 2000).

Other leukemia-associated antigens that were identified by serological screening are RHAMM/CD168 (receptor for hyaluronic acid mediated motility) and MPP11 (m-phase phosphoprotein 11). These antigens induced serological immune responses in AML and CML patients. The naturally processed peptide RHAMM/CD168-R3 was identified as an epitope inducing specific immune responses by $CD8^+$ early effector T cells. There are currently several peptide vaccination trials ongoing, that investigate the clinical significance of peptides derived from proteinase 3, WT-1, and RHAMM (Greiner et al, 2006).

2.3.9 Approaches for antigen identification

2.3.9.1 Biochemical approach using HPLC and MS

This method for the identification of T cell-defined peptide antigens applies the biochemical purification of HLA-associated peptide ligands followed by sequence analysis by tandem mass spectrometry (MS). HLA class I molecules from tumor lysate are first purified by anti-HLA immunochromatography, and the HLA-associated peptides are eluted by acid incubation. The resulting peptide mixture is separated according to solubility by analytical reverse phase-HPLC (high performance liquid chromatography). HPLC fractions are loaded onto antigen-negative, HLA-expressing target cells and are screened in CTL effector assays (^{51}Chromium release, ELISpot) for recognition by CTLs. Positive fractions contain the bioactive peptide epitope. These fractions are further separated by microcapillary liquid chromatography, and are then analyzed by mass MALDI- or ESI-MS (mass spectrometry) to identify the single peptide sequence. Database search is performed to find a candidate gene that contains a coding sequence corresponding to the identified peptide. Minor histocompatibility antigens that were identified by this approach include HA-1, HA-2, HA-8, and several HY-mHAgs (Bleakley/Riddell, 2004; Schetelig et al, 2005), as well as a recently characterized mHAg derived from alternative post-translational splicing of the protein product encoded by the SP110 gene (Warren et al, 2006; Shastri, 2006).

2.3.9.2 cDNA expression cloning

The basis of cDNA expression cloning is a cDNA library, which is prepared from antigen-positive cells. These cDNAs are cloned into an expression vector and are co-transfected with a plasmid encoding the HLA-restricting allele into COS or 293T cells. The transfected cells are then screened by co-culture with antigen-specific T cells to identify those cDNA pools that are recognized by T cells in cytokine ELISpot assay. Positive cDNA pools are sub-cloned to identify those that express the cDNA encoding the antigenic epitope. The antigenic epitope is then localized by transfection of truncated deletions of the gene, or by prediction algorithms for HLA-peptide binding. This method for antigen identification was applied to identify the mHAgs HB-1, UGT2B17, and several mHAgs encoded by the Y-chromosome (Bleakley/Riddell, 2004; Schetelig et al, 2005).

2.3.9.3 Genetic linkage analysis

Genetic linkage analysis uses cell lines which are derived from large pedigrees (e.g. the Centre d'Etude Polymorphism Humain reference family collection, CEPH) that have been

genotyped for highly polymorphic genetic markers. Cell lines are transfected with cDNA encoding the relevant class I HLA-restricting allele, and are tested for recognition by antigen-specific CTLs. Pairwise linkage analysis is then applied to identify the chromosomal region that is involved in the expression of the antigen. This approach has recently been proven for HA-8 to be successful in mHAg identification (Schetelig et al, 2005; Warren et al, 2002).

2.3.9.4 "Reverse Immunology"

The prediction of potential T cell epitopes by special bioinformatic algorithms for HLA-peptide binding (e.g. SYFPEITHI; Rammensee et al, 1999) is referred to as a "reverse immunology" approach for antigen identification. Candidate peptides are pulsed onto antigen-presenting cells carrying the respective HLA allele in order to test recognition by T cells (Viatte et al, 2006).

2.3.9.5 SEREX (serological screening of cDNA expression libraries)

The SEREX approach for identification of tumor-associated antigens utilizes tumor cell expression libraries that are screened with autologous sera from cancer patients containing tumor-reactive antibodies (Sahin et al, 1995). TAAs that have been identified by this method include for example the cancer testis antigen NY-ESOI (Chen et al, 1997).

2.4 Motivation and aim of this work

The efficient *in vitro* generation and expansion of leukemia-reactive CTLs for allogeneic T cell transfer therapies of hematological malignancies has until now shown to be a challenging goal. Most groups isolate them from peripheral blood samples drawn from chimeric leukemia patients after allogeneic HSCT (van Lochem et al, 1992; Den Haan et al, 1995; Wang et al, 1995; Dolstra et al, 1999; Vogt et al, 2000; Brickner et al, 2001). This procedure increases the likelihood that donor-derived CTL precursors have been primed against host leukemia antigens *in vivo*. The major disadvantages of this strategy are that most patients have low T cell counts following transplantation and frequently require continuous treatment with immunosuppressive drugs adversely affecting T cell function. It has also been difficult to predict the exact time points when leukemia-reactive CTLs appear with high frequencies in peripheral blood. Furthermore, allogeneic mixed lymphocyte-leukemia cultures (allo-MLLCs) traditionally applied for the *in vitro* generation of anti-leukemia CTLs are lymphocytic bulk populations that are largely unpredictable with regard to suppressive and stimulatory immune effects.

Due to these limitations of currently used protocols for the generation of leukemia-reactive T cells, it was the aim of this project to establish a novel *in vitro* approach that allows the reliable and efficient generation of anti-leukemia CTLs from peripheral blood lymphocytes of healthy donors. Ideally, these CTLs should recognize antigens that are exclusively expressed by leukemic blasts and leukemia-initiating precursor cells, but not by healthy patient cells. This would allow achieving a beneficial GVL effect without causing GVHD. Due to the enormous diversity of their T-cell receptor (TCR) repertoire, naive $CD8^+$ T cells contain the majority of allo-reactive and tumor-reactive CTL precursors in healthy individuals (Goldrath et al, 1999; Pittet et al, 1999; Foster et al, 2004; Bleakley et al, 2004 abstract; Kausche et al, 2006). This makes naive $CD8^+$ T cells promising "candidates" for the isolation of leukemia-reactive CTLs.

With regard to stimulator cells, we aimed at the use of native, primary leukemic blasts, since they express the whole repertoire of naturally *in vivo* occurring T cell epitopes. Unfortunately, the immunostimulatory properties of leukemic blasts have until now reported to be rather low (Brouwer et al, 2000b).

A successful realization of those challenges should form the methodological basis for the routine generation of specific anti-leukemia T cell products in individual donor-patient pairs and should prepare their clinical testing. Furthermore, leukemia-reactive CTLs

expanded to sufficient quantities in this study could be applied to identify leukemia-associated-antigens that are recognized by T cells of healthy individuals.

3. MATERIALS & METHODS

3.1 Primary cells of patients and healthy donors

Primary AML blasts were isolated from peripheral blood and leukaphereses of patients who were newly diagnosed with M4 (MZ369, MZ574) or M5 (MZ201, MZ418, MZ431, MZ456) subtype AML according to the French-American-British Cooperative Group (FAB) classification (Bennet et al, 1985). For in vitro experiments, peripheral blood of healthy donors with serological HLA class I match was provided by the blood bank of the university clinics, Mainz. *In vitro* systems of AML patients and healthy donors used in this work are summarized in table 3. High-resolution HLA typing was performed from genomic DNA using class I-specific primers (Dr. B. Thiele, Institute for Immunology and Genetics, Kaiserslautern, D). Patient characteristics and results of high-resolution HLA class I typing are described in *chapter 4.1*.

	FAB-Subtype	**HLA class I type**	**Healthy donors**
MZ201-AML	AML M5a	A01/A02, B08/B56, Cw01/Cw07	Donor168 Donor332 Donor650
MZ418-AML	AML M5b	A02/A03, B35/B44, Cw04/Cw06	Donor093 Donor804 Donor482 Donor587
MZ431-AML	AML M5a	A01/A02, B08/B44, Cw05/Cw07	Donor532
MZ574-AML	AML M4eo	A01/A02, B07/B15, Cw03/Cw07	Donor172 Donor247
MZ369-AML	AML M4	A01/A24, B18/B38, Cw07/Cw12	Donor167 (=369-SIB)
MZ456-AML	AML M5a	A02/A66, B14/B18, Cw07/Cw08	Donor116 (=456-SIB)

Table 3: Overview of AML patients and HLA class I matched healthy donors. The FAB-subtype and the serological HLA class I type are given.

PBMCs and AML blasts were isolated from peripheral blood, buffy coat and leukapheresis products by standard Ficoll separation and were stored in liquid nitrogen until use. All leukemia samples tested in this study contained more than 95% leukemia cells as determined by cytology and flow cytometry analyses.

The study protocol was approved by the local Ethics Committee of the Landesärztekammer Rheinland-Pfalz. Healthy donors and leukemia patients participated in the study after informed consent in accordance with the Helsinki protocol.

3.2 Cell lines

3.2.1 K562 cells

K562 cells were obtained from Prof. Dr. E. Weiss, University of Munich. This cell line was originally isolated from a patient with chronic myeloid leukemia (Klein et al, 1976) and serves as a NK cell target, since it does not express any HLA class I and class II molecules on the cell surface (Kaplan et al, 1978). It is therefore used as a negative control cell target for antigen-specific $CD8^+$ T cells.

3.2.2 Tumor cell lines

The following tumor cell lines (table 4) were used for cross-reactivity tests or as stimulator cells: Lung carcinoma (LC) MZ6-LC and MC16-LC, obtained from Dr. S. Horn, Mainz; the melanoma (Mel) cell lines SK29-Mel, D05-Mel, MZ9-Mel, MZ12-Mel, provided by Prof. Dr. T. Wölfel, Mainz.

	HLA class I alleles
MZ6-LC	A02
MZ16-LC	A02
SK29-Mel	A02/28, B44/45, Cw05/06
D05-Mel	A02, B27/44, Cw02/05
MZ9-Mel	B55
MZ12-Mel	B55

Table 4. Used tumor cells lines. Listed are HLA class I alleles that were relevant for their inclusion in cross-reactivity testings.

3.2.3 CD40L-transfected murine fibroblasts

Ltk- huCD40L-transfected L cells were a kind gift of Dr. Pierre Garrone, Schering-Plough (Dardilly, France), to Dr. Catherine Wölfel, Mainz.

3.3 Cell culture

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

3.3.1 Cell culture material

3.3.1.1 Substances used for cell culture

AIM V medium	Gibco/Invitrogen (Karlsruhe, D)
Amphotericine B (Fungizone®)	Gibco/Invitrogen (Karlsruhe, D)
Basic fibroblast growth factor	R&D Systems (Minneapolis, USA)
CSA (Cyclosporine A)	Pharmacy of the University Clinics, Mainz
DMSO (Dimethyle sulfoxide)	Merck (Darmstadt, D)
DNase I	Roche (Grenzach-Wyhlen, D)
PBS (phosphate-buffered saline, -$CaCl_2$, -$MgCl_2$)	Gibco/Invitrogen (Karlsruhe, D)
FCS (fetal calf serum)	PAA Laboratories, Pasching (A)
Ficoll lymphocyte separation medium LSM1077	PAA Laboratories, Pasching (A)
Fluconazole	Pharmacy of the University Clinics, Mainz
Geneticin (G418)	Gibco/Invitrogen (Karlsruhe, D)
Gentamicin	Ratiopharm (Ulm, D)
Heparin (Liquemin® N5000)	Roche (Grenzach-Wyhlen, D)
Human albumin (20% Octalbumin®)	Octapharma (Langenfeld, D)
Human serum (HS)	Isolated after informed consent from blood of healthy donors; pooled, heat-inactivated, sterile filtrated (0.22 µm), and stored at -80°C until use.
Ionophor Calcimycin (A-23187)	Sigma-Adrich (Steinheim, D)
MEM medium	Gibco/Invitrogen (Karlsruhe, D)
Penicillin/Streptomycin (Pen/Strep)	Gibco/Invitrogen (Karlsruhe, D)
Phytohemagglutinine (PHA)	Murex Biotech LTD (Dartford Kent, UK)
RPMI 1640 medium 1x (+ L-Glutamine)	Gibco/Invitrogen (Karlsruhe, D)
T cell activation/expansion kit, human	Miltenyi Biotec (Bergisch Gladbach, D)
Trypane Blue (solution for cell counting)	75 ml stock solution + 25 ml NaCl [150 mM]
Trypane Blue (stock solution)	Trypane Blue 2.0 g ad 1 l H_2O
Trypsin-EDTA (0.05%)	Gibco/Invitrogen (Karlsruhe, D)
EDTA (Versen) 1%	Biochrom (Berlin, D)
X-VIVO 15 medium	Biowhittaker (Viersen, B)

3.3.1.2 Cell culture media

RPMI-medium for culture of cell lines	RPMI 1640 10% FCS 1% Pen/Strep
RPMI-medium for generation of EBV-B lines	RPMI 1640 20% FCS 1% Pen/Strep
AIM-V-medium for T cell culture and AML pre-culture	AIM-V 10% human serum (HS)
Freezing medium	X-VIVO 15 8% human albumine 10 U/ml heparin (Liquemin® 5000 U/500 µl) (10% DMSO added prior to use)
MEM-medium for fibroblast culture	MEM-medium 20% FCS 10 ng/ml basic fibroblast growth factor

All cell culture media were stored at 4°C.

3.3.1.3 Cytokines

Cytokine	**Manufactorer**	**Stock concentration**	**Final concentration**
IL-2 (Proleukin)	Chiron Behring (Marburg, D)	$2.5x10^4$ IU/ml	250 IU/ml
IL-7	R&D Systems (Minneapolis, USA)	5 ng/µl	5 ng/ml
IL-12	R&D Systems (Minneapolis, USA)	1 ng/µl	1 ng/ml
IL-15	R&D Systems (Minneapolis, USA)	5 ng/µl	5 ng/ml
IL-4	Strathmann Biotec (Hannover, D)	500 IU/µl	1000 IU/ml
GM-CSF (Granulocyte-monocyte colony stimulating factor)	Sandoz Biopharmaceuticals (Milano, I)	1000 IU/µl	1000 IU/ml
SCF (Stem cell factor)	R&D Systems (Minneapolis, USA)	5 ng/µl	5 ng/ml
TNF-α (Tumor necrosis factor-α)	R&D Systems (Minneapolis, USA)	10 ng/µl	10 ng/ml
IL-1β	Pharmingen (San Diego, USA)	10 ng/µl	10 ng/ml
IL-6	Strathmann Biotec (Hannover, D)	1000 IU/µl	1000 IU/ml
PGE_2 (prostaglandin 2)	Sigma (Deisenhofen, D)	1 µg/µl	1 µg/ml
IFN-γ (Interferon-γ)	PromoCell (Heidelberg, D)	500 IU/µl	500 IU/ml

Table 5: Cytokines used for cultivation of primary cells and cell lines. Manufacturer, stock concentration and final concentration are listed. Cytokines were dissolved in AIM-V medium and stored at -80°C or -20°C.

3.3.2 Freezing and thawing of cells

For cryo-preservation in liquid nitrogen, cells were frozen in portions of 2-5x10^6 (T cells) or 10-100x10^6 (cell lines, PBMCs) in 1 ml of freezing medium containing 10% DMSO. Cryo tubes were transferred in cryo boxes and stored over night at -80°C, before they were placed into the nitrogen tank.

For thawing of cells, cryo tubes were shortly put into the water bath (37°C) until frozen cells began to thaw. The content of the tube was transferred with fresh RPMI medium in a cell culture tube. In order to remove DMSO, cells were centrifuged, thawing medium was discarded, and cells were counted and seeded in fresh medium.

Clumping cells were treated after centrifugation with 1 to 10 mg/ml DNase I for 2 min at room temperature and washed again with RPMI medium.

3.3.3 Cultivation of cell lines in suspension culture

Suspension cell lines were cultivated at a cell density of 0.2-0.4x10^6/ml in RPMI medium containing 10% FCS and 1% penicillin/streptomycin. Cells were counted twice a week and sub-cultivated, when the cell density reached approx. 1x10^6/ml.

3.3.4 Cultivation of adherent-growing cell lines

Adherent growing cell lines (tumor cell lines) were cultivated in RPMI medium containing 10% FCS and 1% penicillin/streptomycin in cell culture flasks of 80 cm^2 and 175 cm^2. Depending on the growth rate, cells were sub-cultivated once or twice a week. Briefly, used medium was discarded, and adherent cells were rinsed with PBS and incubated for 3-10 min at 37°C with 2-3 ml trypsin-EDTA. Detached cells were resuspended and washed in RPMI medium and seeded in fresh medium with a density of approx. $10^4/cm^2$.

Ltk- huCD40L-transfected L cells were detached with Versen/EDTA (no trypsin to avoid shedding of CD40L) and seeded at 2x10^5 cells/ml in a total volume of 30-50 ml in 150 cm^2 cell culture flasks.

3.3.5 Isolation of PBMCs from buffy coats by Ficoll density centrifugation

For the isolation of PBMCs from buffy coats by Ficoll density centrifugation, blood (approx. 60-70 ml) was first transferred from the bag into a sterile cell culture flask and diluted with PBS (room-temperature) to a total volume of 120 ml. Six 50 ml cell culture tubes or Leucosep® tubes were filled with 15 ml Ficoll lymphocyte separation medium. 20 ml of the

diluted cell suspension was gently pipetted on top. Ficoll tubes were centrifuged without brake for 20 min at 2500 rpm. The white blood cell ring fraction was collected and transferred into new 50 ml tubes, by combining two rings into one tube. For washing, volumes were adjusted to 50 ml per tube with RPMI, and isolated cells were centrifuged at 1800 rpm for 10 min. After discarding the supernatants, cell pellets were resuspended in RPMI, pooled into one 50 ml tube and again spun down (1500 rpm, 5 min). The supernatant was discarded and PBMC were resuspended in RPMI medium and counted.

3.3.6 Cryo-preservation of leukaphereses

For the cryo-preservation of leukapheresis material of AML patients, the content of a leukapheresis bag was first transferred into sterile tubes by using a perfusor syringe. An aliquot of the cell suspension was diluted for counting. Leukemic cells were diluted with MEM medium, 10% DMSO and heparin (5000 U/100 ml) and frozen in approx. 200 portions of 10^8/1 ml in cryo tubes. Residual leukapheresis material was frozen in bags at a cell density of $5x10^8$/ml.

3.3.7 Overnight pre-culture of leukemic blasts

For restimulation of antigen-specific T cell or for effector function assays (IFN-γ ELISpot, cytotoxicity test), allogeneic AML blasts were thawed one day before use, and pre-cultured overnight at 37°C in AIM-V medium supplemented with 10% human serum. For determination of viability, cells were counted after thawing and seeded in uncoated Petri dishes at cell numbers of $1x10^6$ to $3x10^6$ per milliliter. In experiments with HLA-matched sibling donors, pre-culture medium was supplemented with 1000 IU/ml IL-4, 1000 IU/ml GM-CSF, 10 ng/ml TNF-α, and 5 ng/ml SCF, to further increase expression of HLA and costimulatory molecules on AML blasts.

3.3.8 Maturation of leukemic blasts

In experiments using maturated AML blasts, leukemic cells were incubated for 4 days at 37°C in AIM-V medium supplemented with 10% human serum and the following maturation reagents:

a) IL-4 (1000 IU/ml)
b) Cytokine "cocktail" consisting of 1000 IU/ml IL-4, 1000 IU/ml GM-CSF, 10 ng/ml TNF-α, and 5 ng/ml SCF

c) Calcium Ionophore Calcimycin (A-23187), final concentration 180 ng/ml

d) IL-4 (1000 IU/ml) over 4 days, addition of anti-biotin MACSI-Beads (Miltenyi Biotec) preincubated with biotinylated anti-human CD40 mAb (R&D systems) at day 2 with a beads:cells ratio of 1:2

e) IL-4 (1000 IU/ml) over 4 days, cocultviation of AML blasts with CD40L-transfected murine fibroblasts (L-cells) from day 2-4 at an AML:L-cell ratio of 25:1. L cells were irradiated with 15.000 rad prior to cocultivation

3.3.9 Generation of EBV-transformed B cell lines

For the generation of Epstein Barr-Virus (EBV)-transformed B cell lines of patient or donor origin, 10^7 PBMCs were incubated for 4 h at 37°C with 0.5 ml of EBV-containing supernatant of B95.8 cells in a 15 ml cell culture tube. Cells and supernatant were subsequently transferred in small cell culture flasks in a total volume of 5 ml RPMI medium supplemented with 20% FCS and 1% penicillin/streptomycin. For the inhibition of T cells, cyclosporine A (CSA) was added at a final concentration of 1 µg/ml. After 24 h of incubation at 37°C, cells were washed in order to remove virus and CSA, and seeded in 5 ml of fresh medium. Cell growth was checked daily and fresh medium was added when necessary. EBV-B lines with stable growth were transferred in medium size cell culture flasks and cultivated at a cell density of 0.2-0.4x10^6/ml in RPMI medium containing 10% FCS and 1% penicillin/streptomycin.

3.3.10 Generation of PHA-activated PBMC blasts

Phytohemagglutinin (PHA)-activated T cell blasts were generated by seeding 2x10^6 PBMCs of patient or donor origin in 24-well plates in 2 ml AIM-V medium containing 5% human serum, supplemented with 1 µg/ml PHA and 250 IU/ml IL-2. Cells were incubated at 37°C and splitted with fresh IL-2 containing medium. PHA blasts were used as target cells in ELISpot and ^{51}Chromium release assays between day 8 and 12 of culture, when cells had a blast-like morphology.

3.3.11 Generation of dendritic cells ("fastDCs")

The generation of mature dendritic cells (DCs) from blood monocytes was performed by Sylvia Köhler, Mainz, according to the "fastDC" protocol (Dauer et al, 2003). Briefly, monocytes were selected from PBMC by adherence and cultivated for 24 hours in medium

containing GM-CSF and IL-4. These immature MoDCs (monocyte-derived DCs) were further cultivated for another 24 hours in the presence of IL-1β, IL-6, TNF-α and PGE_2 to generate mature dendritic cells. To confirm the phenotype of mature DCs, "fastDCs" were analysed by flow cytometry for expression of the cell surface marker CD80, CD83, CD86, and HLA-DR.

3.3.12 Generation of stromal fibroblasts

The generation of stromal fibroblasts was established and performed by Dr. Marion Nonn, Mainz. Primary stroma cells were isolated from bone marrow aspirates of AML patients according to a previously published protocol (Honegger et al, 2002) with minor modifications. Briefly, adherent bone marrow cells were expanded in MEM-medium containing 20% fetal calf serum supplemented with 10 ng/ml basic fibroblast growth factor following the first culture passage. After the second passage, more than 95% of detached cells reacted with monoclonal antibodies specific for the fibroblast marker prolyl-4-hydroxylase, CD90, and HLA class I in flow cytometry. Less than 5% of cells bound mAbs specific for CD14, CD33, and CD45. Staining results indicated that the bone marrow-derived cells were stromal fibroblasts (Honegger et al, 2002; Min et al, 2002). In experiments using CTLs 1C6 and 4D7, fibroblasts were pre-treated with 500 IU/ml IFN-γ over 3 days before testing.

3.4 Immunological methods

3.4.1 Magnetic cell separation (MACS)

3.4.1.1 Principle

The MACS® technology (Miltenyi Biotec, Bergisch-Gladbach) is based on the use of magnetic MicroBeads that are coupled to monoclonal antibodies, which bind to a specific cell surface antigen on the target cell population. By using a MACS® column placed in a permanent magnet (MACS® Separator), the magnetically labelled target cells are retained. Unlabelled cells are washed out by rinsing the column with MACS-buffer. The labelled cell fraction is obtained by removing the column from the magnet.

3.4.1.2 Materials for magnetic cell separation

CD8 T cell Isolation Kit II, human	Miltenyi Biotec # 130-091-154
CD62L MicroBeads, human	Miltenyi Biotec # 130-091-758
CD14 MicroBeads, human	Miltenyi Biotec # 130-050-201
CD15 Whole Blood MicroBeads, human	Miltenyi Biotec # 130-090-058
CD19 MicroBeads, human	Miltenyi Biotec # 130-050-302
LS columns	Miltenyi Biotec # 120-000-475
MS columns	Miltenyi Biotec # 120-000-472
Preseparation filter	Miltenyi Biotec # 120-002-220
MiniMACS® Separator	Miltenyi Biotec # 130-042-102
MidiMACS® Separator	Miltenyi Biotec # 130-042-302
MACS® Multistand	Miltenyi Biotec # 130-042-303
AutoMACS® Separator	Miltenyi Biotec, Equipment of the Tumor Vaccination Centre, Mainz
MACS buffer	PBS 1x (-$CaCl_2$, -$MgCl_2$) 0.5% BSA, 2 mM EDTA sterile filtrated (0.22 µm), stored at 4°C
Albumine, bovine (BSA)	Carl Roth (Karlsruhe, D)
EDTA (Ethylendiamine-tetra-acetic acid)	Sigma-Aldrich (Steinheim, D)

3.4.1.3 Isolation of $CD8^+$ $CD62L^+$ T cells from healthy donor PBMCs

For the isolation of the $CD8^+$ $CD62L^+$ T cell subset, a two-step separation strategy was applied. Since CD62L is also expressed by $CD4^+$ cells, untouched $CD8^+$ T cells were first purified from PBMCs by depletion of non-CD8 cells using the CD8 T cell Isolation kit II (Miltenyi Biotec). This negative selection technique used a cocktail of biotin-conjugated mAbs against CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCRγδ, and CD235a. After

addition of anti-biotin MicroBeads, magnetically labelled cells were depleted on LS columns. $CD8^+$ $CD62L^+$ cells were subsequently enriched by positive isolation applying CD62L MicroBeads and MS columns. Purity of resulting $CD62L^{(high)+}$ and $CD62L^{(low)+/neg}$ cell fractions was controlled by flow cytometry analysis.

The isolation procedure was performed according to a special protocol by Miltenyi Biotec, with minor modifications. $CD8^+$ untouched isolation was started with approx. 10^8 PBMC per one LS column. Washed cells were incubated for 10 min on ice with 40 µl MACS-Puffer and 10 µl biotinylated antibody cocktail per 10^7 cells. Then, further 30 µl MACS-buffer and 20 µl anti-biotin MicroBeads per 10^7 cells were added and incubated on ice for another 15 min. After washing, cells were resuspended in 1 ml of MACS-buffer and separated on an equilibrated LS-column place in a MidiMACS® magnet. The column was washed three times with 3 ml MACS-buffer to remove unbound ("untouched" $CD8^+$) cells. Bound non-CD8 cells were recovered by removing the column from the magnet and by passing 5 ml MACS-buffer through the column using a plunger.

For the positive isolation of CD62L-expressing cells, the negative fraction ("untouched" $CD8^+$ cells) was counted, and separated on a MS column. Labelling with CD62L Microbeads was performed in a total volume of 100 µl, but with up to 30 µl Microbeads (instead of 20 µl as recommended in the protocol), because several separations showed still high amounts of $CD62L^+$ cells in the negative fraction. For the same reason, incubation time on ice was prolonged up to 30 min (instead of 15 min as recommended). Separation on a MS column was performed according to the standard protocol, but with 2 ml (instead of 1 ml) MACS-buffer for removing the positive fraction from the column.

All isolation and staining steps were performed at 4°C (on ice), and the separation material was stored in advance in the refrigerator to avoid increased shedding of CD62L at higher temperature.

Cell recovery was determined by counting each separation fraction. Purity of each fraction was controlled by flow cytometry. Additional co-stainings were performed for the following markers: CD3, CD4, CD8, CD16, CD56, CD62L, CCR7, CD45RA, CD45R0 CD27, and CD28.

3.4.1.4 Positive isolation of $CD14^+$ monocytes from PBMCs

Isolation of monocytes from healthy donor or patient PBMCs was accomplished with CD14 MicroBeads as recommended in the manufacturer's instruction. Cells were labelled with 20 µl CD14 MicroBeads per 10^7 PMBCs and separated on MS columns. Purity of the isolated monocytes was analysed by FACS staining of CD14.

3.4.1.5 Positive isolation of CD19$^+$ B cells from PBMCs

Isolation of CD19$^+$ B cells from healthy donor or patient PBMCs was accomplished with CD19 MicroBeads as recommended in the manufacturer's instruction. Cells were labelled with 20 µl CD19 MicroBeads per 10^7 PMBCs and separated on MS columns. Purity of the isolated monocytes was analysed by FACS staining of CD19.

3.4.1.6 Isolation of CD15$^+$ granulocytes from whole blood

For the isolation of granulocytes from whole blood, the AutoMACS® Separator (Miltenyi Biotech) and the established protocol of the "tumor vaccination centre" (TVZ, Tumorvakzinationszentrum), Mainz, was used.

One millilitre of whole blood was mixed with 25 µl of anti-CD15 whole blood-MicroBeads (Miltenyi Biotec) and incubated for 15 min at 4-8°C (refrigerator). After addition of 10 ml PBS, the cell suspension was centrifuged for 10 min at 1500 rpm (approx. 445 g) without brake. The supernatant was carefully removed and discarded, and the remaining cell sediment was mixed with PBS in a total volume of 3 ml. Cell separation using the AutoMACS® separator was performed applying the "posseld2" program. Purity of the CD15$^+$ fraction was confirmed by flow cytometry analysis.

3.4.2 Flow cytometry

3.4.2.1 Principle

Flow cytometry (FACS, fluorescence-activated cell sorting) is a method used for the phenotypic analysis of a cell population. A flow cytometer can detect fluorescence on individual cells in a suspension and thereby determine the number of cells expressing the molecule to which a fluorescent probe binds.

Cells are first stained by incubating cell suspensions with a fluorescently labeled antibody specific for a surface antigen of a cell population. The amount of probe bound by each cell in the population is measured by passing the cells one at a time through a fluorimeter with a laser-generated incident beam. The incident laser beam is of a designated wavelength, and the light that emerges from the sample is analyzed for forward and sideward scatter as well as fluorescent light or one or more wavelengths that depend on the fluorochrome labels attached to the antibodies. The forward and side light-scattering properties of measured cells reflect cell size and internal complexity, respectively. The relative amount of a particular cell surface antigen on different cell populations can be

compared by staining each population with the same probe and determining the amount of fluorescence emitted.

3.4.2.2 Monoclonal antibodies used for flow cytometry

For immunophenotyping of cell populations by flow cytometry, the following mouse anti-human monoclonal antibodies (mAbs) were used: Purchased mAbs with direct fluorochrome conjugation (table 6), HLA-specific mAbs from hybridoma supernatants (table 7) in combination with FITC- or PE-conjugated goat-anti-mouse IgG, and combinations of mAbs from the IOTest® Beta Mark Kit (Beckman Coulter) specific for TCR Vβ families (table 8). All antibodies were stored at 4°C and handled under sterile conditions.

Antibody specificity	**Fluorochrome conjugation**	**Manufacturer**
Isotype control mouse IgG1	FITC/PE, PC5, APC	Immunotech/Coulter (Marseille, F)
CD3	FITC, PC5	Immunotech/Coulter (Marseille, F)
CD3/CD4	FITC/PE	Immunotech/Coulter (Marseille, F)
CD3/CD8	FITC/PE	Immunotech/Coulter (Marseille, F)
CD4	FITC, PE, PC5	Immunotech/Coulter (Marseille, F)
CD8	FITC, PE, PC5	Immunotech/Coulter (Marseille, F)
CD16	FITC	Immunotech/Coulter (Marseille, F)
CD56	PE	Immunotech/Coulter (Marseille, F)
CD25	PE	Immunotech/Coulter (Marseille, F)
CD69	PE; FITC	BD Pharmingen (Heidelberg, D)
CD62L	PE	Immunotech/Coulter (Marseille, F)
CCR7	FITC	R&D Systems (Minneapolis, USA)
CD45RA	PC5	BD Pharmingen (Heidelberg, D)
CD45R0	FITC	Immunotech/Coulter (Marseille, F)
CD27	PE	Immunotech/Coulter (Marseille, F)
CD28	FITC	Immunotech/Coulter (Marseille, F)
TCR α/β	PC5	Immunotech/Coulter (Marseille, F)
TCR γ/δ	PC5	Immunotech/Coulter (Marseille, F)
TCR Vβ 12	PE	Immunotech/Coulter (Marseille, F)
TCR Vβ 17	PE	Immunotech/Coulter (Marseille, F)
CD45	FITC	Immunotech/Coulter (Marseille, F)
CD80	FITC	Immunotech/Coulter (Marseille, F)
CD86	PE	Immunotech/Coulter (Marseille, F)
CD40	PE	BD Pharmingen (Heidelberg, D)

CD54	PE	BD Pharmingen (Heidelberg, D)
CD58	FITC	Immunotech/Coulter (Marseille, F)
CD123	PE	Miltenyi Biotec (Bergisch-Gladbach)
CD11c	PE	BD Pharmingen (Heidelberg, D)
CD33	PC5	BD Pharmingen (Heidelberg, D)
CD34	PE	Miltenyi Biotec (Bergisch-Gladbach)
CD14	FITC	Immunotech/Coulter (Marseille, F)
CD19	PE	Immunotech/Coulter (Marseille, F)
CD90	PE	Immunotech/Coulter (Marseille, F)

Table 6: FACS antibodies used for the detection of cell surface antigens. Fluorochrome-conjugations: fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanin 5.1 conjugate (PC5), allophycocyanin (APC).

Antibody	Specificity	Isotype	Reference
w6/32	total HLA class I	mouse IgG2a	Parham et al, 1979
B1.23.2	HLA-B & -C	mouse IgG2b	Rebai/Malissen, 1983
PA2.1	HLA-A2	mouse IgG1	Parham/Bodmer, 1978
SFR8B6	HLA-Bw6, some C-alleles	IgG2b mouse/rat fusion	Radka et al, 1982
GAP-A3	HLA-A3	mouse IgG2a	Berger et al, 1982
C7709A2	HLA-A24	mouse IgG2a	Ikeda et al, 1997
BB7.1	HLA-B7	mouse IgG1	Toubert et al, 1988
HB55	HLA-DR	mouse IgG2a	Lampson/Levy,, 1980

Table 7: HLA-specific monoclonal antibodies. Hybridoma supernatants of murine monoclonal antibodies were used for indirect immunofluorescent staining in combination with FITC- or PE-labeled goat-anti-mouse (gam) IgG (Immunotech, Marseille, F). For HLA-blocking experiments in ELISpot assays, antibodies were purified from hybridoma supernatants, aliquoted and stored until use in PBS at -20°C. Purified antibodies were concentrated between 1 and 2 mg/ml. Isotype-matched IgG (BD Pharmingen) were used as controls.

3.4.2.3 Further materials and equipment for flow cytometry analysis

FACS buffer	PBS 1x (- CaCl2, - MgCl2) 0.1% BSA sterile filtrated (0.22 µm), stored at 4°C
FACS fixation solution	1% PFA in FACS buffer
Albumine, bovine (BSA)	Carl Roth (Karlsruhe, D)
Para-Formaldehyde (PFA, 37% solution)	Merck (Darmstadt, D)
BD FACS™ Lysing Solution	Becton Dickinson Biosciences (Heidelberg, D)
SYTOX® Red nucleic acid stain	Invitrogen/Molecular Probes (Karlsruhe, D)

Flow Cytometer	BD FACSCanto, Becton Dickinson Biosciences (Heidelberg, D) BD FACSCalibur, Becton Dickinson Biosciences (Heidelberg, D)
Software	BD FACS DIVA, Becton Dickinson Biosciences (Heidelberg, D) CellQuest™Pro, , Becton Dickinson Biosciences (Heidelberg, D) EXPO™32, Beckman Coulter (Krefeld, D)

3.4.2.4 Immunofluorescent staining

For direct immunofluorescent staining, 50,000 to 200,000 cells were washed with FACS buffer and incubated for 15-30 min at 4°C with 3-10 µl FITC-, PE- PC5- or APC-conjugated monoclonal antibodies specific for indicated CD antigens (table 6) in a total volume of 50 µl. After washing of unbound antibody, stained cells were resuspended in 0.5 ml fixation solution.

For indirect stainings, cells were first incubated with 50 µl of hybridoma supernatants containing the respective murine monoclonal antibody (table 7), incubated for 30 min at 4°C, washed, and subsequently stained with FITC- or PE-labeled goat-anti-mouse IgG (1:200 in PBS). After further 30 min of incubation at 4°C, cells were washed and resuspended in 0.5 ml of FACS fixation solution. Flow cytometry analysis was performed using the flow cytometer BD FACSCanto. After gating of viable lymphocytes or leukemic blasts, 10,000 events were measured and analysed applying the BD FACS DIVA software. Additional data analysis was performed using the EXPO™32 Software (Beckman Coulter).

For determination of cell viability in adoptive transfer experiments, cells were resuspended after staining in FACS buffer, and incubated with 2.5 nM SYTOX® Red Nucleic Acid Stain (Molecular Probes/Invitrogen) 15 min prior to analysis.

3.4.2.5 T cell receptor V beta chain analysis

T cell receptor (TCR) V beta chain determination was performed using fluorescein-labeled mAbs recognizing 24 different Vβ families from the IOTest® Beta Mark Kit (Immunotech/Beckman Coulter). This kit is a multi-parametric analysis tool for the quantitative determination of the TCR Vβ repertoire of human T lymphocytes and takes advantage of the fact that Vβ specificities may be grouped into mutually exclusive combinations. It allows the detection of three Vβ families in the same FACS tube, by combining three mAbs with only two fluorophores. A first mAb is FITC-conjugated, a

second mAb is PE-conjugated, and a third mAb is a balanced mixture of a PE- and a FITC-conjugated form (www.beckmancoulter.com, table 8).

To determine the TCR Vβ expression of antigen-specific $CD8^+$ T cells, 100,000 cells were incubated at room temperature with 10 µl of one of each anti-Vβ combination (tube A-H) in combination with 5 µl anti-CD8 mAb conjugated with PC5 as a third colour, allowing it to gate on the $CD8^+$ T cell compartment. As compensation controls, cells were stained with CD8-FITC, CD8-PE or CD8-PC5, respectively. After washing, cells were resuspended in 0.5 ml fixation solution and analysed using a BD FACSCanto flow cytometer. Separate confirmatory tests included further fluorescein-conjugated mAbs specific for individual Vβ families.

	Vβ family monoclonal antibodies	**Fluorochrome labeling**
Tube A	Vβ5.3 Vβ7.1 Vβ3	PE FITC/PE FITC
Tube B	Vβ9 Vβ17 Vβ16	PE FITC/PE FITC
Tube C	Vβ18 Vβ5.1 Vβ20	PE FITC/PE FITC
Tube D	Vβ13.1 Vβ13.6 Vβ8	PE FITC/PE FITC
Tube E	Vβ5.2 Vβ2 Vβ12	PE FITC/PE FITC
Tube F	Vβ23 Vβ1 Vβ21.3	PE FITC/PE FITC
Tube G	Vβ11 Vβ22 Vβ14	PE FITC/PE FITC
Tube H	Vβ13.2 Vβ4 Vβ7.2	PE FITC/PE FITC

Table 8: Composition of the IOTest® Beta Mark Kit (Beckman Coulter) used for determination of the TCR Vβ expression on $CD8^+$ T cells. Each tube contains combinations of three monoclonal antibodies with only 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.

3.4.3 Mixed lymphocyte/leukemia culture (MLLC)

3.4.3.1 Mini-MLLC

The mini-MLLC approach was modified from a recently published protocol that was successfully used to generate minor histocompatibility antigen-reactive donor CTLs by primary stimulation with HLA-matched recipient-derived mature dendritic cells (Bleakley et al, 2004, abstract). Mini-MLLCs were performed in round-bottomed 96-well microtiter plates in AIM-V culture medium supplemented with 10% heat-inactivated human serum. Responder cells were healthy donor $CD8^+$ T cells (10^4/well) obtained from the $CD62L^{(high)+}$ and the $CD62L^{(low)+/neg}$ subsets, respectively. Stimulator cells were allogeneic AML blasts (10^4/well) pre-cultured overnight at 37°C in AIM-V culture medium supplemented with 10% heat-inactivated human serum (designated as d1-AML blasts). In experiments with HLA-matched sibling donors, pre-culture medium was supplemented with 1000 IU/ml IL-4, 1000 IU/ml GM-CSF, 10 ng/ml TNF-α, and 5 ng/ml SCF, to further increase expression of HLA and costimulatory molecules on AML blasts (Brouwer et al, 2000a). CD8-negative donor PBMCs (10^4/well) separated from $CD8^+$ T cells during the CD8/CD62L isolation procedure served as feeder cells. Stimulator and feeder cells were irradiated with 35 Gray prior to use. The culture medium was supplemented with 5 ng/ml IL-7, 1 ng/ml IL-12, and 5 ng/ml IL-15. Mini-MLLC responders were re-stimulated weekly with irradiated d1-AML blasts in fresh cytokine-containing medium. IL-12 was replaced by IL-2 (250 IU/ml) on d14 of culture. As a first testing for reactivity against d1-AML blasts, split-well IFN-γ ELISpot assays were performed at day 19 or 26 of culture. Growing mini-cultures were transferred into 96-well flat bottom plates and subsequently in 48-well plates at day 21 or day 28 of culture, respectively. Mini-MLLC populations with consistent reactivity against d1-AML blasts were further expanded in 24-well plates using 10^6 responders and 10^6 stimulators per well.

3.4.3.2 Maxi-MLLC (bulk cultures)

In order to compare the new mini-MLLC approach to traditionally used maxi-MLLCs, bulk cultures were performed in 24-well plates exactly as described for mini-MLLCs, but with $5x10^5$ each of responder, stimulator, and feeder cells per well, respectively.

3.4.3.3 T cell cloning by limiting dilution

At the beginning of this project, the liming dilution method (Schawaller et al, 1980) was applied in order to generate clonal $CD8^+$ T cells from conventional MLLC bulk cultures in the MZ201-AML/Donor168 model. MLLCs were initiated with $0.5x10^5$ $CD8^+$ selected

PBMCs of Donor168 in 24-well plates and stimulated with irradiated MZ201-AML blasts in AIM-V medium supplemented with 10% HS. At day 3 of MLLC, 250 IU/ml IL-2 was added. MLLC responders were re-stimulated weekly with irradiated d1-AML blasts in fresh cytokine-containing medium. At day 28 of bulk culture, T cells were seeded at 0.3/well, 1/well and 3/well in 96-well plates and re-stimulated weekly as described, but in medium contained 250 IU/ml IL-2, 5 ng/ml IL-7, and 5 ng/ml IL-15 and with irradiated Donor168-EBV-B cells as feeders. Growing CTL clones were transferred into 96-well flat bottom, 48-well and 24-well plates, respectively.

3.4.4 IFN-γ ELISpot (Enzyme-Linked Immunosorbent Spot) assay

3.4.4.1 Principle

Interferon-gamma (IFN-γ) is one of the cytokines that is released from T lymphocytes following antigen contact. The principle of the ELISpot assay (figure 3) is the capture and colorimetric detection of a cytokine released by individual T cells, accomplished by a sequential antibody-binding and enzyme-substrate reaction. First, a monoclonal antibody (the primary antibody) directed against one cytokine-epitope binds with the constant region of its heavy chain to a nitrocellulose- or nylon-membrane in 96-well ELISpot plates. Lymphocytes are co-incubated in those wells together with their target cells, and release cytokines, when antigen contact takes place. The cytokine molecules are bound by the variable region of the primary antibody. After cells have been washed from the membrane, a second mAb is added that recognizes a different epitope of the cytokine. This secondary antibody is coupled to biotin, which can on its part be bound by avidin. Avidin in turn is coupled to an enzyme (horseradish peroxidase or alkaline phosphatase) that converts in the last step its substrate in a colour reaction, thereby marking the place of cytokine-release by an individual T cell as a coloured spot. The number of spots can be counted and quantifies thereby the reactivity or frequency of single antigen-specific T lymphocytes.

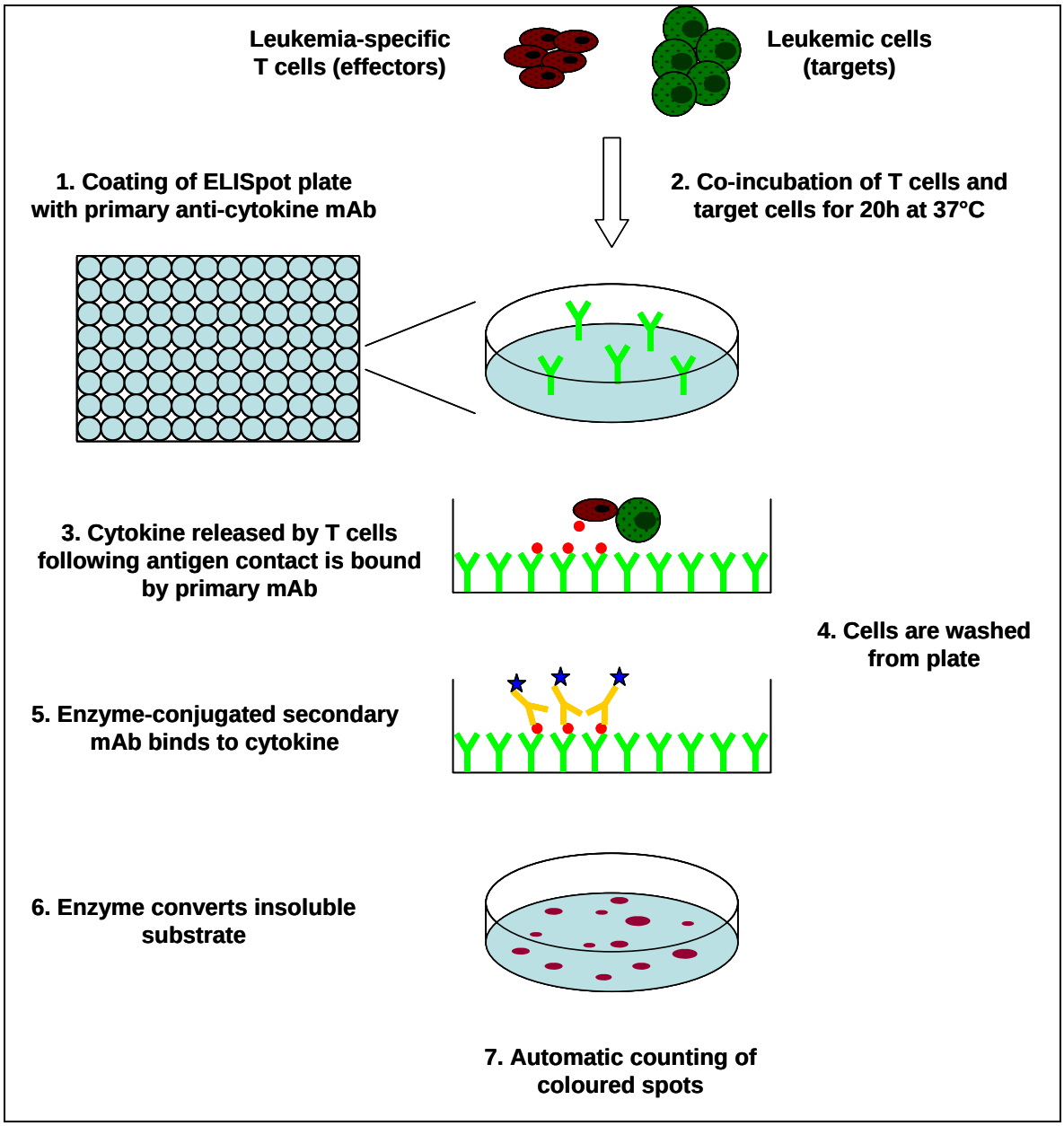

Figure 3. Principle of ELISpot assay.

3.4.4.2 Materials and equipment for IFN-γ ELISpot experiments

Multiscreen HTS™ IP 96well filtration system	Millipore (Eschborn, D)
Anti-hIFN-γ -antibody #1-D1K (mouse IgG)	Mabtech AB (Nacka, S)
Anti-hIFN-γ -antibody #7-B6-1 (mouse IgG), biotinylated	Mabtech AB (Nacka, S)
Vectastain® Elite Kit (reagents A + B)	Vector Laboratories (Burlingame, USA)
Wash buffer	PBS / 0.05% Tween 20
Buffer for secondary mAb	PBS / 0.5% BSA
PBS (phosphate buffered saline)	PBS Instamed® Powder (9.55 g/l), Biochrom (Berlin, D)

Acetate buffer	615.8 ml H_2O 1,8 g Na-acetate 9.2 ml Acetic acid (100%)
Tween 20	Merck-Schuchardt (Hohenbrunn, D)
Albumine, bovine (BSA)	Carl Roth (Karlsruhe, D)
Acetic acid	Carl Roth (Karlsruhe, D)
Na-acetate	Sigma-Aldrich (Steinheim, D)
AEC (3-amino-9-ethylcarbazole) tablets	Sigma-Aldrich (Steinheim, D)
N,N-Dimethylformamide	Sigma-Aldrich (Steinheim, D)
H_2O_2 30% (w/w) solution	Sigma-Aldrich (Steinheim, D)
Ethanol 35% (V/V)	Merck (Darmstadt, D)
Axioplan 2 microscope	Carl Zeiss Vision (Hallbergmoos, D)
Computer-based imaging system and Software KS ELISpot 4.9	Carl Zeiss Vision (Hallbergmoos; D)

3.4.4.3 IFN-γ ELISpot assay for reactivity testing of leukemia-reactive CD8$^+$T cells

ELISpot plates were coated with the anti-IFN-γ primary mAb (10 µg/ml in PBS, 60 µl/well) overnight at 4°C or for 2 h at 37°C. Unbound primary mAb was washed from the membranes with PBS, and free binding spaces on the membrane were blocked by incubation with serum-containing medium. T cells at 0.5-2x10^4/well and target cells at 2-5x10^4/well were seeded in AIM-V medium with 10% human serum in ELISpot plates in a total volume of 100-200 µl. T cells without stimulator cells served as controls for spontaneous cytokine release.

Split-well IFN-γ ELISpot assays were applied in order to analyze mini-MLLC responder populations at day 19 or 26 of culture for reactivity against AML blasts. For this purpose, one part of the responder cells (i.e. 20 µl of 96-well plates, corresponding to approx. 2x10^4 T cells) was used in the assay, whereas the residual cells were further expanded.

For the determination of the HLA-restriction elements, target cells were incubated with saturating concentrations of HLA blocking mAbs for 30 min before addition of effector cells. The following Abs were used: W6/32, an anti-HLA-class-I IgG2a, PA2.1, an anti-HLA-A2 IgG1, GAP-A3, an anti-HLA-A03 IgG2a, C7709A2, an anti-HLA-A24 IgG2a, B1.23.2, an anti-HLA-B and HLA-C IgG2a, and HB55 (L243), an anti-HLA-DR IgG2a (Table 7).

After a culture period of 18-20h at 37°C, plates were washed with PBS containing 0.05% Tween20, and the secondary, biotinylated anti-IFN-γ antibody was added (2 µg/ml in PBS/0.5% BSA, 60 µl/well) and incubated at 37°C for 2 h. After washing again with PBS/0.05% Tween20, plates were incubated with the avidin/peroxidase-complex

(100 µl/well) for 1 h at room temperature in the dark. The avidin/peroxidase-complex solution was prepared 30 min in advance by mixing 10 ml of PBS/0.1% Tween20 with one drop of reagents A and B from the Vectastain® Elite Kit (Vector Laboratories). Plates were again washed (PBS/0.05% Tween20, PBS), and as the final step, the AEC (3-amino-9-ethylcarbazole)-substrate solution (100 µl/well) was added. The substrate solution was prepared by dissolving one tablet of AEC in 2.5 ml dimethyle formamide, filled up with acetate buffer to 50 ml, and addition of 25 µl 30% H_2O_2 solution. The colour reaction was stopped after 10-15 min by rinsing the plates thoroughly with tap water. After the membranes had dried, IFN-γ spots were automatically visualized and counted using an Axioplan 2 microscope combined with the computer-assisted image analysis system KS ELISpot 4.9 (Herr et al, 1997). Results represent means of duplicate or triplicate wells.

3.4.5 ^{51}Chromium release assay

3.4.5.1 Principle

The ^{51}Chromium (^{51}Cr) release assay is used to determine the cytolytic activity of $CD8^+$ T cells towards a target cell as one effector function of cytotoxic T lymphocytes. Live target cells take up radioactively labelled sodium chromate, $Na_2{}^{51}CrO_4$. When these labelled cells are killed, the radioactive chromate is released and its presence in the supernatant can be measured in a gamma-counter. The percentage of specific lysis can be calculated from the amount of released $Na_2{}^{51}CrO_4$, the spontaneous and the maximum release of the target cells.

3.4.5.2 Materials and equipment used for ^{51}Chromium release assays

$Na_2{}^{51}CrO_4$	Amersham Buchler (Braunschweig, D)
Cytotox tubes (0.6 ml)	Greiner Bio-One (Frickenhausen)
96-well plates, conical	Greiner Bio-One (Frickenhausen)
Gamma-Counter	Packard Cobra, GMI Inc. (Ramsey, MN, USA)

3.4.5.3 ^{51}Chromium release assay of leukemia-reactive $CD8^+$ T cells

Target cells (i.e. AML blasts, LCLs, PHA blasts, K562 cells) were incubated for 90-120 min at 37°C with 100-200 µCi $Na_2{}^{51}CrO_4$. After washing, labeled targets (1-1.5x10^3/well) were plated into conical 96-well plates. CTLs were added in duplicates at indicated effector-to-target (E/T) ratios in a total volume of 160 µl/well. After 4 to 5 h of incubation at 37°C, the plates were centrifuged for 5 min at 1000 rpm (without brake) in order to collect 80 µl

supernatant per well for counting of released ^{51}Cr in a gamma-counter. Target cells incubated with detergent solution (1% Tween20/PBS) instead of CTL served as positive control (maximum release). The spontaneous release (negative control) was determined by adding PBS instead of CTL. The percent specific ^{51}Cr-release was calculated by the following formula: (experimental release – spontaneous release) × 100 / (maximum release – spontaneous release). The spontaneous release / maximum release ratios of target cells were generally <25%.

3.5 Adoptive transfer experiments in NOD/SCID IL2Rγ^{null} mice

3.5.1 Cocultivation of primary AML blasts with CD8$^+$ CTLs

MZ201-AML-reactive CTL clones 1C6 and 4D7 were expanded *in vitro* as described in chapter 3.4.3.1. The HLA-A*0201-restricted tyrosinase-reactive CTL IVSB (Wolfel et al, 1994) and the HLA-B*2705-restricted melanoma-reactive CTL clone 3.2/48 (Prof. Dr. T. Wölfel, Mainz, unpublished data) did not recognize MZ201-AML blasts in IFN-γ ELISpot assay (data not shown) and served as controls. All CTLs were used at day 5 after last stimulation with lethally irradiated AML or melanoma cells, respectively. Identical aliquots of d1-AML blasts (5x10^6 per mouse) of patient MZ201 were incubated for 20 hours at 37°C and 5% CO_2 in 3 ml of AIM-V medium supplemented with 10% human serum, either alone or in the presence of CTLs at a T cell/AML ratio of 10:1. Before inoculation into NOD/SCID IL2Rγ^{null} mice, cultures were harvested, washed, and resuspended in 500 µl PBS/0.5% FCS.

3.5.2 Transplantation of AML cells into NOD/SCID IL2Rγ^{null} mice

All experiments handling mice were performed by Dr. Udo Hartwig, Mainz. NOD/SCID IL2Rγ^{null} mice were kindly provided by Dr. L. D. Shultz, Jackson Laboratory, Bar Harbor, ME (USA). Mice were bred and maintained in the Central Animal Facility of Mainz University and were free of commonly tested viruses, parasites, and bacteria. The animal studies were approved by the German state authorities. Eight- to 12-weeks old NOD/SCID IL2Rγ^{null} mice were sublethally irradiated with 2 Gy from a ^{137}Cs source prior to intravenous injection of MZ201-AML cells that had been cultured in either medium alone or with CD8$^+$ CTLs.

3.5.3 Harvest and phenotypic analysis of peripheral blood, spleen and bone marrow cells from transplanted NOD/SCID IL2Rγ^{null} mice

Eight weeks after inoculation, peripheral blood was drawn from all mice transplanted with AML cells alone or AML cells incubated with CD8$^+$ CTLs. Mice were subsequently sacrificed and single cell suspensions of spleen and bone marrow samples obtained from femurs were prepared. Nuclear cells were stained for flow cytometry at 4°C for 30 min with FITC-conjugated anti-human CD45, CD33, CD34, HLA-ABC and PE-conjugated anti-human CD8 mAbs. Isotype-matched FITC-/PE-conjugated mAbs were used as controls. Erythrocyte lysis was performed after staining of peripheral blood samples. For determination of cell viability, cells were resuspended after staining and washing in PBS/0.5% FCS, and incubated with 2.5 nM SYTOX® Red Nucleic Acid Stain (Molecular Probes/Invitrogen)

15 min prior to analysis. After gating of viable cells, 15,000 to 20,000 events were measured on flow cytometer FACSCalibur (BD Biosciences) and analysed using the CellQuest^{TM}Pro Software (BD Biosciences).

3.5.4 DNA analysis of peripheral blood, spleen and bone marrow cells from transplanted NOD/SCID IL2Rγ^{null} mice

Human-specific sequences were detected in genomic DNA prepared from peripheral blood, spleen, and bone marrow samples of transplanted NOD/SCID IL2Rγ^{null} mice by DNeasy tissue kit (Qiagen, Hilden). Preparation of genomic DNA was performed as recommended in the manufacturer's instructions. DNA concentration was measured on a Spectronic Genesys 10 UV spetrophotometer (Thermo Scientific, Dreieich, D).

PCR analysis was performed using oligonucleotide primers (sense, 5′ GGGATAATTTCAGCTGACTAAACAG 3′; antisense, 5′ TTCCGTTTAGTTAGGT GAGTTATC 3′) to amplify an 850 bp fragment of the α-satellite region of human chromosome 17 (Becker et al, 2002). Primers specific for the murine β-actin gene (sense, 5′ AATTAACCCTCACTAAAGGG 3′; antisense, 5′ GAAGCATTTGCGGTGGACGAT 3′) were used to amplify a 330 bp fragment as internal control. The PCR reaction for one sample was performed with 100-150 ng genomic DNA in a total volume of 50 µl:

100-150 ng template DNA
4 x 2.5 µl Primer (hu Chr 17: UH184/UH185; mu β-Actin: UN122/UH123; 1:5 in H_2O)
4 µl dNTP Mix (Qiagen)
5 µl 10x Puffer (Qiagen)
0.5 µl Taq Polymerase (Qiagen)
ad 50 µl ddH_2O

PCR reaction was performed with 30 cycles (94°C/1 min, 55°C/1 min, 72°C/1 min) with a Hybaid PCR cycler (Omnigene, Cambridge, USA). PCR products were analyzed on 1% agarose gel. The MW Plus 1kbp DNA ladder (GIBCO-BRL) served as the molecular size marker.

3.6 Further materials

3.6.1 Laboratory equipment

Autoclave	KSG Sterilisatoren GmbH (Olching, D)
^{137}Caesium source	Gammacell 2000, Molsgaard Medical (Gansloe, DK)
Aluminium foil	Carl Roth (Karlsuhe, D)
Cell culture tube-racks	VWR (Darmstadt, D)
Centrifuge (Megafuge 1.0R)	Hereaus (Langenselbold, D)
CO_2-Incubator	Binder (Tuttlingen, D)
Cryo tube-racks	VWR (Darmstadt, D)
Examination gloves	Semperet (Wien, A)
FACS tube-racks	VWR (Darmstadt, D)
Ice machine	Ziegra (Isernhagen, D)
Laboratory water purification system	Elga Bergfelde (Celle, D)
Minishaker MS2	IKA (Staufen, D)
Nitrogen cryo bank	Air Liquide DMC (Marne-la-Vallée, F)
Nitrogen reservoir tank	Taylor Wharton XL-180, Tec Lab (Königstein, D)
Phase contrast microscope	Axiovert 25, Carl Zeiss AG (Jena, D)
Pipette device	Pipetus® Hirschmann Laborgeräte (Eberstadt, D)
Pipettes: 1-10 µl 20-200 µl, 200-1000 µ Mulitichannel 5-50 µl, 25-200 µl	 Eppendorf (Hamburg, D) Gilson (Villiers-le-Bel, F) Dunn Labortechnik GmbH (Asbach, D)
Precision scale	Precisa, PAG Oerlikan AG (Zürich, CH)
Refrigerator, Freezers (+4°C, -20°C, -80°C)	Hereaus, Kendro (Langenselbold, D)
Sterile work bench	NuAire (Plymouth, MN, USA)
Water bath	GFL (Burgwedel, D)
Water-jet vacuum pump	Brand (Wertheim, D)

3.6.2 Plastic material

Cryo tubes (1.8 ml)	Nunc (Wiesbaden, D)
Cell culture flasks (30 cm^2, 80cm^2, 175 cm^2)	Greiner (Nürtingen, D)
Cryo boxes	Nalgene® Freezing Containers, Nunc (Wiesbaden, D)
Cull culture tubes (15 ml, 50 ml)	Greiner (Nürtingen, D)
FACS tubes	Sarstedt, (Nürnbrecht, D)
Ficoll separation tubes	Leucosep® tubes, Greiner (Nürtingen, D)
Microtiter plates (96-well flat-, round-, V-bottom)	Greiner (Nürtingen, D)

Perfusor syringes (50 ml)	Braun (Melsungen, D)
Petri dishes (9 cm, 13 cm)	Greiner (Nürtingen, D)
Pipette tips (0.5-10 µl, 1-200 µl, 200-1000 µl)	TipOne Pipette Tips, Graduated Filter Tips, Starlab GmbH (Ahrensburg, D)
Pipettes single use (1/2/5/10/25/50 ml)	Greiner (Nürtingen, D)
Reaction tubes (1.5 ml)	Eppendorf (Hamburg, D)
Sterile filter (0.22 µm, 0.45 µm)	Steritop®, Millipore (Eschborn, D)
Syringes (10 ml, 20 ml)	Braun (Melsungen, D)
Tissue culture plates (6-/24-/48-/96-well)	Greiner (Nürtingen, D)

3.6.3 Glass material

Beaker	Schott (Mainz, D)
Duran glass flasks	Schott (Mainz, D)
Cell counting chamber (Fuchs-Rosenthal)	Schreck (Hofheim, D)

4. RESULTS

4.1 Characterization of HLA class I-matched donor/AML models

4.1.1 Unrelated donor/AML models

4.1.1.1 MZ201-AML

Patient MZ201-AML was a female 74 year-old patient diagnosed with AML FAB M5b (WHO: acute monocytic/monoblastic leukemia) in August 2004. She had a normal female karyotype (44,XX), no chromosomal aberrations or known AML-related genomic mutations. The patient had an initial white blood cell (WBC) count of $2x10^5$/µl and died nine months after diagnosis due to progressive disease that was refractory to chemotherapy.

For *in vitro* experiments, the blood bank of the university clinics, Mainz, provided buffy coats of three HLA class I-identical healthy unrelated donors (Donor168, Donor332, Donor650). High-resolution HLA typing from genomic DNA using class I-specific primers revealed a complete HLA class I match at the suballelic level (table 9).

	HLA-A	HLA-B	HLA-Cw
MZ201-AML	*0101 *0201	*0801 *5601	*0102 *0701
Donor168	*0101 *0201	*0801 *5601	*0102 *0701
Donor332	*0101 *0201	*0801 *5601	*0102 *0701
Donor650	*0101 *0201	*0801 *5601	*0102 *0701

Table 9: HLA class I type of patient MZ201-AML and three healthy unrelated donors. High resolution typing was performed from genomic DNA using class I-specific primers (Dr. B. Thiele, Institute for Immunology and Genetics, Kaiserslautern).

4.1.1.2 MZ418-AML

MZ418-AML was a male 49 year-old patient diagnosed in June 2005 with AML (FAB M5b, WHO: monocytic/monoblastic AML); the initial WBC was $3x10^5$/µl. Cytogenetic analysis revealed a 46,X,del(y) karyotype. Molecular diagnostics did not detect any known AML-related genomic mutations. Patient MZ418 died 11 months after diagnosis due to treatment-refractory disease (third AML relapse) and infiltration of the central nervous system. There were PBMCs of four healthy unrelated donors with serological HLA class I

match available (HLA-A02/03, B35/44, Cw04/05). The patient was suballele-typed for HLA-A*0201.

4.1.1.3 MZ431-AML

The 69 year-old female patient MZ431-AML was diagnosed with an FLT-ITD-positive AML FAB M5a (WHO: acute monoblastic/monocytic leukemia) in June 2006. The WBC count at diagnosis was $2.2x10^5$/µl. Complete remission was achieved after standard chemotherapy, but the patient died four month later from recurrent disease. The HLA class I type of MZ431-AML was HLA-A*0101/*0201, -B*0801/*4402, -Cw*0501/*0702. For *in vitro* experiments, blood lymphocytes of healthy donor 532 were used, who was serologically HLA class I-matched (HLA-A01/02, -B08/44, -Cw05/07).

4.1.1.4 MZ574-AML (Cw07 suballele mismatch)

Patient MZ574 was a 43 year-old male diagnosed with AML FAB M4eo (WHO: AML with recurrent cytogenetic aberrations) in March 2006. The initial WBC count was $1x10^5$/µl. Cytogenetic analysis revealed a normal karyotype (46,XY) with an inv16(p13q23)[23] inversion. Molecular diagnostics additionally detected a FLT3 D835 point mutation. Occurring complications were affection of liquor and questionable of testes. Patient MZ574 was not transplanted and died 3 months after diagnosis due to refractory disease.
For *in vitro* experiments, we obtained from the blood bank buffy coats of two healthy unrelated donors, which were serologically matched for HLA class I (HLA-A01/02, B07/15(62), Cw03/07). However, high-resolution HLA-typing detected a suballele mismatch for Cw07 between patient and donors (table 10).

	HLA-A	HLA-B	HLA-Cw
MZ574-AML	*0101 *0201	*0702 *1501	*0304 *0701
Donor172	*0101 *0201	*0702 *1501	*0304 *0702
Donor247	*0101 *0201	*0702 *1501	*0304 *0702

Table 10: HLA class I type of patient MZ574-AML and two healthy unrelated donors. High resolution typing was performed from genomic DNA using class I-specific primers (Dr. B. Thiele, Institute for Immunology and Genetics, Kaiserslautern), revealing a suballele-mismatch between patient (Cw*0701) and donors (Cw*0702).

4.1.2 Sibling donor/AML models

4.1.2.1 MZ369-AML

Patient MZ369-AML was a 30 year-old female patient diagnosed in November 2005 with FAB M4 AML (WHO: myelomonocytic AML). She had an initial WBC count of $2x10^5$/µl. Cytogenetic analysis revealed a normal female karyotype (46,XX); AML-related genomic mutations have not been detected. Complete remission could be achieved after induction chemotherapy. She received an allogeneic HSC transplantation from her HLA-identical brother. Unfortunately the disease relapsed three months after HSCT and was refractory to several salvage therapies (chemotherapy, anti-CD33 mAb, $3x10^7$ CD3 T cells/kg DLI). The patient died nine months after HSCT. Graft-versus-host disease did not occur at any time point. The serological HLA class I type of patient MZ369-AML was HLA-A01/24, -B18/38, -Cw07/12.

4.1.2.2 MZ456-AML

The 51 year-old female patient MZ456 was diagnosed with AML M5a (WHO: acute monoblastic/monocytic leukemia) in May 2005 with a WBC count of 66,000/µl. The karyotype was determined as 47,XX +8[2/18] (trisomy 8). Induction chemotherapy achieved a complete remission. Patient MZ456 developed moderate acute GVHD following allogeneic HSCT from her HLA-identical sister in December 2005 and is currently free of AML. The HLA class I type was determined as A02/66, B14/18, Cw07/08.

4.2 Expression of HLA, costimulatory and adhesion molecules on primary AML blasts

4.2.1 Phenotypic effect of 24 h pre-culture on MZ201-AML blasts

An appropriate recognition of leukemic cells by T cells requires an efficient interaction between target and effector cell involving several cell surface molecules. It has been previously shown that a low expression of HLA class I or II, costimulatory (CD80, CD86, CD40) and adhesion molecules (CD54, CD58) on malignant cells may lead to an insufficient T cell immune response. It was also reported that a low expression of multiple costimulatory and adhesion molecules on AML cells could be up-regulated by 24 hours of culture; this was associated with increased recognition of the AML blasts by allogeneic T cells (Jansen et al, 1992; Brouwer et al, 2000b; Brouwer et al, 2002). For that reason we first investigated the expression of HLA class I and II and of costimulatory and adhesion molecules on leukemic blasts in model MZ201-AML, and determined whether a 24 hour-culture of the cells was associated with up-regulation of these molecules. Accordingly, immunophenotyping was performed directly after thawing of MZ201-AML cells and after a 24 hour incubation period at 37°C/5%CO_2 in AIM-V medium supplemented with 10% human serum.

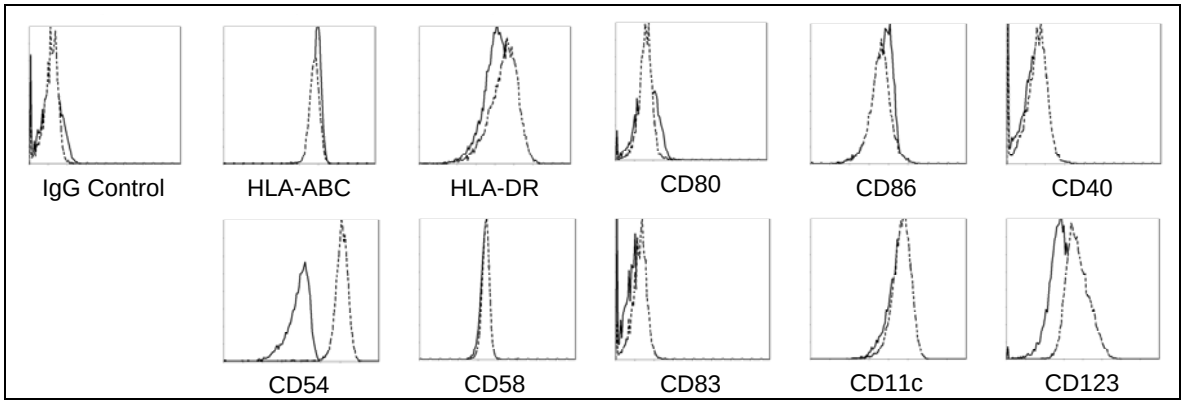

Figure 4. Flow cytometric analysis showing histograms of HLA, costimulatory and adhesion molecules on leukemic blasts of patient MZ201-AML. To identify the leukemic population, cells were gated on the myeloid marker CD33. Blasts were analyzed immediately after thawing (continuous line) or after a culture period of 24 hours (dashed line).

Flow cytometric analysis showed a high expression of HLA class I and II, CD86, CD58 and CD11c, that was not further increased by incubating the AML blasts for 24 hours. The pre-incubation period resulted in a significant upregulation of the adhesion molecule CD54, as well as in an increased expression of CD123 (IL-3 receptor alpha chain). The costimulatory

molecules CD80 and CD40, as well as the mature DC marker CD83 could not be detected on MZ201-AML cells in this analysis (figure 4).

4.2.2 Phenotypic effect of different maturation treatments on MZ201-AML blasts

To study the feasibility of generating leukemic dendritic cell (DC)-like antigen presenting cells (APCs) from leukemic blasts and hence to improve the immunogenicity of these cells, MZ201-AML blasts were cultured for 4 days at 37°C/5% CO_2 in the presence of 1000 IU/ml IL-4, a mixture of several DC maturation cytokines (a "cocktail" composed of 1000 IU/ml IL-4, 1000 IU/ml GM-CSF, 10 ng/ml TNF-α and 5 ng/ml SCF), huCD40L (CD154)-transfected mouse fibroblasts (Ltk- huCD40L-transfected L cells), or a biotinylated anti-human CD40 antibody cross-linked with anti-biotin MACSi-Beads®, both in combination with 1000 IU/ml IL-4, or the calcium ionophore Calcimycin (A-23187, 180 ng/ml). Those reagents have been described to differentiate AML blasts into APCs with morphology and phenotypic markers typically found on mature DCs (Waclavicek et al, 2001; Westers et al, 2003). Immunophenotypic analysis (figure 5) in comparison to AML blasts that had been incubated for 24 hours in medium without stimulatory supplements, showed a comparably high expression of HLA class I as well as of the costimulatory molecule CD86 for all conditions tested. HLA class II expression determined by staining with an anti-HLA-DR antibody was elevated from approximately 50% positive cells, when leukemic blasts were incubated without supplement, to approximately 90% under the different stimulatory conditions. Surface expression of CD80 was significantly increased (up to 30-50%) when cells were stimulated over four days with the calcium ionophore (Calcimycin, A-23187) or CD40L-transfected fibroblasts in combination with IL-4. The mature DC marker CD83 could only be detected on MZ201-AML blasts by using CD40L-transfected fibroblasts plus IL-4 as maturation agents. Induction of the costimulatory molecule CD40 on MZ201-AML blasts could be achieved by each of the applied maturation treatments, but was highest (50-60% positive cells) when the cytokine "cocktail", the calcium ionophore or CD40L-transfected fibroblast in combination with IL-4 were used. Only CD40L-transfected fibroblasts used as stimuli for MZ201-AML blasts consistently induced all of the characteristics of mature DCs (expression of CD80, CD83 and CD40 in addition to CD86 and HLA class I and II).

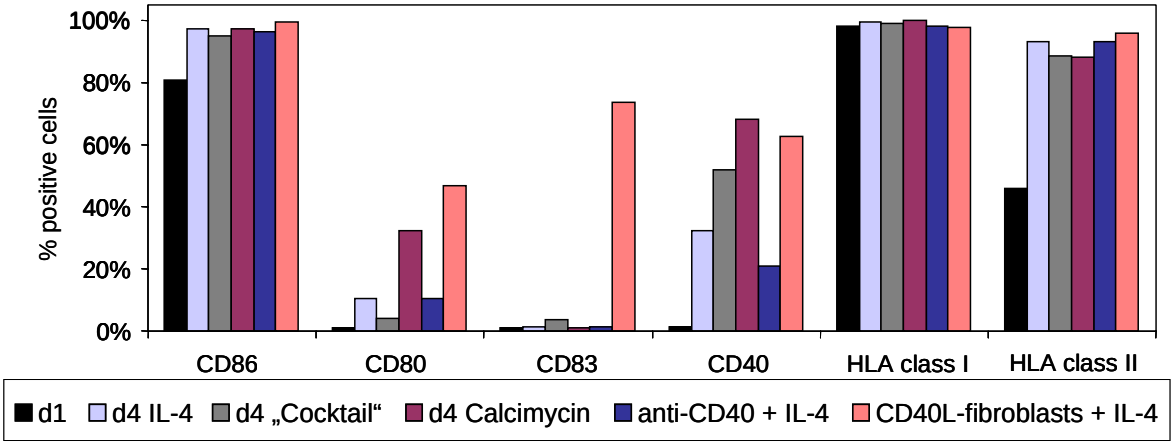

Figure 5. Immunophenotyping of MZ201-AML blasts after different maturation treatments. Flow cytometry analysis of CD86, CD80, CD83, CD40 and HLA class I and II was performed after incubating MZ201-AML blasts for 24 hours in AIM-V medium supplemented with 10% HS (d1), for 4 days in medium containing IL-4, a cytokine "cocktail" (IL-4, GM-CSF, SCF, TNF-α), calcium ionophore (Calcimycin, A-23187), anti-CD40 mAb + IL-4, or in the presence of CD40L-transfected murine fibroblasts + IL-4.

4.2.3 Impact of AML maturation on recognition by allogeneic CD8$^+$ T cells

In order to analyze whether the recognition of MZ201-AML by established allogeneic CD8$^+$ T cell lines is improved by pre-incubating leukemic cells for 24 hours or by AML maturation applying the cytokine "cocktail" (IL-4, GM-CSF, TNF-α, SCF) or the calcium ionophore (Calcimycin, A-23187), MZ201-AML blasts prepared in this way were used as targets in IFN-γ ELISpot assays. An increased recognition of MZ201-AML by CD8$^+$ MLLC responder lymphocytes could be observed when target cells were cultured *in vitro* for 24 hours, in comparison to freshly thawed leukemic cells. Application of the cytokine "cocktail" or the calcium ionophore did not further augment target recognition by allogeneic CD8$^+$ T cells in IFN-γ ELISpot assay (figure 6). Thus, pre-cultured leukemic cells appeared to be better targets for CD8$^+$ T cells compared to their freshly thawed counterparts. This effect can most likely be explained by an increased expression of the adhesion molecule CD54, as observed in flow cytometric analysis (figure 4). Maturation of MZ201-AML blasts using the tested agents was not necessary for an improved recognition by allogeneic CD8$^+$ T cell lines.

Analyses of AML blasts from three further patients confirmed the upregulation of CD54 and CD123, as well as of CD86, CD11c and HLA class II molecules after 24 hours of pre-incubation or IL-4 treatment. Similarly, recognition of pre-cultured AML blasts by allogeneic CD8$^+$ MLLC responders was improved in comparison to freshly thawed leukemic cells (data not shown).

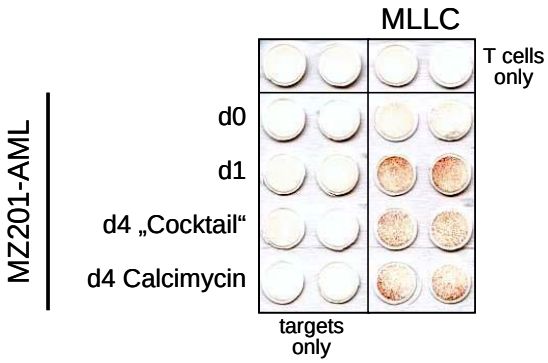

Figure 6. Recognition of MZ201-AML blasts by MLLC responders (Donor168). Leukemic blasts of patient MZ201 were used as target cells (d0, freshly thawed; d1, pre-incubated for 24 h; d4 Cocktail, pre-incubated for 4 days with IL-4, GM-CSF, TNF-α, SCF; d4 Calcimycin, pre-incubated for 4 days with calcium ionophore A-23187) in an IFN-γ ELISpot assay with $1.3x10^4$ $CD8^+$ T cells (MLLC Donor168 d43+5) as responders.

In subsequent MLLC experiments, we did not use murine CD40L-fibroblasts despite their superior effects with regard to AML maturation. The reason for this decision was our concern that this may lead to unwanted xenogenic T cell responses against cells or processed cellular material of murine origin. We also preferred the cytokine "cocktail" over Calcimycin because of the availability of all "cocktail" components in good manufacturing practice (GMP) quality, facilitating future clinical translation. The cytokine "cocktail" was applied only in sibling donor/AML combinations, since its strong inducing effect on HLA class II led to dominant anti-class II mismatch responses in unrelated donor/AML pairs that were matched only for HLA class I, but not for class II molecules (data not shown).

4.3 Generation of CTL clones from MLLC bulk cultures in model MZ201-AML/Donor168

At the beginning of this project, we attempted to generate leukemia-reactive $CD8^+$ CTLs by the traditionally used method of limiting dilution cloning from T cell bulk cultures. For this purpose, mixed lymphocyte/leukemia cultures (MLLCs) were initiated with $CD8^+$ blood lymphocytes from healthy donor 168 and stimulated over four weeks with irradiated MZ201-AML d1-blasts in AIM-V medium supplemented with 10% human serum. The healthy donor 168 and the AML patient MZ201 were completely matched for HLA class I in high resolution HLA typing (table 9). As cytokines, only IL-2 (250 IU/ml) was administered from day three on. Limiting dilution cloning was performed at day 28 of culture. Only five CTL clones (1F12, 4E3, 9B10, 9C12, 10C4) emerging from ten limiting dilution 96-well plates showed sufficient growth to be transferred into 24-well plates. An expansion of these five CTLs was attempted by weekly stimulation with irradiated AML d1-blasts and the cytokine combination IL-2 (250 IU/ml), IL-7 (5 ng/ml) and IL-15 (5 ng/ml). Of these, two CTLs (1F12, 10C4) showed only very weak reactivity against patient-derived AML blasts in IFN-γ ELISpot assay (<50 spots/10^4 T cells; data not shown). With exception of CTL 9C12, all other CTLs recognized patient-derived PHA-activated T cell blasts in IFN-γ ELISpot assays (data not shown). Three CTLs (9C12, 9B10, 4E3) were further analyzed for HLA restriction, using anti-HLA blocking antibodies in IFN-γ ELISpot assays. CTL 9B10 was HLA-A02-restricted, whereas CTL 4E3 was restricted by HLA-B or -C alleles. Cross-reactivity tests showed recognition of one further AML, with only Cw07 as a matched HLA allele, suggesting HLA-Cw07 as the restriction element for CTL 4E3 (data not shown). CTL clone 9C12 was HLA-A02-restricted (figure 7 A) and showed strong lysis of primary AML blasts of patient MZ201, but only minor lysis of patient-derived PHA-activated T blasts or the NK cell target K562 (figure 7 B). Flow cytometric analysis of PHA-activated T blasts of patient MZ201 compared with primary AML blasts confirmed a comparably high cell surface expression of HLA-A02 (data not shown). Accordingly, the residual lysis (10-20%) of PHA-blasts of patient MZ201 appeared to be nonspecific, as it could not be explained by a decreased expression of the HLA restriction element A02. EBV-transformed B cells of patient MZ201 to be included in specificity testings were not available at that time point.

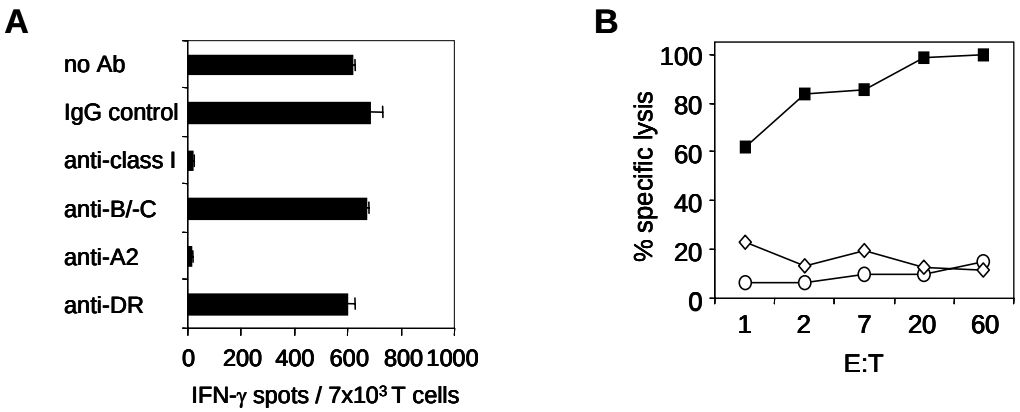

Figure 7. CTL clone 9C12 is HLA-A02 restricted and specifically lyses MZ201-AML blasts. CTL 9C12 generated by limiting dilution cloning of MLLC MZ201-AML/Donor168 was tested in an IFN-γ ELISpot assay using different anti-HLA blocking antibodies to determine the HLA-restricting element (A). Cytolytic activity of CTL 9C12 against patient-derived AML blasts (■), patient-derived PHA-activated T blasts (◇), and the NK cell target K562 (○) was tested in a 5 h ^{51}Cr-release assays (B). E:T, effector to target ratio

Proliferation of CTL clones isolated from MLLC MZ201-AML/Donor168 was measured weekly by cell counting. Total cell numbers of CTLs 9C12, 9B10, 4E3 and 1F12, determined from d33 to d61 after limiting dilution cloning, are illustrated in figure 8. Unfortunately, only one CTL (9C12) could be expanded to a cell number exceeding ten million (11,6x10^6 at day 47 after limiting dilution cloning). All other CTL clones did not reach cell numbers higher than two to three million. Attempts to further expand CTL clone 9C12 applying biotinylated antibodies against CD2, CD3, and CD28 in combination with anti-biotin MACSiBeads® (T cell activation/expansion kit, Miltenyi) were not successful. In conclusion, the traditionally used method of T cell limiting dilution cloning from bulk cultures was regarded as inappropriate for the *in vitro* generation and expansion of leukemia-reactive CD8$^+$ cytotoxic T lymphocytes from healthy donor/AML pairs.

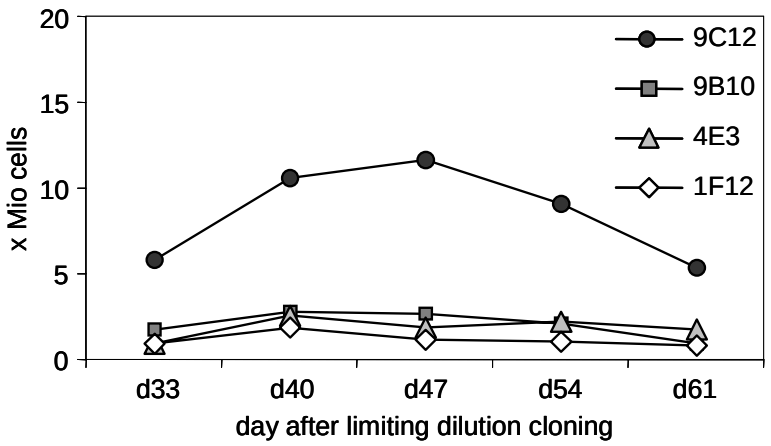

Figure 8: Weak proliferation of CTL clones derived from Donor168. CTLs were generated by limiting dilution cloning at day 28 of MLLC bulk culture. Cell numbers were determined by weekly counting when cells numbers reached the 24-well stage.

4.4 Characterization of AML-reactive CTL clones generated and expanded by a novel allo-MLLC protocol: mini-MLLC

4.4.1 Rationales for a new allo-MLLC protocol

The traditional strategy for the *in vitro* expansion of leukemia-reactive CTLs is to isolate them from peripheral blood samples drawn from leukemia patients after allogeneic HSCT (Den Haan et al, 1995; Wang et al, 1995; Dolstra et al, 1999; Vogt et al, 2000; Brickner et al, 2001). This procedure allows the *in vivo* priming of donor-derived CTL precursors against host leukemia antigens. A disadvantage of this method is that most patients have low T cell counts following transplantation due to continuous treatment with immunosuppressive drugs that impair T cell function. Another difficulty is to predict the exact time points when leukemia-reactive CTLs appear with high frequencies in peripheral blood. Traditionally used allo-MLLC protocols (Bleakley/Riddell, 2004) with subsequent limiting dilution cloning of leukemia-reactive CTLs *in vitro* are of relatively low efficiency, as also demonstrated in the MZ201-AML/Donor168 model (*chapter 4.3*). Those allo-MLLC bulk populations are largely unpredictable with regard to suppressive and stimulatory immune effects, may show changing anti-leukemia reactivities, or may be predominantly reactive against lymphoblastoid cell lines (LCLs) of patient and/or donor origin.

Due to these limitations of conventional protocols, it was the aim of this project to establish a new method that allows the efficient and reliable *in vitro* generation and expansion of AML-reactive $CD8^+$ cytotoxic T lymphocytes from healthy donors. Current allo-MLLC protocols were modified in the following aspects: As responder cells, naive $CD8^+$ T cells of healthy donors were used, selected by the expression of CD62L, with only 10^4 responder cells per well in 96-well microtiter plates ("mini-MLLC"). The rationale for this procedure was on one hand to achieve clonal T cell populations, since the precursor frequency of AML-reactive CTLs in healthy donor PBMCs is estimated to be lower than 10^{-5} (Smit et al, 1998). On the other hand, it was intended to create multiple different responder-stimulator compositions in replicate wells in order to provide for the growth of anti-leukemia CTLs optimized culture conditions by chance. Irradiated AML d1-blasts were regularly used as stimulator cells (10^4/well). In AML/sibling donor combinations, however, AML blasts were pre-incubated in medium containing the cytokine "cocktail" to further increase the expression of costimulatory and adhesion molecules (see *chapter 4.2*). Irradiated CD8-negative cells of the T cell donor served at day 0 as feeder cells (10^4/well). For an effective priming of naive $CD8^+$ T cells, IL-12 was administered to the culture until

day 14, and was then replaced by IL-2 for T cell expansion. The cytokines IL-7 and IL-15 were used throughout the culture period.

Since naive T cells can be characterized by the expression of several different cell surface markers (e.g. CD45RA, CD62L, CCR7), preliminary experiments were performed in order to establish an efficient strategy for the isolation of naive $CD8^+$ T cells from healthy donor PBMCs. Flow cytometry analyses of donor PBMCs in the MZ201-AML model showed that the expression of CD62L did not completely coincide with staining results for CD45RA (table 11). As the CD45RA-positive subset of $CD8^+$ T cells comprised much more cells than the CD62L-positive subset, it could not be excluded that the former also contained more differentiated $CD8^+$ T cells re-expressing CD45RA. Due to this observation, CD62L was chosen as the more suitable marker for isolating naive $CD8^+$ T cells from healthy donor PBMCs.

	Donor168	**Donor332**	**Donor650**
$CD3^+$ / $CD8^+$	27.0%	29.6%	24.6%
$CD8^+$ / $CD62L^+$	10.6%	10.8%	6.7%
$CD8^+$ / $CD45RA^+$	22.1%	24.5%	13.8%
$CD8^+$ / $CD45R0^+$	9.7%	8.4%	10.3%

Table 11. Flow cytometric analysis of healthy donor PBMCs. The percentages of naive and memory $CD8^+$ T cells in PBMCs of healthy donors were determined by staining with fluorochrome-conjugated mouse-anti-human mAbs against CD3, CD8, CD62L, CD45RA, and CD45R0.

Further experiments included the depletion of CD45R0-positive memory cells prior to the positive isolation of CD62L CD8 T cells. This three-step isolation strategy ("untouched" CD8 isolation / CD45R0 depletion / CD62L positive isolation) had the disadvantages of a reduced cell yield as well as increased stress for the isolated cell populations. Since this strategy also did not further improve the purity of the CD62L-enriched subset, the CD45R0-depletion step was omitted in cell separations for mini-MLLC experiments (data not shown).

The homing molecule CD62L is known for increased shedding with higher temperature, which would be disadvantageous for magnetic cell separation experiments. To analyze the impact of storage temperature – because healthy donor buffy coats might be stored over night before cell isolations – different buffy coat preparations were compared for CD62L expression after overnight storage at 4°C, 37°C, and at room temperature. These analyses confirmed that the CD62L cell surface expression decreases with increasing temperature (data not shown). In subsequent cell separation experiments, all isolation steps were

performed on ice and materials were stored at 4°C prior to use in order to avoid shedding of CD62L at higher temperature.

4.4.2 Separation of CD8$^+$ T cells into CD62L$^{(high)+}$ and CD62L$^{(low)+/neg}$ subsets

Allogeneic MLLCs were established using primary FAB M4 or M5 AML blasts as stimulator cells. The leukemic blasts were isolated from high-risk AML patients MZ201, MZ369, MZ418, MZ431, and MZ456. MLLC lymphocytes were peripheral blood CD8$^+$ T cells derived from healthy related and unrelated donors. CD8$^+$ T cells were obtained by negative isolation from healthy donor PBMCs using a cocktail of biotinylated mAbs against CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCRγδ, and CD235a. After incubation with anti-biotin MicroBeads, magnetically labeled non-CD8 cells were depleted on LD or LS columns (Miltenyi Biotec). Untouched CD8$^+$ T cells were subsequently enriched for CD62L-expressing cells (CD62L$^{(high)+}$ fraction), containing naive and central memory T cells. The negative fraction, containing effector memory T cells, showed CD62L$^{(low)+/neg}$ expression (figure 9). Purity of both fractions was determined by flow cytometry, as shown in figure 9 for Donor332.

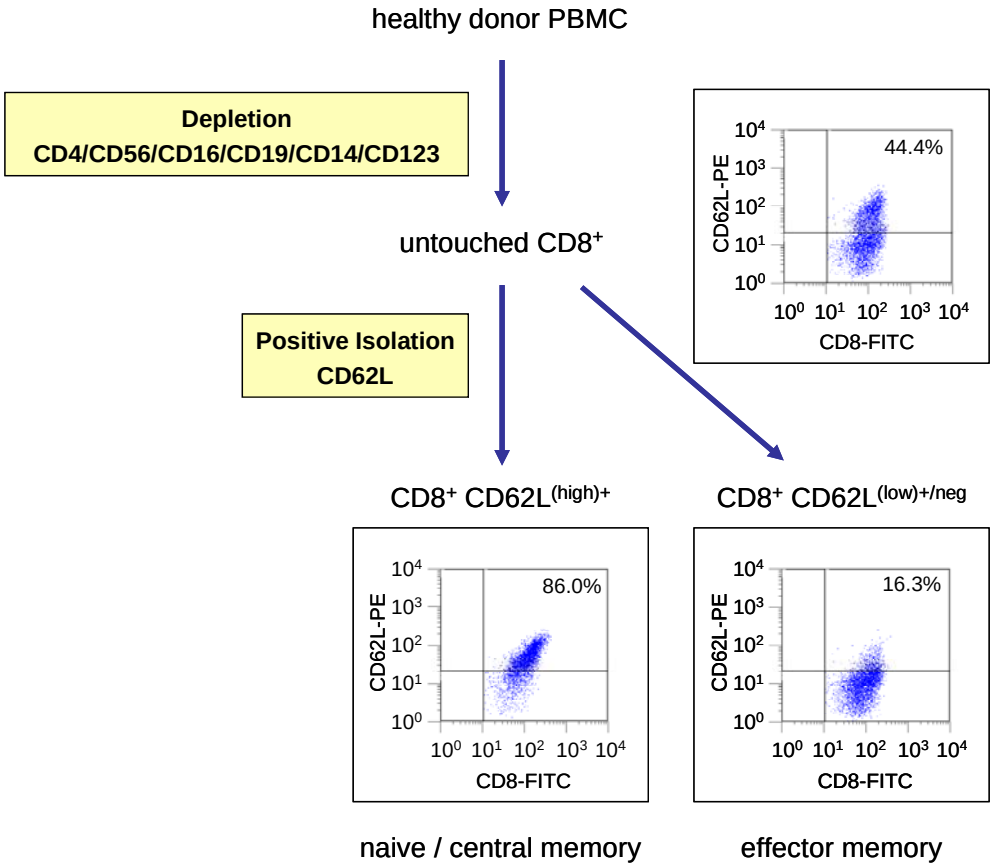

Figure 9. Isolation strategy for the separation of peripheral blood CD8$^+$ T cells according to CD62L expression. Healthy donor PBMCs were enriched for CD8$^+$ CD62L$^+$ cells by a two-step magnetic separation strategy. "Untouched" CD8$^+$ T cells, isolated in the first step by depletion of non-CD8 cells, were in the second step positively isolated for CD62L. The positive fraction was enriched for T cells that expressed CD62L at high level (CD62L$^{(high)+}$). The negative fraction contained T cells with either low or absent CD62L expression (CD62L$^{(low)+/neg}$). Representative flow cytometry data of healthy donor 332 are shown.

Further separation results are summarized in table 12. The differences in CD8/CD62L percentages between total lymphocyte populations and gating on CD8$^+$ cells can be attributed to the purity of the CD8$^+$ fraction after the first isolation step. The application of LS-columns for CD8 untouched isolation, as recommended by the manufacturer, yielded CD8$^+$ purities of only approx. 80%. In contrast, the use of LD depletion columns in the case of Donor332 resulted in a nearly pure CD8$^+$ fraction (>95%). But since the cell yield was significantly lower compared to LS-columns, the latter were used for the subsequent separations.

CD62L-enrichment resulted in almost pure positive fractions (approx. 90% in average). The negative fractions still contained significant amounts of CD62L$^+$ cells, which can be attributed to the positive isolation strategy that regularly favors cell enrichment over cell depletion. To further improve the removal of CD62L$^+$ cells from the negative fraction, the latter was in some experiments subjected to a second positive isolation step for CD62L.

		ungated lymphocyte population		gated on CD8$^+$ lymphocyte population	
		CD8$^+$ / CD62L$^{(high)+}$ fraction	CD8$^+$ / CD62L$^{(low)+/-}$ fraction	CD8$^+$ / CD62L$^{(high)+}$ fraction	CD8$^+$ / CD62L$^{(low)+/-}$ fraction
		% CD8 / CD62L positive cells			
MZ201	**Donor168**	78.2%	44.8%	92.5%	53.5%
	Donor332	82.8%	15.0%	86.0%	16.3%
	Donor650	82.1%	35.5%	93.3%	41.5%
MZ369	**SIB369**	90.2%	21.0%	99.9%	24.7%

Table 12. Purity of CD8$^+$ / CD62L$^{(high)}$ and CD8$^+$ / CD62L$^{(low)+/-}$ fractions. Results of flow cytometry analysis for CD8 and CD62L expression of both isolation subsets in the MZ201-AML and MZ369-AML models, gated on the complete lymphocyte population (left) and on CD8$^+$ lymphocytes (right), respectively.

Further flow-cytometric analysis of both subsets were performed to determine the co-expression of other cell surface markers, which were reported to characterize naive/central memory and effector memory CD8$^+$ T cells, respectively (Weninger et al, 2002; Sallusto et al, 2004). Co-staining results for Donor532 (MZ431-AML) are shown in figure 10. The CD62L$^{(high)+}$ fractions showed stronger co-expression of CCR7 and CD45RA compared with the CD62L$^{(low)+/neg}$ counterparts in flow cytometry, indicating an effective separation of naive/central memory and effector memory CD8$^+$ T cells.

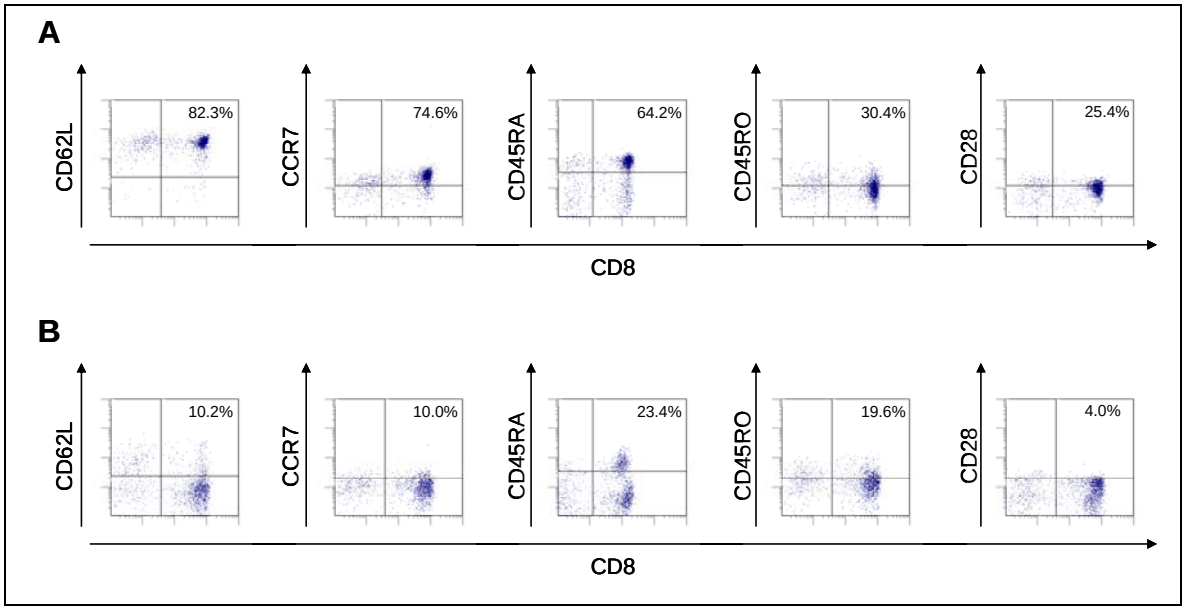

Figure 10. Expression of cell surface markers on CD62L$^{(high)+}$ and CD62L$^{(low)+/neg}$ subsets, characterizing naive/central memory and effector memory cells, respectively. The CD62L$^{(high)+}$ (A) and the CD62L$^{(low)+/neg}$ (B) subpopulations were stained in flow cytometry for co-expression of CCR7, CD45RA, CD45RO, and CD28 immediately after the isolation procedure. Isotype-matched IgG were used as controls. Results for Donor532 are shown.

Both subsets were subsequently used for the initiation of MLLCs. $CD62L^{(high)+}$ and $CD62L^{(low)+/neg}$ $CD8^+$ T cells were seeded at 10^4 and $5x10^5$ per well into 96-well mini-MLLC and 24-well maxi-MLLC plates, respectively.

4.4.3 Most AML-reactive $CD8^+$ T cells derive from $CD62L^{(high)+}$ precursors

MLLC responder populations were stimulated weekly with irradiated d1-AML blasts in fresh cytokine-containing medium. When necessary, mini-cultures were transferred at day 14 from U-bottom in flat-bottom 96-well plates. Reactivity against primary AML blasts was tested for the first time after 2 or 3 re-stimulations on d14+5 or d21+5, respectively, depending on the growth of mini-cultures. By application of split-well IFN-γ ELISpot assay, reactivity was determined with one part of the responder cells (approx. $2x10^4$ cells per replicate well) tested against d1-AML blasts; the same cell number was tested without target cells to determine spontaneous IFN-γ spot production. The residual cells were used for further expansion. Numerous mini-MLLC populations obtained from 96-well plates were strongly reactive with AML blasts as illustrated for the Donor332/MZ201-AML pair in figure 11 A.

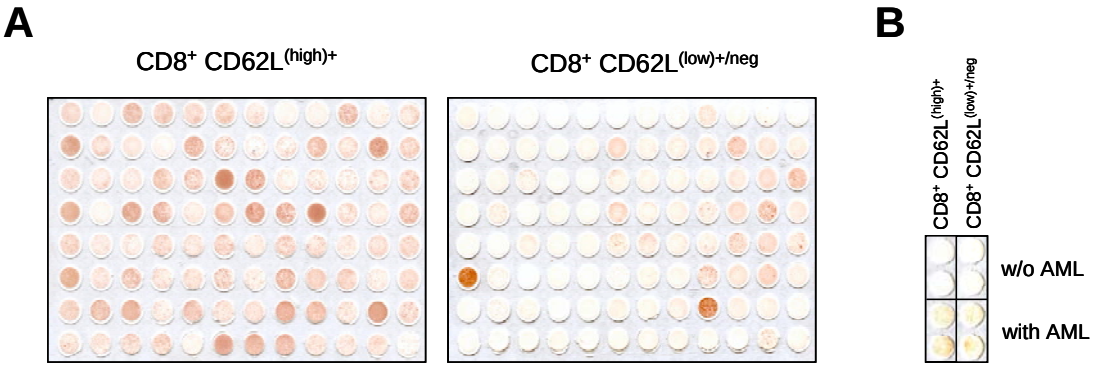

Figure 11. AML reactivity of mini- and maxi-MLLC responder populations in IFN-γ ELISpot assay. $CD62L^{(high)+}$ and $CD62L^{(low)+/neg}$ $CD8^+$ T cells were weekly stimulated with primary AML blasts in 96-well mini-MLLCs and 24-well maxi-MLLCs, respectively. AML reactivity of individual culture units was determined on day 19 or day 26 by split-well IFN-γ ELISpot assay. (A) Original ELISpot results obtained with an averaged number of $2x10^4$ AML-stimulated mini-MLLC responder cells per test unit in the Donor332/MZ201-AML pair are illustrated. AML reactivity of each individual culture unit was calculated by subtracting background spot numbers (not shown) from spot numbers obtained upon AML stimulation. Summary of evaluated data from the Donor332/MZ201-AML pair is included in figure 12. (B) Original ELISpot results obtained with $2x10^4$ maxi-MLLC responders per test unit in the Donor332/MZ201-AML pair. The numbers of AML-reactive spot-producing cells were lower than 200 per $2x10^4$ for both populations.

Up to one-third of seeded mini-cultures contained more than 500 spot-producing lymphocytes above background. The number of AML-reactive responder populations was higher in plates initiated with $CD62L^{(high)+}$ compared with $CD62L^{(low)+/neg}$ $CD8^+$ T cells.

This result was consistently observed in 8 out of 8 donor/AML combinations analyzed (figure 12), with IFN-γ spot numbers between 200 and 1000 over background per $2x10^4$ seeded effector cells. In contrast, maxi-MLLC populations generated from $CD62L^{(high)+}$ and $CD62L^{(low)+/neg}$ $CD8^+$ T cells in 24-well plates demonstrated an overall lower AML reactivity compared with the strong anti-leukemia IFN-γ spot production by individual mini-cultures. Representative data obtained from the Donor332/MZ201-AML pair are shown in figure 11.

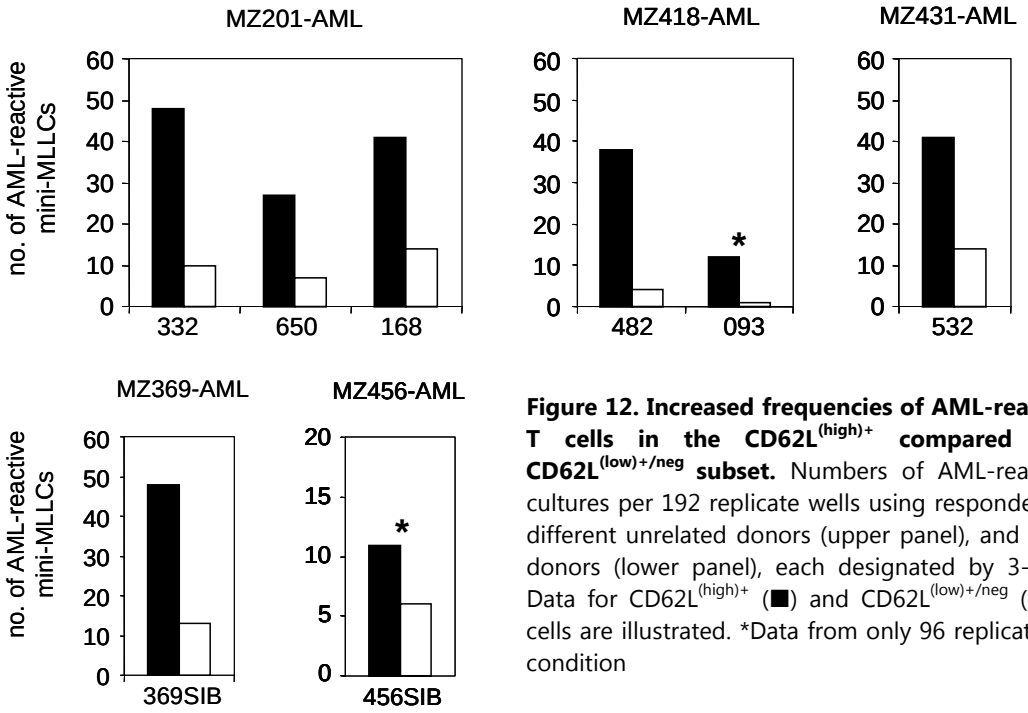

Figure 12. Increased frequencies of AML-reactive $CD8^+$ T cells in the $CD62L^{(high)+}$ compared with the $CD62L^{(low)+/neg}$ subset. Numbers of AML-reactive mini-cultures per 192 replicate wells using responder cells of 6 different unrelated donors (upper panel), and two sibling donors (lower panel), each designated by 3-digit code. Data for $CD62L^{(high)+}$ (■) and $CD62L^{(low)+/neg}$ (□) $CD8^+$ T cells are illustrated. *Data from only 96 replicate wells per condition

The AML reactivity of maxi-MLLC responders varied considerably in weekly re-analyses. Several populations developed an attenuated anti-leukemia response during prolonged culture. Most of them were cross-reactive with B-LCL of donor and patient origin, as shown for Donor650 (system MZ201-AML) in figure 13.

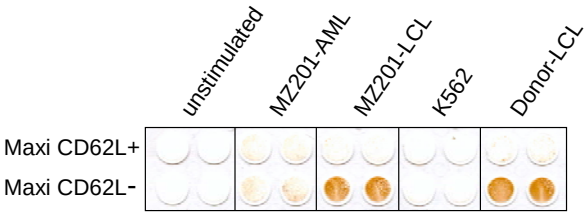

Figure 13. Reactivity of bulk cultures derived from the Donor650/MZ201-AML pair. Maxi-MLLC responders ($5x10^4$/well) were tested at day 14+5 of culture in an IFN-γ ELISpot assay for recognition of leukemic blasts of patient MZ201-AML, patient- and donor-derived EBV-transformed B cell lines, and NK target K562.

4.4.4 Cytolytic activity of mini-MLLC responders

A total of 246 AML-reactive mini-MLLC populations were transferred into 24-well plates and were stimulated weekly with AML blasts. Eighty-four of them could be further expanded and retained AML reactivity in confirmatory ELISpot tests. Due to the limited availability of leukemia material, only 26 populations were subsequently expanded in large-scale cultures. They showed intermediate to high levels of cytolytic activity against primary AML blasts in ^{51}Cr-release assays, as illustrated for Donor332 and Donor650 (stimulated with MZ201-AML) in figure 14, and for the sibling donor 369-SIB (stimulated with MZ369-AML) in figure 16. These AML-reactive CTL populations did not lyse natural killer target K562 and B-LCLs of patient and donor origin, which excluded that they contained significant numbers of natural killer and alloreactive effector cells.

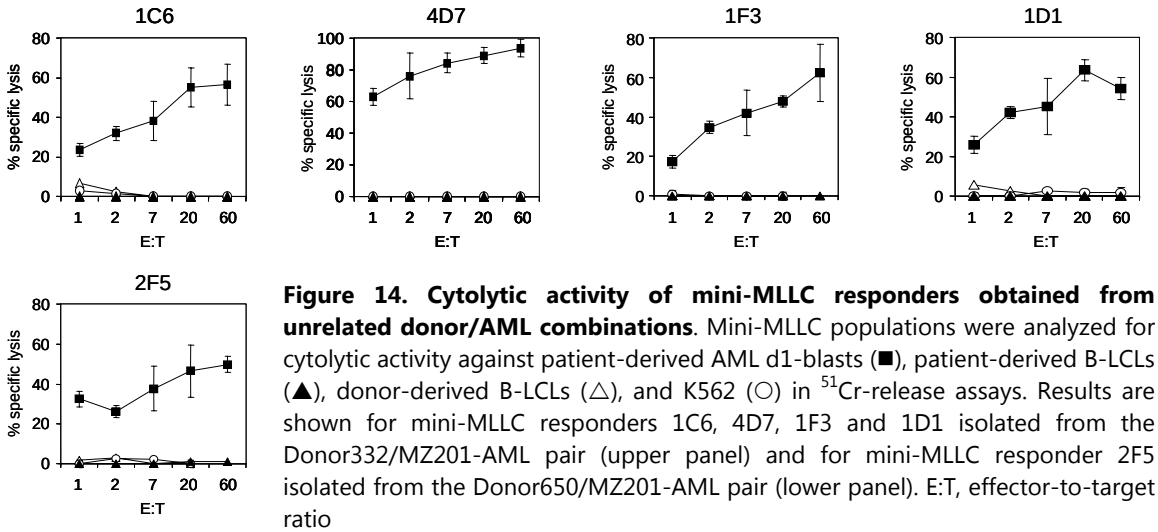

Figure 14. Cytolytic activity of mini-MLLC responders obtained from unrelated donor/AML combinations. Mini-MLLC populations were analyzed for cytolytic activity against patient-derived AML d1-blasts (■), patient-derived B-LCLs (▲), donor-derived B-LCLs (△), and K562 (○) in ^{51}Cr-release assays. Results are shown for mini-MLLC responders 1C6, 4D7, 1F3 and 1D1 isolated from the Donor332/MZ201-AML pair (upper panel) and for mini-MLLC responder 2F5 isolated from the Donor650/MZ201-AML pair (lower panel). E:T, effector-to-target ratio

Mini-MLLC populations derived from Donor168, which was already used for isolating CTL clones by conventional limiting dilution cloning from T cell bulk cultures (*chapter 4.3*), were also tested in cytotoxicity assays (figure 15). CTL population 1H9 showed intermediate specific lysis of patient-derived AML blasts, but not of patient- or donor-derived B-LCLs or K562 cells. In contrast, mini-MLLC population 1E1 demonstrated also minor lysis against the B-LCLs. It was possible to expand Donor168-derived mini-MLLC populations to 21.4x10^6 (1H9) and 3.3x10^6 (1E1) cells, clearly exceeding numbers obtained by the traditionally used limiting dilution cloning procedure in this donor (figure 8).

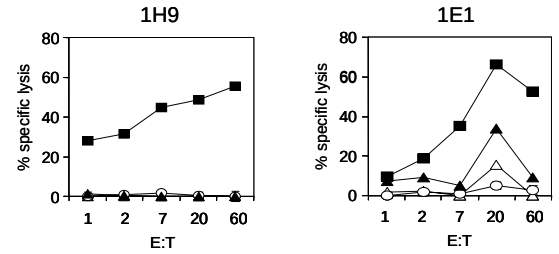

Figure 15. Cytolytic activity of mini-MLLC responders isolated from the Donor168/ MZ201-AML pair. Mini-MLLC responder populations 1H9 and 1E1 were analyzed for cytolytic activity against patient-derived AML d1-blasts (■), patient-derived B-LCLs (▲), donor-derived B-LCLs (△), and K562 (○) in ^{51}Cr-release assays. E:T, effector-to-target ratio

The CTL 1E3 isolated by mini-MLLC from the sibling donor of patient MZ369-AML mediated stronger cytolytic activity against AML blasts isolated from patient MZ369 following terminal leukemia relapse, compared with original AML blasts used for primary stimulation (figure 16). In the same donor/AML pair, CTL 1E7 showed minor if any cytotoxicity against relapsed AML compared with primary AML (figure 16). This indicated that leukemia cells can simultaneously up-regulate and down-regulate individual CTL target structures (Dermime et al, 1997). The impact of the cytokine "cocktail" used during the pre-incubation phase of AML blasts on the expression on HLA-restricting elements was analyzed in more detail in *chapter 4.4.5*.

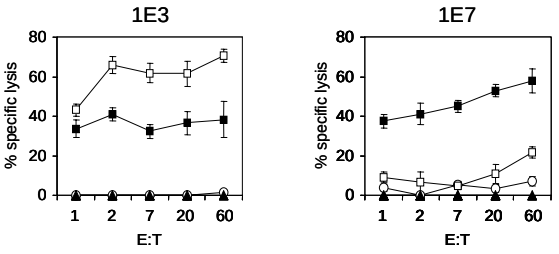

Figure 16. Cytolytic activity of mini-MLLC responders derived from sibling donor/MZ369-AML combination. Mini-MLLC responder populations 1E3 and 1E7 were analyzed for cytolytic activity against patient-derived primary MZ369-AML blasts (■), AML blasts isolated from patient MZ369 during post-transplant leukemia relapse (□), patient-derived B-LCLs (▲), donor-derived B-LCLs (△), and K562 (○) in ^{51}Cr-release assays. Leukemic blasts were pre-incubated for 24 hours in medium supplemented with the cytokine "cocktail". E:T, effector-to-target ratio

4.4.5 HLA class I–restriction and cross-reactivity pattern of mini-MLLC-derived CTLs

To identify restricting HLA alleles of mini-MLLC-derived CTLs, AML reactivity was determined in the presence of mAbs that bind to allele-specific or shared HLA determinants. As mAbs with blocking activity for each individual HLA class I allele were not available, CTLs were also analyzed for cross-reactivity with primary myeloid and lymphatic leukemia cells. These targets were matched with the original AML stimulator cells for single HLA class I alleles.

Of the 26 AML-reactive CTL populations analyzed, HLA-A2, HLA-B56, HLA-A24, HLA-Cw12, and HLA-Cw7 were identified as the restricting HLA alleles. Except for the latter allele, representative examples are illustrated in figures 17-19. The AML-reactive CTLs cross-reacted with a significant proportion of acute and chronic myeloid leukemias carrying the respective HLA alleles. In accordance with the failure to recognize donor and patient derived B-LCLs, these CTLs did not react with HLA-matched B-cell leukemias. The target cell panel also included monocytes, monocyte-derived mature dendritic cells, granulocytes, PHA-activated T cell blasts, and primary bone marrow derived stromal fibroblasts. Of the tested CTLs, only 4D7 showed low reactivity with IFN-γ treated fibroblasts that were generated from bone marrow stroma cells of the corresponding AML patients *in vitro* (figure 18).

CTL 1C6 was restricted by HLA-A*0201, as determined by HLA-blocking in IFN-γ ELISpot assay (figure 17 A). Cross-reactivity analysis showed a superior recognition of HLA-A02-positive myeloid leukemias, whereas acute and chronic lymphoid leukemias were not recognized (figure 17 B). In total, 11 of 19 tested primary AML blasts and 2 of 5 tested primary CML cells of HLA-A*0201-positive patients were recognized by CTL 1C6. Reactivity of CTL 1C6 against monocytes was observed at very low level (figure 17 C).

The identification of the peptide epitope recognized by CTL 1C6 – performed in a separate project – suggested a myeloid leukemia-associated antigen with some co-expression in lung tissues. For that reason, the HLA-A*0201-positive lung carcinoma cell lines MZ6-LC and MZ16-LC were included as target cells, but were not recognized by CTL 1C6 in IFN-γ ELISpot assay (not shown).

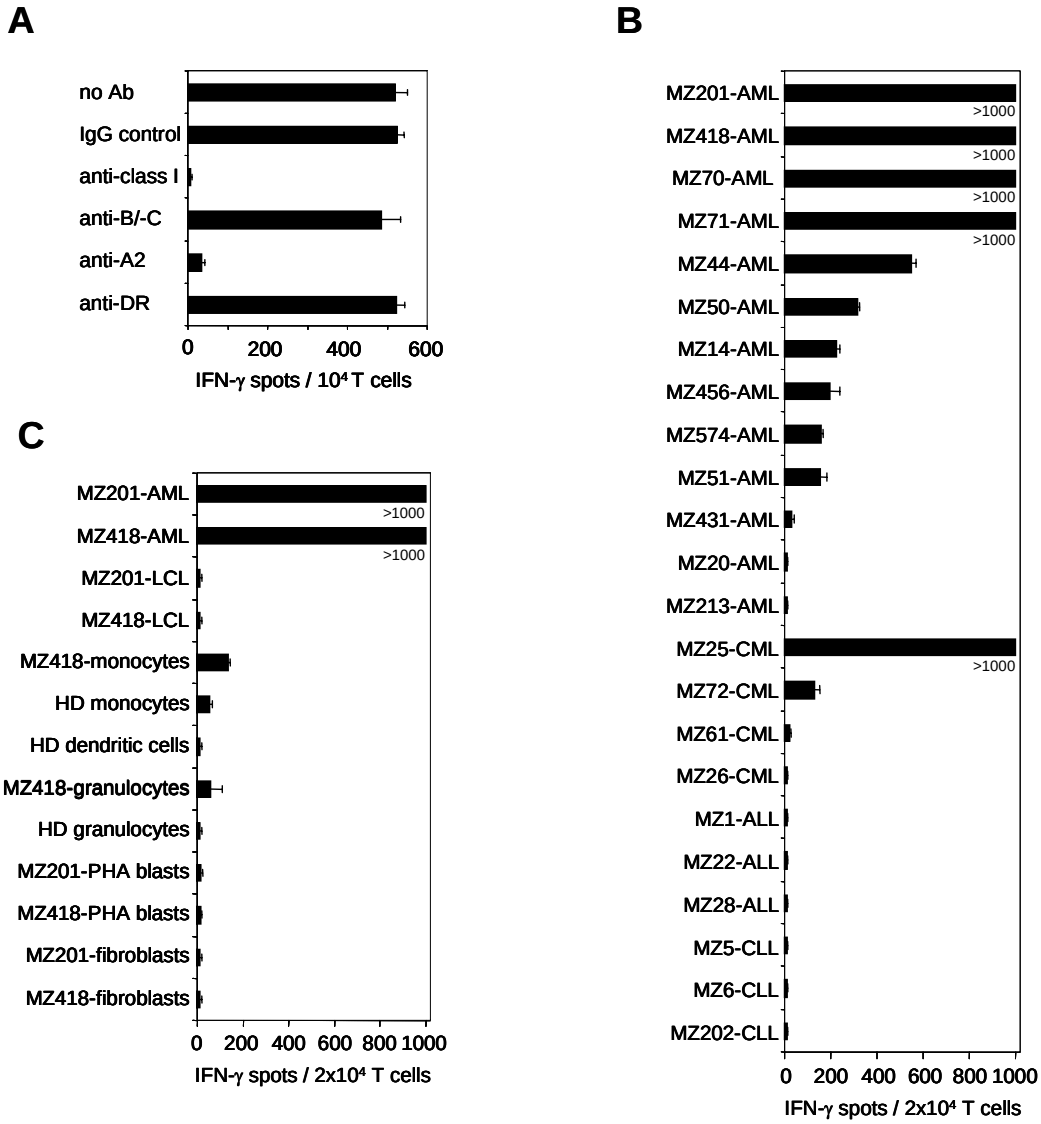

Figure 17. CTL 1C6 recognizes a myeloid leukemia-associated antigen in association with HLA-A*0201. AML-reactive CTL 1C6 generated by mini-MLLC from the Donor332/MZ201-AML pair was analyzed in IFN-γ ELISpot assays for blocking by anti-HLA antibodies and for cross-reactivity with partially HLA compatible cells. The shared HLA class I type of Donor332 and patient MZ201 was A*0101/*0201, B*0801/*5601, Cw*0102/*0701. (A) Effect of mAbs blocking total HLA class I, total HLA-B/-C, HLA-A2, or HLA-DR on CTL-mediated recognition of primary MZ201-AML blasts. (B) Cross-reactivity pattern against a panel of primary myeloid and B-cell leukemia cells. All targets expressed HLA-A*0201, except for the HLA-A*0202-positive MZ25-CML. (C) Cross-reactivity pattern against HLA-A*0201-positive B-LCLs, monocytes, monocyte-derived mature dendritic cells, granulocytes, PHA blasts, and bone marrow-derived stromal fibroblasts. Monocytes and granulocytes of patient MZ418 were isolated when AML was in complete remission. Monocytes and granulocytes of patient MZ201 were not available. CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; HD, healthy donor

CTL clone 4D7, also isolated from mini-MLLC of the Donor332/MZ201-AML combination, was clearly restricted by a shared HLA-B or -C allele, as recognition of MZ201-AML blasts could by blocked by using a mAb against a common epitope of HLA-B and C-alleles in IFN-γ ELISpot assay (figure 18 A). Cross-reactivity testings against several myeloid and

lymphoid leukemias expressing shared HLA-B/-C alleles revealed HLA-B*5601 (member of the Bw22 family) as the restriction element for CTL 4D7 (figure 18 B). This could be confirmed by results obtained from cDNA expression cloning, which was performed in a separate project to identify the peptide epitope recognized by CTL 4D7 (data not shown).

While CTL 4D7 did not react with autologous monocytes, it showed significant recognition of monocytes from 1 out of 2 further HLA-B*5601-positive healthy donors tested (Donor650, figure 18 B). In order to further analyze the expression pattern of the epitope recognized by CTL 4D7, PBMC subpopulations ($CD19^+$, B cells; $CD14^+$, monocytes; $CD3^+$, T cells) of Donor650 were tested as targets in an ELISpot assay (figure 18 C). This experiment demonstrated that only $CD14^+$ monocytes, but not B cells, T cells or residual PBMCs of Donor650 were recognized, suggesting an monocytic antigen as the target structure of CTL 4D7. Since AML blasts were recognized to much higher levels compared with monocytes, the antigen seems to be over-expressed in AML. HLA-Bw22-positive melanoma cell lines were not recognized by CTL 4D7 (data not shown).

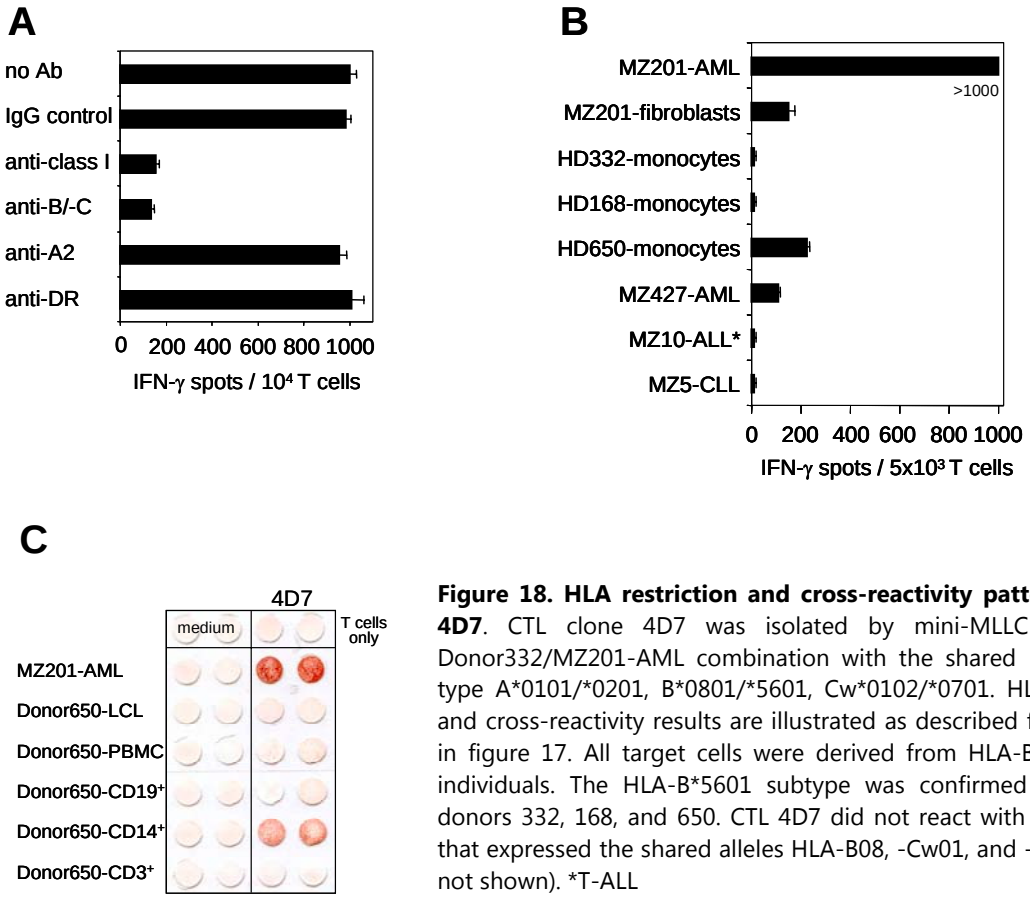

Figure 18. HLA restriction and cross-reactivity pattern of CTL 4D7. CTL clone 4D7 was isolated by mini-MLLC from the Donor332/MZ201-AML combination with the shared HLA class I type A*0101/*0201, B*0801/*5601, Cw*0102/*0701. HLA blocking and cross-reactivity results are illustrated as described for CTL 1C6 in figure 17. All target cells were derived from HLA-B56-positive individuals. The HLA-B*5601 subtype was confirmed in healthy donors 332, 168, and 650. CTL 4D7 did not react with target cells that expressed the shared alleles HLA-B08, -Cw01, and -Cw07 (data not shown). *T-ALL

Mini-MLLCs initiated in the unrelated donor/MZ574-AML combinations that later on proved to be mismatched for Cw07 suballeles (patient MZ574: Cw*0701; Donor247 and Donor172: Cw*0702) yielded multiple responder populations with alloreactivity against the mismatched Cw07-allele, as confirmed by analyzing the recognition of COS7 cells transfected with Cw*0701 (data not shown). Nevertheless, it was possible to isolate $CD8^+$ T cell populations by mini-MLLC that were restricted by the shared allele A*0101 and that showed specific reactivity against patient's AML blasts at intermediate level (data not shown).

In the sibling donor/MZ369-AML pair with the shared HLA class I type A01/24, B18/38, Cw07/12, the mini-MLLC derived CTL clones 1E3 and 1E7 were characterized for HLA restriction and cross-reactivity patterns (figure 19).

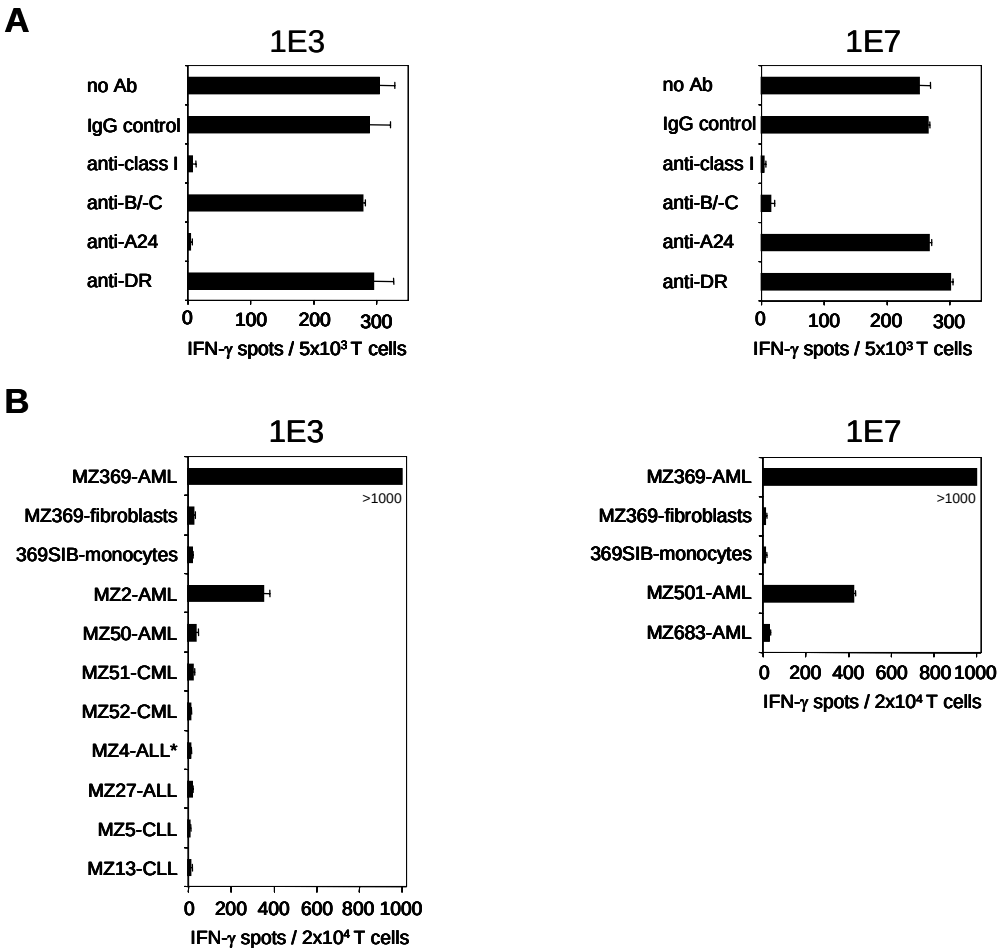

Figure 19. HLA restriction and cross-reactivity pattern of CTLs 1E3 and 1E7. CTL clones 1E3 and 1E7 were isolated by mini-MLLC from the sibling donor/MZ369-AML combination with the shared HLA class I type A01/24, B18/38, Cw07/12. HLA blocking (A) and cross-reactivity results (B) are illustrated as described for CTL 1C6 in figure 17. (B, left panel) All target cells expressed HLA-A24. Monocytes of patient MZ369 were not available. (B, right panel) All target cells expressed HLA-Cw12. CTL 1E7 did not recognize target cells expressing the shared alleles HLA-B18, -B38, and -Cw07 (data not shown). *T-ALL

CTL 1E3 was restricted by HLA-A24, as demonstrated in an IFN-γ ELISpot assay using a mAb for blocking HLA-A24 on MZ369-AML blasts as target cells (figure 19 A, left). One further AML, only matched for HLA-A24, was also recognized by 1E3 (figure 19 B, left). CTL 1E7 was restricted by a shared B- or C-allele, as demonstrated by a complete blocking of MZ369-AML as targets, when a mAb specific for a common epitope of HLA-B and -C alleles was added to the assay medium (figure 19 A, right). With the use of leukemic cells, which were only matched with the AML/sibling donor combination for single HLA-B/-C alleles, CTL 1E7 showed cross-reactivity against the Cw12-positive MZ501-AML (figure 19 B, right). This confirmed that HLA-Cw12 was the HLA restriction element of CTL clone 1E7. Patient-derived primary stromal fibroblasts or autologous monocytes were neither recognized by CTL 1E3 nor by 1E7.

As already mentioned, MZ369-AML blasts used for restimulation and reactivity testing of mini-MLLC responders, were regularly pre-incubated overnight in medium containing the cytokine "cocktail". We further investigated if this cytokine combination would have an influence on CTL-mediated recognition of leukemic blasts isolated from primary AML and AML relapse (figure 20). The HLA-A24-restricted CTL clone 1E3 recognized cytokine-pretreated relapsed AML even stronger than primary AML blasts, as already observed in the lysis assay (figure 16). Omitting the cytokines did not significantly influence the strength of primary MZ369-AML blast recognition by CTL 1E3. In contrast, blasts from relapsed AML were only recognized when these target cells were pre-incubated with the cytokine combination. The same phenomenon was observed when CTL 1E7 was used as effector cells. Reactivity of 1E7 in IFN-γ ELISpot assay was much stronger against leukemic blasts of the primary AML compared to the AML relapse, confirming the result of the ^{51}Chromium release assay (figure 16). Application of the cytokine cocktail did not change the reactivity of 1E7 against primary AML blasts of patient MZ369, but recognition of relapsed AML by 1E7 decreased remarkably when target cells were not pre-treated with the cytokines (figure 20).

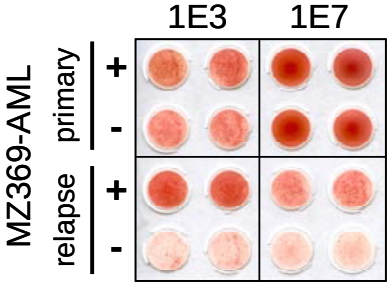

Figure 20. Differential recognition of primary and relapsed MZ369-AML blasts with or without cytokine "cocktail" pre-incubation. The mini-MLLC-derived HLA-A24 and -Cw12 restricted CTL clones 1E3 and 1E7 ($2x10^4$/well) were tested in an IFN-γ ELISpot assay for recognition of primary and relapsed AML blasts of patient MZ369, that had been pre-cultured for 24h in medium with (+) or without (-) the cytokine cocktail (IL-4, GM-CSF, TNF-α, SCF).

Immunophenotypic analyses were performed to investigate a possible effect of the pre-incubation cytokine "cocktail" on the cell surface expression of HLA class I alleles on blasts of the primary AML and the AML relapse. Staining of cytokine-pretreated cells using an HLA-A24-specific mAb showed a selectively up-regulated surface expression for HLA-A24 on relapsed MZ369-AML compared with primary MZ369-AML blasts (figure 21). This observation might explain the enhanced recognition of relapsed AML by the HLA-A24-restricted CTL 1E3 in the lysis assay and the IFN-γ ELISpot assay (figures 16, 20). Due to the unavailability of an allele-specific mAb, it was not possible to perform a similar study on HLA-Cw12, which was used by CTL 1E7 as the restricting HLA element (figure 19). Flow cytometric analysis using a mAb against a common epitope of HLA-B and -C alleles did not show a significant difference in cell surface expression of these HLA alleles on primary and relapsed MZ369-AML blasts, both pre-incubated with cytokines (figure 21).

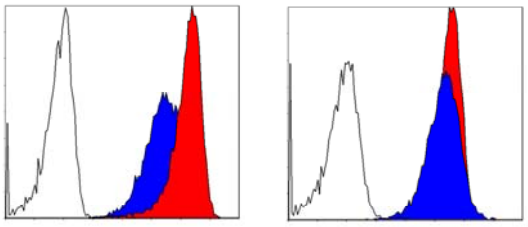

Figure 21. Expression of HLA class I molecules on MZ369-AML. Primary (blue) and relapsed (red) AML blasts of patient MZ369 were pre-incubated for 24 h in medium containing the cytokine "cocktail" (IL-4, GM-CSF, SCF, TNF-α), and analyzed in flow cytometry using mouse-anti-human mAbs against HLA-A24 and against a common epitope of HLA-B and -C alleles in combination with a PE-conjugated goat-anti-mouse IgG. An isotype-matched mAb (continuous line) was used as control.

4.4.6 TCR Vβ chain analysis of mini-MLLC populations

It has been previously demonstrated that the precursor frequency of leukemia-reactive $CD8^+$ CTLs is lower than 10^{-5} in PBMCs of healthy individuals (Smit et al, 1998). This observation suggests that AML-reactive CTLs growing from a pool of 10^4 *ex vivo* $CD8^+$ T cells should be of clonal origin. To investigate this hypothesis, mini-culture derived CTL populations were characterized using TCR Vβ family-specific mAbs in flow cytometry. This analysis demonstrated that mini-MLLC-derived CTLs expressed TCR Vβ chains of single Vβ families, indicating their clonality (figure 22).

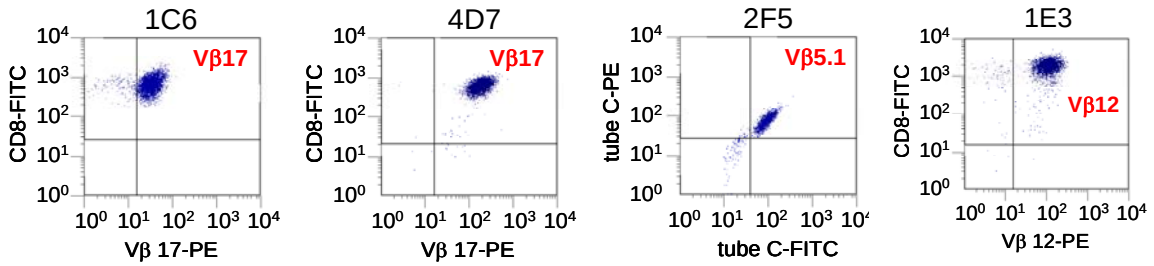

Figure 22. AML-reactive CTLs use TCR Vβ chains of single Vβ families. Multiparametric flow cytometry analysis with mAbs recognizing 24 distinct TCR Vβ families (BetaMark Kit, Beckman Coulter) was applied to determine Vβ usage of CTLs 1C6, 4D7, 2F5 (stimulated with MZ201-AML), 1E3, and 1E7 (stimulated with MZ369-AML) between d42 and d63 of cultures. Confirmatory test results by co-staining CTLs with mAbs specific for individual Vβ families and for CD8 are shown, except for CTL clone 2F5, since there was no anti-Vβ5.1 mAb available. The TCR Vβ chain of CTL 1E7 could not be detected by this approach.

In further staining analyses, AML-reactive CTLs were CD45RA$^-$, CD45RO$^+$, CCR7$^{(low)+/neg}$, CD27$^-$, CD28$^-$, CD25$^+$, and CD69$^+$, consistent with the phenotype of activated mature effector cells. Long-term cultured CTLs showed down-regulation of CD62L expression, as shown for CTL 1C6 (MZ201-AML/Donor332) in figure 23. In contrast, sustained expression of CD62L was observed on CTL 4D7 (not shown).

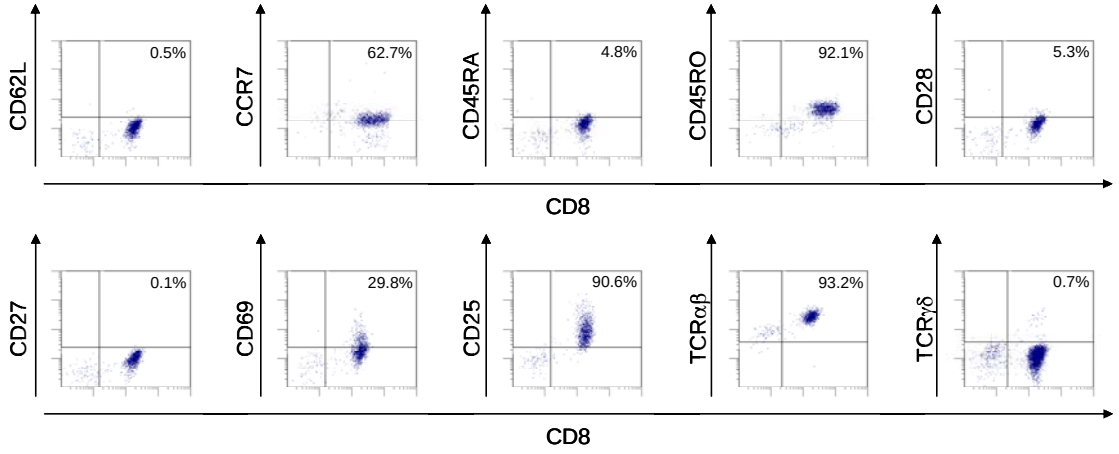

Figure 23. Flow-cytometric analysis of long-term cultured CTL clone 1C6. Co-staining of mini-MLLC-derived CTL clone 1C6 at day 63 of culture with mAbs against CD8 and cell surface markers distinguishing naive and memory cells (CD62L, CCR7, CD45RA, CD45R0, CD27, CD28), activation markers (CD69, CD25) and mAbs against αβ- and γδ-TCR, respectively.

4.4.7 Expansion of AML-reactive CTL clones by antigen-specific stimulation

Mini-culture derived CTLs were amplified by weekly adding fresh irradiated AML blasts and cytokines. Mitogenic agents such as PHA and anti-CD3 antibodies, or feeder cells of other allogeneic donors were not used to further augment T cell proliferation. The CTL populations demonstrated a vigorous and durable expansion, as determined by calculating the proliferation factor in weekly restimulations. More than 10^8 to 10^9 cells could be expanded by antigen-specific stimulation within 6 to 8 weeks, if total cell amounts were restimulated each week (figure 24).

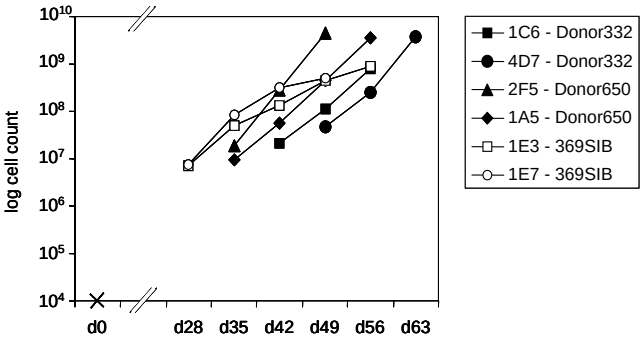

Figure 24. Proliferation of mini-MLLC populations. Proliferation was determined by calculating the proliferation factors in weekly restimulations. Shown are theoretically possible total cell numbers, if expanded cells were totally restimulated each week. Due to limited amounts of AML material, expanded CTLs were frozen in aliqots after reaching cell numbers of approx. $2x10^8$.

Several mini-MLLC derived CTL clones were expanded to cell numbers sufficient for identification of CTL-defined antigens by cDNA expression cloning. CTLs were frozen and stored in aliquots, when cell numbers of approximately $2x10^8$ were reached (table 13). Upon thawing and stimulation with AML blasts, CTLs retained growth, IFN-γ spot production, and cytolytic activity already in the first week of re-culture.

In a separate project, performed in collaboration with Dr. Catherine and Prof. Dr. Thomas Wölfel, Mainz, the peptide epitope of CTL clone 4D7 has been successfully defined by cDNA expression cloning. We are currently in the process to identify the peptide epitope of CTL clone 1C6 using the same procedure.

	Mini-MLLC-derived CTL clone	HLA-restriction element	frozen
MZ201-AML / Donor332	1C6 ✓	A*0201	$>2x10^8$
	4D7 ✓	B*5601	$>2x10^8$
	1F3	B / C	$2.4x10^8$
	2H5*	B / C	$>2x10^8$
	4E6	B / C	$1.7\ x10^8$
	4C11	A02	$8x10^7$
	4B12	B / C	$2x10^8$
MZ201-AML / Donor650	2F5	B / C	$3x10^8$
	1A5	B / C	$1.2x10^8$
	3F10	B / C	$2.6x10^8$
	3B12	B / C	$1x10^8$
MZ369-AML / SIB	1E3	A24	$6x10^7$
	1E7	Cw12	$1.2x10^8$

Table 13. Mini-MLLC-derived CTL clones expanded from unrelated donor/MZ201-AML and sibling donor/MZ369-AML combinations. Shown are the HLA-restricting elements, as determined by anti-HLA blocking ELISpot assays or cross-reactivity tests with partially matched myeloid leukemias, and the amount of cryo-preserved cells. ✓, CTL clones 1C6 and 4D7 were already used for antigen identification by cDNA expression cloning. *, CTL 2H5 seems to recognize the same peptide epitope as CTL 4D7

4.5 Adoptive transfer experiments in NOD/SCID IL2Rγ^{null} mice

4.5.1 AML-reactive CD8$^+$ CTL clones prevent AML engraftment in NOD/SCID IL2Rγ^{null} mice

To investigate the biological significance of the mini-MLLC derived AML-reactive CTL clones, their capability to inhibit the engraftment of human primary AML blasts in NOD/SCID IL2Rγ^{null} mice was analyzed (Shultz et al, 2005; Shultz et al, 2007). These mice have been reported to show a superior engraftment for human hematopoietic stem cells compared with traditionally used NOD/SCID mice (Ito et al, 2002; Shultz et al, 2005). In preliminary experiments, different doses of primary AML blasts were tested for leukemia engraftment. It was observed that a dose of 5x10^6 primary AML blasts per mouse was sufficient to obtain leukemic engraftment during 4 to 8 weeks, as determined by flow cytometry and PCR analysis of peripheral blood (data not shown). Subsequently, it was investigated whether the anti-MZ201-AML CTL clones 1C6 and 4D7 could inhibit AML engraftment in these mice. Using an experimental procedure, similar as previously described (Bonnet et al, 1999), MZ201-AML blasts were cultured for 20 hours *in vitro* in medium alone, with AML-reactive CTLs, or with anti-melanoma CTLs to exclude non-specific effects of T cells on leukemic engraftment. The mixtures were then inoculated into cohorts of sublethally irradiated NOD/SCID IL2Rγ^{null} mice. The numbers of mice receiving AML blasts without CTLs, with anti-melanoma CTLs, with CTL 1C6, and with CTL 4D7 were 5, 3, 2, and 3, respectively. Eight weeks after inoculation, the AML engraftment was evaluated by sacrificing the mice and analyzing peripheral blood, spleen, and bone marrow samples for human CD33$^+$, CD34$^+$ and CD45$^+$ CD8$^-$ cells using flow cytometry, and for human chromosome 17 DNA by PCR analysis.

Human cells were detected in 4 out of 5 mice inoculated with AML blasts that had been cultured without CTLs, and in 3 out of 3 mice receiving AML blasts pre-incubated with melanoma-reactive CTLs (figure 25). The proportion of human CD45$^+$ CD8$^-$ cells in peripheral blood, spleen, and bone marrow ranged from 10-22% of total cells (figure 25 A). CD45$^+$ CD8$^-$ human cells detected in mice did not show co-expression of CD33 or CD34 as it would have been expected for MZ201-AML blasts, suggesting a down-regulation of these myeloid markers on leukemic cells upon inoculation in mice.

In addition to leukemic blasts, individual mice also contained a small fraction of human $CD8^+$ cells (range, 1-4%) that most likely represented residual $CD8^+$ T cells from the primary AML preparation. In contrast, human cells were detected in none of the NOD/SCID IL2Rγ^{null} mice inoculated with AML blasts that had been cultured with anti-leukemic CTL clones 1C6 or 4D7, neither by flow cytometry nor by PCR analyses (figure 25). These results indicated that the AML cells had been completely eliminated by the leukemia-reactive CTLs.

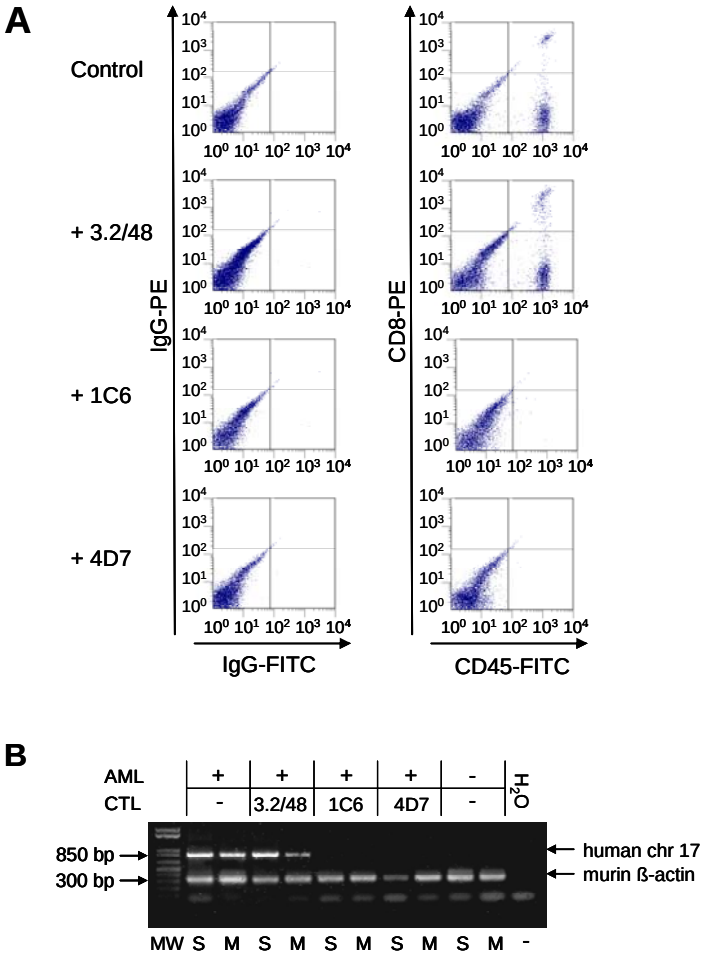

Figure 25. $CD8^+$ CTL clones 1C6 and 4D7 inhibit AML engraftment in NOD/SCID IL2Rγ^{null} mice. Spleen and bone marrow cells were isolated 8 weeks after transplant from NOD/SCID IL2Rγ^{null} mice receiving 5x10^6 MZ201-AML blasts that had been cultured in either medium alone (control), with anti-melanoma CTL clone 3.2/48, or with anti-MZ201-AML CTL clones 1C6 or 4D7 at a CTL/AML cell ratio of 10:1. Representative results obtained from a single mouse per each condition are shown. (A, right panel) Flow cytometric analysis for human $CD45^+$ and $CD8^+$ cells in the spleen of transplanted mice. (A, left panel) Staining results using isotype control mAbs. (B) Detection of human-specific DNA within spleen (S) and bone marrow (M) cells of NOD/SCID IL2Rγ^{null} recipients by PCR amplifying an 850 bp fragment of human chromosome (chr) 17. The annotation above each lane indicates whether the mouse had received AML cells cultured in medium alone or with CTL clones. DNA prepared from corresponding samples of untreated mice was included as control. Primers specific for the murine β-actin gene were used to amplify a 330 bp fragment as internal control.

5. DISCUSSION

The major limitations of donor lymphocyte infusions after allogeneic hematopoietic stem cell transplantation of relapsed acute leukemias are the insufficient graft-versus-leukemia effect and the high incidence of graft-versus-host disease (Kolb et al, 2004). In the present work, a new allo-MLLC method was established that allows the efficient generation and expansion of AML-reactive, HLA class I-restricted cytotoxic T cell clones from blood lymphocytes of the unprimed donor prior to transplantation. The protocol uses CD62L-selected naive and central memory $CD8^+$ T cells of healthy donors that were stimulated in 96-well microtiter plates with HLA-matched primary AML blasts of the patient. Thus, it is a procedure that does not depend on the availability of *in vivo* primed memory T cells from chimeric patients after allogeneic hematopoietic stem cell transplantation (Den Haan et al, 1995; Wang et al, 1995; Dolstra et al, 1999; Vogt et al, 2000; Brickner et al, 2001). The feasibility of generating primary leukemia-reactive CTLs from HLA-matched healthy donors *in vitro* independent from an allo-transplantation procedure has already been demonstrated by other groups (Faber et al, 1992; Smit et al, 1998; Falkenburg et al, 1999; Mutis et al, 1999; Montagna et al, 2003; Barbui et al, 2006). Compared to these previous approaches, the newly established mini-MLLC combines several potential advantages, in particular the set-up with purified $CD8^+$ $CD62L^+$ naive and central memory cells instead of total PBMCs as responders. Further differences were the use of low responder cell numbers to promote CTL clonality, the *in vitro* stimulation with primary AML blasts expressing the full spectrum of minor histocompatibility and leukemia-associated antigens, and the composition of exogenous cytokines resulting in remarkably efficient *in vitro* expansion of leukemia-reactive CTLs.

***In vitro* priming of naive CD8 T cells from healthy donors.** The use of healthy donors as the source for allogeneic T cells has the advantage that expanded T cells could be already infused together with the stem cell graft. However, the precursor frequency of leukemia-reactive CTLs in healthy individuals is low (Smit, et al, 1998). Naive $CD8^+$ T cells were chosen as responders, since this subset most likely contains the majority of anti-leukemic CTL precursors in healthy individuals. Furthermore, studies in murine models of adoptive cell transfer demonstrated that antitumor efficacy of adoptively transferred T cells is dependent on the differentiation status of the cells, with lymphocyte differentiation inversely correlated with *in vivo* antitumor effectiveness (Gattinoni et al, 2006; Hinrichs et al, 2006). Naive T cells can be phenotypically distinguished from effector and memory cells

by the expression of distinct cell surface markers, such as CD45RA, CD62L, and CCR7 (Sallusto et al, 1999). CD62L and the chemokine receptor CCR7 are both involved in homing of naive and central memory $CD8^+$ T cells to secondary lymphoid organs (Weninger et al, 2002). The superior antitumor immunity mediated by $CD62L^+$ $CCR7^+$ $CD8^+$ T cells in murine adoptive immunotherapy studies can be attributed to their increased proliferative and migratory abilities, compared to $CD62L^-$ $CCR7^-$ effector memory $CD8^+$ T cells (Gattinoni et al, 2005).

On the basis of preliminary experiments, the cell adhesion molecule CD62L (L-selectin; Kishimoto et al, 1990) was evaluated as an appropriate marker for selecting naive $CD8^+$ T cells. CD8 CD62L double-positive T cells were easily isolated by a two-step magnetic cell separation procedure. "Untouched" $CD8^+$ T cells were first purified by depletion of non-CD8 cells. This isolation step was implemented to remove other PBMC subpopulations that might compete with leukemia-reactive T cell precursors of the targeted naive cell compartment for cytokines, nutrition, and space. Since CD62L is also found on a subset of $CD4^+$ T cells mediating potent immunosuppressive effects (Ermann et al, 2005; Hoffmann et al, 2006), it was also aimed at reducing the number of those regulatory $CD4^+$ T cells that might suppress the *in vitro* generation of anti-tumor CTLs (Zou et al, 2006).

Most AML-reactive CTLs were isolated from the $CD8^+$ $CD62L^{(high)+}$ PBMC compartment. The numbers of established CTLs were significantly lower within the $CD8^+$ $CD62L^{(low)+/neg}$ cell fractions. These results confirm previous reports demonstrating that naive $CD62L^+$ T cell subpopulations contain higher frequencies of allo-reactive and tumor-reactive T cell precursors compared with the $CD62L^-$ memory subset in healthy individuals (Bleakley et al, 2004, abstract; Foster et al, 2004; Kausche et al, 2006), likely due a greater TCR repertoire in naive T cells. Since the CD62L isolation strategy did not yield absolutely pure cell subsets, AML-reactive CTLs could have derived from residual $CD62L^+$ contaminants within the CD62L-depleted populations. However, it can not be definitively excluded that leukemia-reactive CTLs have originated from $CD8^+$ $CD62L^-$ precursors. To verify that AML-reactive CTL precursors derive exclusively from the $CD62L^+$ subset, further studies should include the separation of responder cells by FACS sorting. This procedure should result in almost pure $CD62L^+$ and $CDL62^-$ subsets as starting responder populations for MLLC.

The influence of putative $CD8^+$ regulatory T cells on immune responses in humans is up to now not well characterized. A very recent publication reports the identification of human $CD8^+$ $LAG\text{-}3^+$ $CD25^+$ $FOXP3^+$ regulatory T cell subset. Those $CD8^+$ T_{regs} co-expressed CD62L and CD28, but not CD27, CD57, CD127 (IL7Rα) and IFN-γ (Joosten et al, 2007). If the expression of CD62L on regulatory $CD8^+$ T cells will be confirmed in further studies, the

$CD8^+$ CD62L-enriched PMBC subset used as responders should be furthermore analyzed for a broad panel of markers, in order to clearly separate true anti-leukemia from putative immunosuppressive $CD8^+$ T cells.

Mini-MLLC versus bulk culture. Conventional allo-MLLC approaches are traditionally based on the enrichment of leukemia-reactive CTLs in bulk culture before cloning them by limiting dilution assay (LDA; Bleakley/Riddell, 2004). Initial experiments in this study also attempted to isolate AML-reactive CTLs by this procedure, but proved to be inefficient. Only a small number of CTL clones could be isolated, showing low to intermediate reactivity against leukemic blasts. Furthermore, these CTL demonstrated only very weak proliferative capacities. This failure to efficiently clone and expand leukemia-reactive CTLs from bulk cultures may have different reasons. Such a procedure may preferentially select for CTL precursors with the capacity to survive and proliferate in bulk culture, and may not accurately reflect the diversity of T cell specificities involved in the primary anti-AML response. It has been previously reported that CML-reactive CTLs derived from bulk allo-MLLCs are more prone to lose their specific reactivity during long-term culture compared with CTLs that were generated from allo-MLLC responders initially seeded under LDA conditions (Smit et al, 1998). This may in part be due to the increased overgrowth of leukemia-reactive CTLs in bulk cultures by non-specific responder cells, probably also promoted by the rather early administration of IL-2. In our study, initial allo-MLLC experiments were performed in 24-well plates under comparable conditions as mini-MLLCs in 96-well microtiter plates. Although the same ratio of responders to stimulators and the same cytokine combination was applied as in mini-cultures, bulk cultures predominantly generated CTL populations with lower and changing AML reactivity compared to more robust and stable anti-leukemia CTLs obtained from individual mini-cultures. In addition, bulk cultures, particularly those initiated with $CD8^+$ $CD62L^{(low)+/neg}$ T cells, frequently developed major reactivity with EBV-transformed B-cell lines of patient or donor origin. This suggests an overgrowth of leukemia-reactive CTLs with memory T cells recognizing EBV antigens. In contrast, only a minority of mini-MLLC-derived CTL populations reacted with the EBV-B cell lines, whereas the majority specifically recognized the AML blasts used for primary stimulation. These observations suggest that the use of low responder cell numbers in 96-well replicates provides randomly optimized culture conditions for multiple different stimulator-responder combinations, promoting the isolation of leukemia-specific CTLs.

Clonal expansion. The initiation of mini-MLLCs with responder T cells seeded at a comparably low cell number (i.e. 10^4/well) also aimed at achieving the outgrowth of single AML-reactive CTL clones, since the estimated CTL precursor frequency in healthy donor PBMCs is lower than 10^{-5} (Smit et al, 1998). This hypothesis was confirmed by flow cytometric analyses, revealing that mini-MLLC-derived CTLs expressed TCRs of single Vβ chain families. Further support for the clonality of mini-MLLC responders came from the observation that AML-reactive CTLs were regularly isolated from less than one-third of seeded wells. The latter indicates either - according to the Poisson distribution - a CTL precursor frequency of lower than 10^{-4} within CD62L-selected $CD8^+$ T cells, or a CTL cloning efficiency below 100% (Taswell, 1981). By the application of antigen-specific stimulation with irradiated AML blasts and the cytokine combination IL-2, IL-7, and IL-15, it was possible to rapidly expand several CTLs within six to eight weeks to cell numbers between 10^8 to 10^{10}. In contrast to previous approaches, it was not necessary to use mitogenic agents such as PHA, CD3 and CD28 ligation, or allogeneic feeders for expansion (Riddell/Greenberg, 1990; Montagna et al, 2003; Montagna et al, 2006).

Primary AML blasts as stimulators. Primary leukemic cells were reported to have only limited immunostimulatory capacities, mainly due to a reduced expression of costimulatory and adhesion molecules (Brouwer et al, 2000b). Previously published procedures for stimulating leukemia-reactive T cells therefore mainly used leukemia-derived APCs generated by maturation with cytokines or calcium ionophore (Brouwer et al, 2000a; Westers et al, 2003; Barbui et al, 2006), donor-derived DCs loaded with leukemic blasts (Montagna et al, 2003; Montagna et al, 2006), CD40 ligation of loaded DCs (Montagna et al, 2001) or artificial APCs (Oosten et al, 2004; Schilbach et al, 2005) as stimulator cells. Beside their laborious generation, DC preparations or artificial APCs might have the disadvantage that they do not present the full repertoire of leukemia-associated antigens naturally occuring *in vivo*. Stimulator cells used in the established mini-MLLC protocol were irradiated primary leukemic blasts of AML patients. Those AML blasts were pre-cultured for 24 hours prior to their use as stimulator cells. The pre-incubation period was implemented to further increase the expression of costimulatory and adhesion molecules, which was associated with an increased recognition by AML-reactive CTLs, as already previously demonstrated (Brouwer et al, 2000b). Even though the application of maturation treatments such as CD40L-transfected murine fibroblasts, the calcium ionophore Calcimycin, or the maturation cytokines IL-4, GM-CSF, TNF-α, and SCF led to an even stronger upregulation of costimulatory and adhesion molecules, it was not

mandatory to promote such a professional APC phenotype for generating AML-reactive CTLs.

Stimulator cells used in MLLCs of this study were monocytic FAB M4 and FAB M5 AML blasts isolated from high-risk patients with treatment-refractory disease. In order to evaluate if monocytic AMLs have a superior immunostimulatory capacity, further studies should include AML blasts of different FAB subtypes as stimulators concomitant with their phenotypic analysis. An increased expression of single costimulatory and adhesion molecules on FAB M4 and M5 AMLs compared to other subtypes has been reported (Brouwer et al, 2001). Surprisingly, this study also concluded an association of high CD40 and CD11a expression with poor prognosis predominantly in M4 and M5 subtypes.

The immunostimulatory capacity of leukemic cells also depends on the expression of HLA molecules presenting antigens to T cells. In contrast to solid tumors, HLA class I antigen expression has been investigated only to a limited extent in hematologic malignancies (Elkins et al, 1984; Wetzler et al, 2001; Demanet et al, 2004). Defects in HLA expression have been found only in a low percentage of the samples analyzed. Total HLA class I antigen loss has been associated with an aggressive clinical course of the disease in non-Hodgkin lymphoma (Amiot et al, 1998) and with an increased expansion of the leukemic clone at diagnosis in AML (Savoia et al, 1992). In this study, a selective up-regulation of HLA-A24 in relapsed compared with primary AML blasts was observed in the MZ369-AML/sibling donor model and corresponded with increased *in vitro* lysis by the HLA-A24-restricted CTL 1E3. This finding confirms previous reports about single AML patients who developed a relapse after allogeneic HSCT, concomitant with an up-regulation, down-regulation or even loss of cell surface expression of HLA class I molecules on relapsed AML blasts (Dermime et al, 1997; Brouwer et al, 2002). Most notably, decreased HLA expression levels were immunologically relevant leading to impaired recognition by HLA-restricted CTLs (Brouwer et al, 2002). In this study, experiments in the sibling donor/MZ369-AML model included the regular pre-culture of AML cells with the maturation cytokines IL-4, GM-CSF, SCT, and TNF-α. Interestingly, the application of these supplements on relapse AML cells resulted in an upregulated expression of HLA-A24 and total HLA-B and –C molecules. In contrast, this cytokine effect was not detectable on the primary AML preparation.

Cytokines. The high efficiency of the established mini-MLLC protocol may also rely to some extent on the sequential use of cytokines promoting CTL priming and proliferation. IL-12 is known to favor the generation of antigen-specific CTLs (Trinchieri, 1995), while IL-7

and IL-15 act primarily by supporting the survival and growth of naive and memory $CD8^+$ T cells (Tan et al, 2001; Lu et al, 2002; Berard et al, 2003; Waldmann, 2006). Although IL-2 has been the preferred cytokine for the *ex vivo* expansion of CTLs, it also drives regulatory T cell proliferation and activation (Thornton et al, 2004; Ahmadzadeh et al, 2006), triggers apoptosis in activated T cells (Zheng et al, 1998), and might lead to an overgrowth with unspecific T cells. Therefore, IL-2 was not administered during the first 2 weeks of MLLC. IL-2 and IL-15 have recently also been demonstrated to induce the transcription factor FOXP3 in $CD8^+$ T cells, which is considered as a marker for regulatory T cells (Ahmadzadeh et al, 2007). Another common gamma chain cytokine that might be interesting to explore in future MLLC experiments is IL-21 (Leonard/Spolski, 2005), since it has been described to promote antigen-specific CTLs (Li et al, 2005).

Antigens. The AML-reactive CTLs isolated in this study cross-reacted with a significant proportion of HLA-matched acute and chronic myeloid leukemias, but not with lymphatic leukemias. In single cases, minor reactivity was observed against monocytes of individuals carrying the respective HLA class I alleles. These data suggest that the leukemia-reactive CTLs may recognize antigens that are overexpressed in myeloid leukemias. Particularly, the antigens might be derived from non-polymorphic proteins that are expressed by leukemia cells at much higher levels than by normal cells. Antigens of this category that have been investigated as targets for T cells are for example proteinase 3 and WT-1 (Molldrem et al, 1996; Gao et al, 2000). Circulating memory T cells reactive against these antigens can be found in healthy donors and in patients after allogeneic HSCT (Molldrem et al, 1996; Molldrem et al, 2000; Rezvani et al, 2003). However, because patient-derived normal myeloid cells such as monocytes or granulocytes were not available for testing, polymorphic minor H antigens with a myeloid expression pattern can not be excluded as the target structures for CTLs. The latter represent the majority of currently known T cell-defined leukemia antigens and have been discovered with CTLs isolated from primed leukemia patients after allogeneic HSCT (Bleakley/Riddell, 2004). The antigens recognized by CTL clones 1C6 and 4D7 in system MZ201-AML/Donor332 are currently being identified by cDNA expression cloning in collaboration with Dr. Catherine and Prof. Dr. Thomas Wölfel, Mainz.

Biological significance of AML-reactive CTLs. AML is considered to arise from a leukemic precursor cell that is capable of establishing malignant hematopoiesis. The putative AML stem cell, termed SCID leukemia-initiating cell (SLIC), has been identified by

transplanting human primary AML cells into immunodeficient mice (Lapidot et al, 1994). Leukemic stem cells are present at very low frequency in the AML cell population and are regarded as essential for leukemic hematopoiesis (Bonnet/Dick, 1997; Dick, 2005). AML-reactive CTLs with biological significance should not only be able to eliminate leukemic blasts, but also leukemic stem cells. Otherwise the disease would not be completely eradicated. In this study, the biological relevance of AML-reactive CTL clones was analyzed in an experimental mouse model by a similar strategy as previously described (Bonnet et al, 1999). Instead of the NOD/SCID mouse, the NOD/SCID IL2Rγ^{null} derivative strain that lacks the functional common IL-2 receptor γ-chain was used (Ito et al, 2002; Shultz et al, 2005). These mice showed an excellent engraftment of human primary AML blasts, most likely because they carry severe defects in innate and adaptive immunity. In addition to the lack of T and B cells, NOD/SCID IL2Rγ^{null} mice also have impaired NK cell function (Shultz et al, 2007). As experimental setting, AML blasts of patient MZ201 were pre-incubated for 24 hours with CTLs 1C6 or 4D7 that lyse MZ201-AML, or with anti-melanoma control CTLs. Those cell co-cultures were inoculated into NOD/SCID IL2Rγ^{null} mice; mice receiving only AML blasts served as engraftment controls. Mice were sacrificed eight weeks after inoculation, and were analyzed by FACS and PCR for human cells. The leukemic engraftment was specifically inhibited if inoculated AML blasts had been pre-incubated with AML-reactive CTLs, but not with anti-melanoma control CTLs. These data suggest that the AML-reactive CTL clones were capable of directly eliminating leukemic precursor cells. An alternative explanation would be that the inhibitory effect on AML engraftment was mediated indirectly by cytokines released from activated CTLs following recognition of more differentiated cells in the AML population. In conclusion, our data provide the first evidence that CD8$^+$ CTLs raised from healthy donors against AML blasts using primary *in vitro* stimulation might have biologically significant anti-leukemic activity. Future experiments should aim at the analysis of the anti-leukemia effects of AML-reactive CTLs in a therapeutical model *in vivo*. In particular, the establishment of leukemic hematopoiesis in mice prior to the adoptive transfer of CTLs capable of eradicating the disease *in vivo* would be the definitive proof for the biological significance of these CTLs.

6. CONCLUSION & OUTLOOK

The results of this project demonstrate that myeloid leukemia-reactive CTL clones capable of preventing AML engraftment in mice can be rapidly isolated from $CD8^+$ $CD62L^{(high)+}$ T cells of healthy donors *in vitro*.

Ongoing work include the identification of peptide epitopes recognized by AML-reactive CTLs, as well as the further optimization of mini-MLLC conditions. Particularly, the knowledge of essential protocol parameters will facilitate the translation into an *in vitro* protocol that is compatible with GMP guidelines. In that context, we will try to further optimize the culture conditions, for example by reducing the IL-2 concentration, which might be important for improving the *in vivo* survival of transferred CTLs, as well as the investigation of the newly characterized common gamma chain cytokine IL-21 in promoting antigen-specific CTLs. The use of unfractionated $CD8^+$ cells of healthy donors in comparison to FACS-sorted $CD8^+$ $CD62L^+$ T cells as responder cells in mini-MLLCs might further clarify if AML-reactive CTLs arise exclusively or just preferentially from T cells of the naive compartment. We also plan to perform comparative analyses with leukemia-reactive CTLs isolated from *in vivo* primed patients after allogeneic HSCT. Concerning the use of primary AML blasts as stimulator cells in mini-MLLC experiments, we will investigate the influence of different FAB subtypes, as well as the impact of different maturation treatments. It is also intended to establish similar protocols for other applications, such as the generation of AML-reactive $CD4^+$ T cells, since an optimal adoptive immunotherapy should include the co-transfer of $CD4^+$ helper T cells. The use of acute lymphatic leukemia blasts as stimulator cells might create an additional application of the mini-MLLC approach, in case that ALL blasts demonstrate appropriate immunostimulatory capacity. The aim of an associated project is to isolate and characterize putative leukemic stem cells, described to reside within the $CD34^+$ $CD38^-$ subset, that could be used as target cells in effector cell assays and for the engraftment in immunodeficient mice. The establishment of a therapeutic mouse model of human AML involving the engraftment of leukemic hematopoiesis prior to the transfer of leukemia-reactive CTLs should help to further elucidate the biological significance of the observations made within this work.

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8. ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
AIRE	autoimmune regulator
ALL	acute lymphatic leukemia
AML	acute myeloid leukemia
APC	antigen-presenting cell
APC	allophycocyanin
approx.	approximately
BSA	bovine serum albumine
BTLA (=CD272)	B and T lymphocyte attenuator
CD	cluster of differentiation
cDNA	complementary DNA
CDR	complementarity-determining region
CEPH	Centre d'Etude Polymorphism Humain
CLIP	class II associated invariant peptide
CLL	chronic lymphatic leukemia
CML	chronic myeloid leukemia
CMV	cytomegalovirus
CSA	cyclosporine A
cSNP	coding single nucleotide polymorphism
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DLI	donor lymphocyte infusion
DNA	desoxyribonucleic acid
E:T	effector to target ratio
EBV	Epstein Barr-Virus
EDTA	Ethylendiamine-tetra-acetic acid
EGFR	epidermal growth factor receptor
ELISpot	Enzyme-linked Immunosorbent Spot assay
ER	endoplasmatic reticulum
ERAPI	ER aminopeptidase I
FAB	French American British Cooperative Group
FACS	fluorescence-activated cell sorting
FasL	Fas ligand
FcR	Fc receptor
FCS	fetal calf serum
FITC	fluorescein-iso-thio-cyanat

FLT3	fibroblast-macrophage stimulating factor receptor (FMS)-like tyrosine kinase receptor 3
FOXP3	forkhead box P3
G-CSF	granulocyte-colony stimulating factor
GITR	glucocorticoid-induced TNFR family-related gene
GM-CSF	granulocyte/macrophage-colony stimulating factor
GMP	Good Manufacturing Practice
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
HEV	high endothelial venules
HLA	human leucocyte antigen
HPLC	high performance liquid chromatography
HPV	human papilloma virus
HS	human serum
HSCT	hematopoietic stem cell transplantation
Id	inhibitor of DNA binding
IDO	idoleamine-2,3-deoxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
inv	inversion
ITD	internal tandem duplication
IU	international unit
KIR	killer inhibitory receptor
LAG-3	lymphocyte activation gene-3
LC	lung carcinoma
LCL	lymphoblastoid cell line
LDA	limiting dilution assay
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic cell sorting
MDS	myelodysplastic syndrome
Mel	melanoma
MEM	Minimal Essential Medium
mHAg	minor histocompatibility antigen
MHC	major histocompatibility complex
MLLC	mixed lymphocyte/leukemia culture
MPD	myeloproliferative disease
MPP11	m-phase phosphoprotein 11
MS	mass spectrometry

NK	natural killer
NOD	nonobese diabetic
NS	nonsynonymous
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cell
PC5 (=PeCy5)	Phycoerythrin-Cyanin-5
PCR	polymerase chain reaction
PD-1	programmed death-1
PE	phycoerythrin
PHA	phytohemagglutinin
PRR	pattern recognition receptor
RHAMM	receptor for hyaluronic acid mediated motility
RNA	ribonucleic acid
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
RTK	receptor tyrosine kinase
Sca-1	stem cell antigen-1
SCF	stem cell factor
SCID	severe combined immuno deficient
SEREX	serological screening of cDNA expression libraries
SLIC	SCID leukemia-initiating cell
TAA	tumor associated antigen
TAP	transporter in antigen processing
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EM}	effector memory T cell
TGF-β	transforming growth factor-β
TIL	tumor infiltrating lymphocyte
TLR	Toll-like receptor
TNFR	tumor necrosis factor receptor
TNF-α	tumor necrosis factor-α
TPPII	tripeptidylpeptidase II
T_{reg}	regulatory T cell
VEGF	vascular endothelial growth factor
WBC	white blood cell count
WHO	World Health Organization
WT-1	Wilms' tumor antigen 1