Generation and characterisation of acute myeloid leukaemia-reactive $CD4^+$ T cells and their potential to eliminate human leukaemia blasts in NSG mice

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1 Abstract

Acute myeloid leukaemia (AML) is a cancer of the haematopoietic system, which can in many cases only be cured by haematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) (Burnett *et al.*, 2011). This therapy is associated with the beneficial graft-versus-leukaemia (GvL) effect mediated by transplanted donor T and NK cells that either recognise mismatch HLA molecules or polymorphic peptides, so-called minor histocompatibility antigens, leukaemia-associated or leukaemia-specific antigens in the patient and thus eliminate remaining leukaemic blasts. Nevertheless, the mature donor-derived cells often trigger graft-versus-host disease (GvHD), leading to severe damages in patients' epithelial tissue, mainly skin, liver and intestine (Bleakley & Riddell, 2004). Therefore, approaches for the selective mediation of strong GvL effects are needed, also in order to prevent relapse after transplantation. One promising opportunity is the *in vitro* generation of AML-reactive $CD4^+$ T cells for adoptive transfer. $CD4^+$ T cells are advantageous compared to $CD8^+$ T cells, as HLA class II molecules are under non-inflammatory conditions only expressed on haematopoietic cells; a fact that would minimise GvHD (Klein & Sato, 2000). In this study, naive $CD4^+$ T cells were isolated from healthy donors and were successfully stimulated against primary AML blasts in mini-mixed lymphocyte/leukaemia cell cultures (mini-MLLC) in eight patient/donor pairs. After three to seven weekly restimulations, T cells were shown to produce T_H1 type cytokines and to be partially of monoclonal origin according to their TCR Vβ chain usage. Furthermore, they exhibited lytic activity towards AML blasts, which was mediated by the release of granzymes A and B and perforin. The patient/donor pairs used in this study were fully HLA-class I matched, except for one pair, and also matched for HLA-DR and -DQ, whereas -DP was mismatched in one or both alleles, reflecting the actual donor selection procedure in the clinic (Begovich *et al.*, 1992). Antibody blocking experiments suggested that the generated $CD4^+$ T cells were directed against the HLA-DP mismatches, which could be confirmed by the recognition of donor-derived lymphoblastoid cell lines (LCLs) electroporated with the mismatched DP alleles. Under non-inflammatory conditions primary fibroblasts did not express HLA-DP and were thus not recognised, supporting the idea of a safer application of $CD4^+$ T cells regarding induction of GvHD. For the assessment of the biological significance of these T cells, they were adoptively transferred into NSG mice engrafted with human AML blasts, where they migrated to the bone marrow and lymphoid tissue and succeeded in eliminating the leukaemic burden after only one week. Therefore, AML-reactive $CD4^+$ T cells expanded from the naive compartment by *in vitro* stimulation with primary leukaemia blasts appear to be a potent tool for DLI in HSCT patients and promise to mediate specific GvL effects without causing GvHD.

2 Introduction

2.1 An overview of the immune system

Within the human body a great variety of effector molecules and cells reside that are responsible to provide protection from infections. The cellular components of this immune system can be divided into two parts: the lymphoid and myeloid branch. All cells originate from a pluripotent haematopoietic stem cell but either develop into erythrocytes, platelets or granulocytes (derived from a common myeloid progenitor) or into dendritic cells (DCs), B, T and natural killer (NK) cells (derived from a common lymphoid progenitor). Erythrocytes circulate within the blood and are responsible for oxygen transport, whereas platelets mediate blood clotting and the healing of lesions in the vasculature. All remaining cells constitute leukocytes. Granulocytes that can further be categorised into neutrophils, eosinophils and basophils together with mast cells and macrophages are involved in the response to parasites or mediate phagocytosis of invading microorganisms as a first-line defence of the unspecific but fast innate immunity. Dendritic cells link the innate to the adaptive immunity. They can also phagocytose intruders and then act, among others, as antigen-presenting cells (APCs) by displaying pathogenic antigens on their surface for T-cell recognition. NK cells are also part of the innate immunity due to their ability to kill abnormal or infected cells in an unspecific manner. The lymphocytes' antigen-specific recognition through highly diverse antigen-binding sites of their receptors, however, is what distinguishes the adaptive immunity. Naive lymphocytes, that have not yet met their specific antigen, circulate through the blood and lymphoid system in a resting state. Once they encounter their respective antigen, they become activated, differentiate and start to proliferate. Therefore, the adaptive immunity is a slower process compared to the innate immunity and needs several days. Nevertheless, activated lymphocytes form a protective memory for future infections that can then be fought much faster. The lymphocytes can be divided into B cells and T cells. The first carry a specific B-cell receptor on their surface. When an antigen binds to this receptor, the cells become activated, differentiate into antibody-secreting plasma cells and show a monoclonal expansion. A similar structure can be found on T cells. They express a T-cell receptor (TCR) that is responsible for antigen-recognition and thus triggers activation of the cells. T cells are categorised into three subtypes with distinct yet overlapping functions. Within the body the cytotoxic T cells (CTLs, $CD8^+$ T cells) are responsible for the killing of cells infected with intracellular pathogens like

viruses, whereas T helper cells (T_H, $CD4^+$ T cells) interact with B cells, CTLs and macrophages to render them more effective. The third subset, the regulatory T cells (T_{reg}), have suppressive functions in controlling the remaining lymphocytes. Nevertheless, all those cells only work if they are supplemented by a great variety of cytokines and chemokines that are needed for cell migration, activation or cell-to-cell communication in general (Delves & Roitt, 2000; Parkin & Cohen, 2001).

2.2 Antigen-recognition by α:β T cells

T cells recognise their antigen with the aid of T-cell receptors. The TCR is a heterodimeric transmembrane protein that consists of one α- and one β-chain, each being composed of a variable (V) and constant (C) domain, with the variable regions of both chains together forming the antigen-binding site. The huge diversity of the TCRs is achieved by gene rearrangement that takes place in the thymus. Here, numerous V and J (joining) segments in the α-chain and V, J and D (diversity) segments in the β-chain are recombined to form the final exons that contain only one version of each segment. During this process additional nucleotides are added to the single-stranded DNA, further increasing diversity (Parkin & Cohen, 2001).

The TCR does usually not bind directly to an entire protein but to short peptide fragments that are presented on APCs by major histocompatibility complexes (MHC), in the human setting also called human leukocyte antigens (HLA). HLA class I molecules, namely HLA-A, -B and -C, which are mainly recognised by $CD8^+$ T cells, consist of a long α-chain that contains the transmembrane region and also the peptide-binding cleft and a non-covalently associated β_2-microglobulin. HLA class II molecules (HLA-DR, -DP, -DQ), however, mainly interact with $CD4^+$ T cells and are made of an α- and β-chain that are also associated non-covalently but both span the membrane. Distribution also differs, as class I complexes can be found on all nucleated cells, whereas class II expression is restricted to haematopoietic cells and thymic stromal cells under non-inflammatory conditions (Klein & Sato, 2000). Both kinds of HLA molecules are only stable if a peptide is bound. Nevertheless, peptides need to fulfil certain characteristics. The HLA alleles carry different amino acids within the antigen-binding cleft and therefore, they bind different peptides. For HLA class I certain anchor residues within the binding cleft, which is closed at both ends, accounting for the high peptide-specificity and only allow the binding of peptides containing eight or nine amino acids (AA).

Class II peptides, on the other hand, have to contain at least 12 AAs. The binding cleft is open at the ends and anchor residues lie in the core of the peptide sequence (Biddison & Martin, 2001). These peptide:HLA complexes can then be bound by the TCR. Nevertheless, the TCR does not work alone but is associated with two CD3 complexes and a ζ dimer, all containing intracellular signal transduction domains (Call *et al.*, 2002). Furthermore, the co-receptors CD4 on T_H cells and CD8 on CTLs, which bind antigen-independently to invariant sites of the HLAs, are needed to enhance the sensitivity of the T cells and modulate antigen recognition (Davis *et al.*, 1998).

Peptides that are presented by HLA class I molecules originate from the cytosol where they can be degraded by the proteasome. These peptides are then transported into the endoplasmatic reticulum (ER) by two proteins called transporter associated with antigen processing TAP1 and TAP2, where they are loaded onto the HLA class I molecules. Finally, the peptide:HLA complex is exported to the cell surface.

Pathogens that enter the vesicular compartment of APCs end up in HLA class II molecules. After taking up extracellular antigens, the pH of the cells' endosomes decreases and they fuse with lysosomes. Acid proteases within these endosomes can then cleave the proteins. HLA class II molecules are assembled in the ER and are packed in vesicles. When these vesicles fuse with the ones containing the peptide antigens, the HLA class II molecules can be loaded with peptides and are subsequently transported to the cell surface (Klein & Sato, 2000).

However, the exception to these two mechanisms is the so-called cross-presentation. Here, macrophages or DCs phagocytose extracellular proteins and load their peptides onto HLA class I molecules. Two different theories are hypothesised. The first one states that proteins are transported from the phagosome to the cytosol in a way that has not been identified yet. In the second one, it is assumed that vesicles from the ER containing HLA class I molecules fuse with the phagosomes and can then be loaded with peptides (Rock & Shen, 2005). Cross-presentation also works the other way round, as proteins that originate from the cytosol can also be presented by HLA class II molecules after degradation by autophagy (Neefjes *et al.*, 2011).

In addition to the TCR binding to the peptide:HLA complex, co-stimulatory molecules like CD80 and CD86 are needed for the activation of naive T cells. They engage the co-stimulatory receptor CD28, which leads to an enhanced T-cell receptor signalling (Delves & Roitt, 2000; Parkin & Cohen, 2001; Acuto & Michel, 2003).

2.3 T-cell development

The common lymphoid progenitor can be found in the bone marrow but precursor T cells migrate into the thymus for maturation and later on into peripheral lymphoid organs like lymph nodes, spleen and lymphoid mucosa-tissue. Within the thymus the bone marrow-derived precursors receive a Notch signal from thymic stromal cells that drives commitment to the T-cell instead of the B-cell lineage. T cells start thymic maturation as $CD3^-/CD4^-/CD8^-$ cells and soon commit either to the γ:δ or α:β T-cell lineage. Part of the T-cell receptor rearrangement takes place and a pre-TCR is expressed on the cell surface of double-negative thymocytes ($CD4^-/CD8^-$). Cells then start to proliferate and pass a stage where they express both CD4 and CD8 (double-positive thymocytes). Subsequently, cells have to undergo positive selection where low-affinity recognition of self-peptide:self-HLA complexes is tested and cells die if they fail. Otherwise, their maturation continues and they start to express high levels of the now finalised TCR and lose either CD4 or CD8 expression (Borowski *et al.*, 2002). In a negative selection step the cells that respond to self-antigens are eliminated, leaving 2 % of the cells that originally entered the thymus to migrate into the periphery. T cells, which are now called recent thymic emigrants (RTEs), then traffic through the blood and enter the lymphoid system via the spleen or lymph nodes where they are directed by ligands binding to the receptor CCR7 on the T-cell surface (Cyster, 2000). RTEs show a distinct phenotype by expressing higher levels of CD24 and the TCR-CD3 complex but lower levels of CD45RA, IL-7Rα and CD28 when compared to mature naive T cells, which they turn into after a post-thymic maturation phase of approximately three weeks (Fink & Hendricks, 2011). To ensure survival, mature naive T cells need to continuously interact with their particular MHC restriction element in the periphery and the cytokine IL-7 (Seddon & Zamoyska, 2002).

2.4 $CD4^+$ T cells and their effector functions

2.4.1 Subsets among $CD4^+$ T cells

Among the $CD4^+$ T cells eight specific subtypes have been identified. Depending on the cytokine milieu that drives their differentiation and the effector functions they fulfil, T helper cells can be divided into T helper 1 (T_H1), T helper 2 (T_H2), T helper 9 (T_H9), T helper 17 (T_H17), T helper 22 (T_H22), induced regulatory (iT_{reg}) , regulatory type 1 (T_r1) and follicular T helper cells (T_{fh}). As soon as a T cell commits to one specific lineage, the alternative fates are

supressed by cross-regulation among the transcription factors involved (Zhu & Paul, 2010; Luckheeram *et al.*, 2012).

Particularly IL-12 and IFN-γ drive T_H1 lineage commitment through activation of the transcription factors T-bet and STAT4. The cells then start to produce IFN-γ themselves together with IL-2 and TNF-α and lead to an enhanced phagocytic activity of macrophages and therefore an elimination of intracellular pathogens (Luckheeram *et al.*, 2012). Furthermore, IL-2 induces proliferation of $CD8^+$ T cells and drives acquisition of a cytolytic phenotype and the formation of memory $CD8^+$ cells (Williams *et al.*, 2006). IL-4 and IL-2, on the other hand, are the important factors for T_H2 differentiation. Upon upregulating the major transcription factor GATA-3, T_H2 proliferation is induced whereas T_H1 differentiation is inhibited (Zhu *et al.*, 2006). T_H2 cells produce IL-4, IL-5, IL-9, IL-13 and IL-25 and are responsible for the elimination of extracellular parasites like helminthes and play an important role in allergies and asthma by activating mast cells, B cells, eosinophils and neutrophils (Del Prete, 1992). T_H9-cell differentiation is triggered by TGF-β in combination with IL-4. They mainly produce IL-9, which was shown to play an important role in the immune response against helminthes as well as in allergies and asthma, as it promotes mast cell proliferation and accumulation (Tan & Gery, 2012). For the induction of T_H17 cell differentiation, TGF-β and IL-6 and the key regulator RORγt play the primary roles, whereas IL-21 and IL-23 are needed for the later self-amplification and stabilisation. The secreted cytokine IL-17 mediates immune responses against extracellular bacteria and fungi. T_H17 cells also produce IL-21 and IL-22, which lead to an inflammatory response by inducing pro-inflammatory cytokines and chemokines and by activating other immune cells (Ivanov *et al.*, 2006). The subset of T_H22 cells was described to show an overexpression of the transcription factors BNC2 and FOXO4. Their differentiation can be stimulated by Langerhans cells in the skin. There they are responsible for the induction of small antimicrobial peptide production. In addition to IL-22, which is associated with both, anti-inflammatory and pro-inflammatory activity, they produce IL-10 and TNF (Coghill *et al.*, 2011). Regulatory T helper cells develop in the periphery after antigen contact through the cytokine TGF-β, which induces the transcription factor FOXP3 (Chen *et al.*, 2003). They are needed for the induction of immunological tolerance towards self and foreign antigens, which is mainly mediated by IL-10, a suppressive cytokine that also inhibits pro-inflammatory cytokine expression in the recovery phase of infections (Fujio *et al.*, 2010; Ouyang *et al.*, 2011). The last subset

among the $CD4^+$ T cells consists of T_{fh} cells, which are characterised by their expression of CXCR5 and the lack of CCR7. They can be found in germinal centres where they interact with B cells and induce their differentiation into antibody-secreting plasma cells and memory B cells (Fazilleau *et al.*, 2009).

2.4.2 Cytotoxicity mediated by $CD4^+$ T cells

A popular paradigm in immunity is that while $CD8^+$ T cells are responsible for the direct killing of their target cells, $CD4^+$ T cells provide helper functions by the release of cytokines that either trigger a cellular response and its memory (mediated by T_H1 cells) or support the humoral immune response of secreted antibodies, antimicrobial peptides and complement proteins (mediated by T_H2 cells). Nevertheless, HLA class II restricted cytolytic $CD4^+$ T cells of the T_H1 type could be generated against poliovirus (Mahon *et al.*, 1995) and Epstein Barr virus (Nikiforow *et al.*, 2003) and were isolated directly *ex vivo* in healthy individuals (Appay *et al.*, 2002). Lysis can be mediated by Fas/FasL interaction. Fas is a membrane-bound protein that belongs to the TNF-receptor family of death receptors and the interaction with its ligand leads to the activation of caspases and therefore cell death through apoptosis. Other death receptors can be activated by TNF-related apoptosis-inducing ligand (TRAIL), which leads to a similar killing through caspase activation. An alternative way of target cell killing is the calcium-dependent release of cytotoxic granules. They predominantly contain perforin, granzymes and granulysin that are released into the cytolytic synapse. Initially, perforin was thought to form a pore in the membrane of the target cell through which granzymes could enter and again lead to caspase activation or DNA strand brakes. Nevertheless, recent findings showed that granzymes were also able to enter target cells in a perforin-independent manner, rendering the role of perforin unclear. Granulysin showed anti-microbial activity as it was able to form pores in bacterial lipid membranes. On the whole, granule-mediated killing of target cells proves to be the preferred way as a much faster target-cell lysis can be achieved by the already pre-loaded cytotoxic cells, whereas Fas upregulation needs time (Barry & Bleackley, 2002; Brown, 2010).

2.4.3 $CD4^+$ T-cell differentiation

When an infection occurs, the innate immunity provides a first-line defence to confine the pathogens. The tissue becomes inflamed and dendritic cells that have taken up antigen migrate to peripheral lymphoid tissue where they meet circulating naive T cells (T_N). Those T cells recognise their antigen on the surface of the dendritic cells and become activated, proliferate and enter the circulation. Chemokines and cytokines guide them to the site of inflammation, where they can enter through the vessels that have been activated by TNF-α and now express adhesion molecules. After antigen encounter, the naive T cells differentiate into effector cells that can mediate an immune response by secreting cytokines and cytotoxic molecules like perforin and granzymes (Fearon & Locksley, 1996; Appay *et al.*, 2008). When the infection is cleared, memory cells remain and form a protective long-lived cell subset to fight a secondary infection. It is not clear yet, whether this differentiation occurs on a linear pathway or might be branched or reversible (Seder & Ahmed, 2003; Appay *et al.*, 2008; Lanzavecchia & Sallusto, 2005).

The various stages of $CD4^+$ T cells not only correlate with different functions and therefore roles in the immune response, but also with expression patterns of distinct surface markers. One important cell surface marker for distinguishing the subsets is CD45RA, an isoform of the tyrosine phosphatase CD45 that is strongly expressed on naive $CD4^+$ T cells but downregulated upon antigen contact (Henson *et al.*, 2012). Naive T cells are further characterised by the expression of the co-stimulatory molecules CD27 and CD28, the adhesion molecules L-selectin (CD62L) and PECAM-1 (CD31) as well as the chemokine receptors CCR7 and CXCR4 for lymph node homing. After antigen encounter, naive T cells can give rise to at least two memory subsets that can be distinguished by the expression of CCR7. The $CCR7^+$ central memory (T_{CM}) cells can be found in blood, spleen and lymphoid organs and it takes them some time after antigen contact to produce cytokines, whereas effector memory (T_{EM}) cells circulate between blood, spleen and non-lymphoid tissue and are fast in producing effector molecules. After antigenic stimulation, T_{CM} ($CCR7^+$, $CD62L^+$, $CD27^+$, $CD28^+$) can migrate to peripheral lymphoid organs and turn into T_{EM} ($CCR7^-$, $CD62L^-$, $CD27^+$, $CD28^-$) that upregulate the CD45 splice variant CD45RO. Recently a new subset of memory cells with naive characteristics, named memory stem cells (T_{SCM}), was described. They can be generated by triggering Wnt signalling *in vitro* and show a $CD45RA^+$/ $CCR7^+$/$CD62L^+$ phenotype with specific expression of $CD95^+$ and IL-2Rβ^+. They are associated

with an increased proliferative capacity, enhanced self-renewal capacity and stronger anti-tumour effects in a humanised mouse model compared to T cells of other differentiation states (Gattinoni *et al.*, 2011). A co-expression of CD45RO and CD45RA can be found on effector (T_{EFF}) and end-stage memory cells (Demeure *et al.*, 1996; Sallusto *et al.*, 1999; Andrian & Mackay, 2000; Seder & Ahmed, 2003; Henson *et al.*, 2012). T_{EFF} cells also upregulate the surface inhibitory receptor CD57, the death receptor CD95 (Fas) and secrete cytokines and cytotoxic molecules. They can be found in a situation where the immune system faces actual antigen stimulation. When the antigen is cleared, T_{EFF} undergo apoptosis, whereas memory T cells remain with the potential to rapidly expand when a secondary infection has to be faced (Hildeman *et al.*, 2002; Seder & Ahmed, 2003). The member of the TNF-receptor family CD134 together with CD28 and IL-7 are essential for memory T-cell homeostasis, preventing apoptosis and therefore for the formation of a long-lived immunological memory (Rogers *et al.*, 2001; Seddon *et al.*, 2003). It is debated whether effector cells can downregulate activation associated-genes and therefore develop into memory cells as well when the antigenic stimuli is withdrawn. Nevertheless, this seems to be impossible for IFN-γ producing T_H1 cells, which rapidly undergo apoptosis but has to be discussed further for IFN-γ^-, uncommitted cells and T_H2 cells (Zhang *et al.*, 1997; Hu *et al.*, 2001; Seder & Ahmed, 2003). An overview of the $CD4^+$ T-cell differentiation stages and their respective markers and characteristics is shown in Figure 2.1.

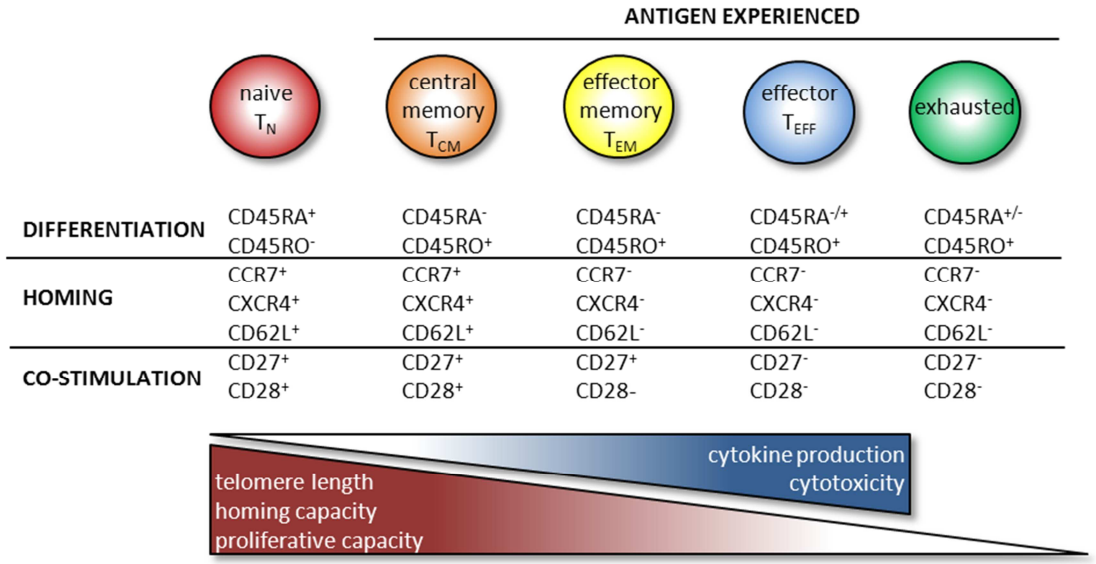

Figure 2.1: Surface marker expression on the $CD4^+$ T cell differentiation stages. $CD4^+$ T cells from various differentiation stages can be distinguished by the surface markers they express. They can be categorised in differentiation markers (CD45RA, CD45RO), homing (CCR7, CXCR4, CD62L) and co-stimulatory molecules (CD27, CD28). Furthermore, after antigen contact, teleomere length as well as the homing and proliferative capacity is reduced. Cytokine production and cytotoxicity on the other hand are increased (derived from Appay *et al.*, 2008 and Seder & Ahmed, 2003).

2.5 Leukaemia

Leukaemia is a cancer of the haematopoietic system and is characterised by strongly elevated numbers of immature leukocytes. Leukaemic blasts start to grow out of control in the bone marrow and then migrate into the blood and supersede healthy and functional blood cells over time leading to symptoms like anaemia, reduced blood clotting and increased susceptibility to infections. This type of cancer is mainly associated with genetic predispositions, ionising radiation and adverse effects resulting from chemotherapy, chemicals or by pre-leukaemic disorders of the haematopoietic system. Even though leukaemias comprise only about 2.4 % of all tumours in Germany, about 11,400 patients are newly diagnosed each year and over 7,000 die annually.

Leukaemias can be subdivided into myeloid and lymphoid types, depending on the precursor cell that is affected. They can further be categorised in acute and chronic forms according to how fast they progress. In acute leukaemias, which make up about 50 %, patients die within several weeks or months. In chronic forms however, the disease progresses much slower and can persist unnoticed for a long time. The risk of suffering from the different forms varies with age. Acute lymphoid leukaemia (ALL) is predominantly diagnosed in three to seven year old kids and young adults, whereas acute and chronic myeloid leukaemia (AML and CML) can often be found in middle-aged persons. Chronic lymphoid leukaemia (CLL) on the other hand, is rare before the age of 50. Patients are diagnosed by assessing their high white blood count (WBC), cytogenetic and immuno-phenotypic characterisation of the malignant cells (Zentrum für Krebsregisterdaten - Robert Koch Institut, 2012; Deutsche Krebsgesellschaft, 2012; American Cancer Society, 2013).

2.5.1 Acute myeloid leukaemia (AML)

The acute myeloid leukaemia is with 25 % in men and 29 % in women the second most common form of leukaemia among newly diagnosed patients. Malignant cells usually expand within the bone marrow and in some cases also spread into the spleen, liver and cerebrospinal fluid. AML blasts can usually be identified by the expression of the most commonly used surface markers CD33 and CD13 (Estey, 2013). The prognosis of survival depends on the subtype of AML a patient suffers from as well as from his or her age. A first categorisation of AML was suggested in 1976 based on the morphological and cytochemical characteristics of the malignant cells and their maturation state and is summarised in the

French-American-British (FBA) classification (Table 2.1) (Bennett *et al.*, 1976). The AML subtypes differ in the precursor cells that are affected. M0 through M5 originate from leukocyte precursors, whereas M6 descends from erythrocyte and M7 from platelet precursors. AML is diagnosed when myeloid blasts populate >30 % of the bone marrow or blood.

Table 2.1: French-American-British (FAB) classification of AML subtypes. Nine different forms of AML can be distinguished according to the appearance and maturation state of the affected cells. The incidences of the subtypes strongly vary (American Cancer Society, 2013).

FAB subtype	definition	incidence
M0	undifferentiated acute myeloblastic leukaemia	5 %
M1	acute myeloblastic leukaemia with minimal maturation	15 %
M2	acute myeloblastic leukaemia with maturation	25 %
M3	acute promyelocytic leukaemia	10 %
M4	acute myelomonocytic leukaemia	20 %
M4 eos	acute myelomonocytic leukaemia with eosinophilia	5 %
M5	acute monocytic leukaemia	10 %
M6	acute erythroid leukaemia	5 %
M7	acute megakaryoblastic leukaemia	5 %

Later on, a new classification was introduced by the World Health Organisation (WHO) that takes into account prognostic factors and is therefore more helpful in regard to a patient's outlook (Table 2.2). Prognostic factors in AML therapy include chromosomal aberrations like translocations (t(8:21), t(15:17)), inversions on chromosome 16 (inv(16)) or deletions on chromosome 5 and 7, genetic mutations, WBC, age, infections and anamnesis. According to the WHO guidelines, AML is diagnosed when myeloid blasts populate >20 % of bone marrow or blood (Vardiman *et al.*, 2002; Vardiman *et al.*, 2009; American Cancer Society, 2013; Estey, 2013).

Patients can further be categorised into different risk groups, namely favourable, intermediate or adverse. In the favourable group the probability for complete remission is high and the relapse rates are low. If combined with a normal caryotype, nucleophosmin (NPM1) mutation, which is associated with the cytoplasmic delocalisation of nuclear proteins, leads to a patient's classification as low risk (Falini et al., 2011). Unfavourable for the patient's outcome are mutations in the FMS-like tyrosine kinase 3 (FLT3) gene, which are found in 33 % of all AML cases and correlate with a poor survival rate of only 30 to 40 %.

Table 2.2: World Health Organisation (WHO) classification of AML subtypes and related neoplasms. In 2002 the WHO introduced a new system for the classification of AMLs according to prognostic factors, which was revised in 2008 (Vardiman *et al.*, 2002; Vardiman *et al.*, 2009; American Cancer Society, 2013).

WHO group	definitions/examples
AML with recurrent genetic abnormalities	t(8:21)(q22;q22), t(15;17)(q22;q12), inv(16)(p13.1q22)
AML with myelodysplasia-related changes	following myelodysplastic syndromes
Therapy-related myeloid neoplasms	alkylating agent, radiation
AML not otherwise specified	/
Myeloid sarcoma	extramedullary proliferation of blasts that disrupts tissue architecture
Myeloid proliferations related to Down syndrome	*GATA1* mutations
Blastic plasmacytoid dendritic cell neoplasm	derived from DC precursors

Naturally, a ligand-induced stimulation of FLT3 induces the differentiation and proliferation of progenitor cells. Alternations like internal tandem repeats (ITDs) can lead to an autonomous receptor activation and therefore to myeloid pathogenesis (Meshinchi & Appelbaum, 2009). Also mutations in the CCAAT enhancer binding protein alpha (CEBPA) are taken into account for assessing the risk status of an individual patient. CEBPA plays a critical role in granulocyte development and is mutated in about 10 % of all AML patients (Pabst & Mueller, 2009). There are several more genes that have been shown to be mutated in AML patients and that might be helpful as diagnostic factors but are not considered so far. These epigenetic regulators include TET2, IDH1 and IDH2 as well as ASXL1 (Abdel-Wahab & Levine, 2013). In addition to cytogenetics, a WBC of $>1x10^5/\mu l$ also correlates with an unfavourable outcome.

2.5.2 Leukaemia therapy

First line treatment of leukaemia consists of chemotherapy, including agents like cytarabine combined with anthracyclines, fludarabine and cyclophosphamide, sometimes combined with a low dose total body irradiation. Even though this first treatment of AML leads to complete remission with <5 % blasts in the bone marrow with recovery of marrow function in 60 to 80 % of the patients, more than half of them relapse and die within a year. For patients over the age of 65 these statistics look even worse (Kubal & Lancet, 2013). Also the minimal residual disease (MRD) status of patients can be taken into account. If patients were

MRD negative, meaning less than 3 % AML blasts in the bone marrow, after consolidation therapy, they face a lower relapse risk (Buccisano *et al.*, 2010). After induction therapy, haematopoietic stem cell transplantation (HSCT) can be performed in patients classified as high risk, which are prone to relapse after a first clinical remission. Before transplantation takes place, the patient receives a conditioning regimen consisting of myeloablative chemotherapy, sometimes combined with radiotherapy. Most HSCTs are performed in an allogeneic setting, which means that the patient receives the graft from another human being, in contrast to an autologous transplantation, where the patient's stem cells are rescued before the conditioning treatment and re-infused afterwards. The cells for the transplant originate from the bone marrow or can be obtained from cytokine-mobilised peripheral blood of a healthy donor, in rare cases also from umbilical cord blood and reconstitute the haematopoietic system of the patient. Together with the transplant, patients receive an immunosuppressive treatment, which prevents graft-versus-host disease but also impairs the graft-versus-leukaemia effect and immune reconstitution (see chapter 2.5.3) (Li & Sykes, 2012; Estey, 2013). In this vulnerable phase, opportunistic infections often occur and latent infections with cytomegalovirus, herpes simplex virus, Eppstein-Barr virus (EBV) or varicella zoster virus can be reactivated and lead to the death of the patient (Hofmann & Greiner, 2011).

2.5.3 Graft-versus-leukaemia effect and graft-versus-host disease

Associated with allogeneic HSCT are two immune-mediated effects: the beneficial graft-versus-leukaemia (GvL) effect and the unwanted graft-versus-host disease (GvHD). GvHD occurs when the transplanted T cells recognise allogeneic peptide:HLA complexes of the recipient that they have not encountered during their thymic selection processes, most frequently leading to damages in the skin, liver and gastrointestinal tract. This is either caused by transplanted T cells recognising mismatched HLA molecules or polymorphic peptides, so-called minor histocompatibility antigens (mHag), presented by the matched HLA molecules. Minor antigens can also derive from Y-chromosomal genes that are recognised in male patients upon transplanted grafts from a female donor (Bleakley & Riddell, 2004). In contrast to the normal recognition, alloreactive immune responses occur with high frequencies and are caused by TCR-interaction either with the allogeneic HLA molecule alone in a peptide-independent manner (direct allorecognition) or with the peptide

irrespective of the HLA restriction element (indirect allorecognition) (Figure 2.2) (Felix & Allen, 2007).

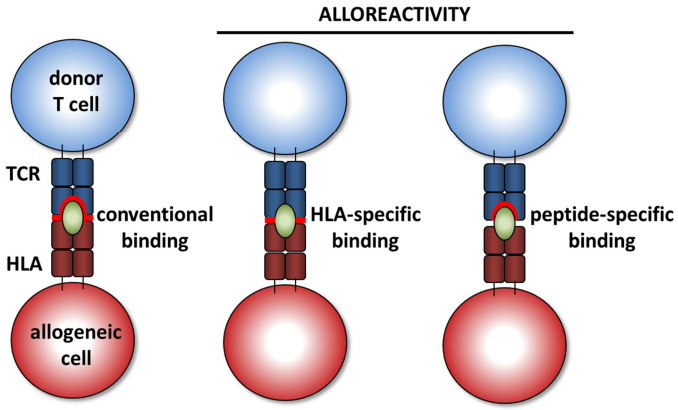

Figure 2.2: **Allorecognition of transplanted T cells.** T cells usually recognise peptides that are bound to self HLA molecules via T-cell receptors (TCR). After allogeneic transplantations they can also interact with allogeneic HLA molecules presenting peptides. Therefore, the TCR can either directly interact with the peptide or solely with the HLA molecule (derived from Felix & Allen, 2007).

The GvL effect is also caused by donor T cells that recognise these non-self structures on leukaemic blasts and lead to tumour eradication (Figure 2.3) (Bleakley & Riddell, 2004). The role T cells play in both of these events was shown previously as patients, who received T-cell depleted transplants, did indeed show reduced GvHD rates but also dramatic increases in the likelihood of graft failure and relapse (Marmont *et al.*, 1991). Among $CD4^+$ T cells, both T_H1 and T_H2 were shown to contribute to GvHD. In murine studies, where either STAT4, associated with T_H1 development, or STAT6, involved in T_H2 development, was deficient, less severe GvHD was observed compared to wild-type T cells. The main effects of GvHD in mismatched transplantations are not necessarily mediated by direct interactions of donor T cells with host tissue, but by pro-inflammatory cytokines like TNF and IL-1, which are released by alloreactive T cells. Furthermore, T cells use various effector mechanisms for the induction of GvHD, including Fas/FasL and granzyme/perforin-mediated cytotoxicity (Shlomchik, 2007). In addition to T cells, also NK cells can be involved in GvHD and GvL effects. In mismatched transplantation settings, where the patient lacks the appropriate killer inhibitory receptors (KIR), alloreactive NK cells can facilitate engraftment and lead to a significantly reduced relapse rate (Ruggeri *et al.*, 1999; Ruggeri *et al.*, 2007). Ideally, therapeutic strategies have to be developed to segregate GvL from GvHD to be beneficial for the patient. This can be achieved by T cells, which only recognise mHags that are expressed only in low amounts on normal tissue or are solely present on haematopoietic cells or

leukaemia-associated antigens that arise from genetic mutations in the malignant, leukaemic cells (Bleakley & Riddell, 2004).

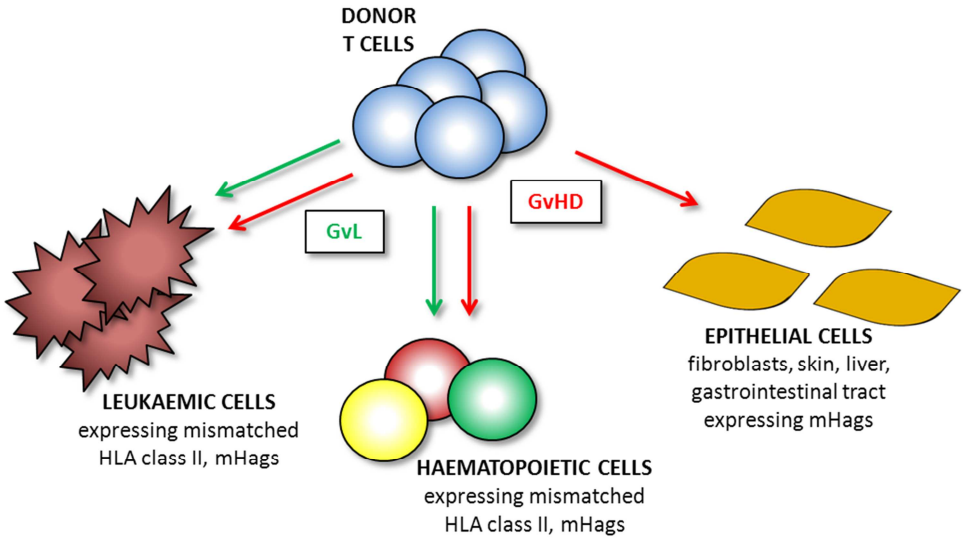

Figure 2.3: **Graft-versus-leukaemia (GvL) effect and graft-versus-host-disease (GvHD).** Donor T cells that are transplanted during allogeneic haematopoietic stem cell transplantation or later in donor lymphocyte infusions can mediate GvHD if they recognise ubiquitously expressed antigens. A selective GvL effect can be induced if the T cells are directed against leukaemia-specific or haematopoiesis-restricted minor histocompatibility antigens (mHags) or mismatched HLA class II molecules (derived from Bleakley & Riddell, 2004 and Riddell *et al.*, 2006).

2.5.4 HLA compatibility in transplantation

Allogeneic HSCT is of course only possible if a suitable donor is found. Siblings are the preferred donors, as there is a 25 % chance they inherited the same HLA haplotypes. If an appropriate relative is not available, a matched unrelated donor needs to provide the graft. Finding a matched donor proves to be difficult as there is a high polymorphism among HLA molecules. For HLA-DRB1 (encoding the DR β-chain), the highest polymorphic locus among class II genes, 1,355 alleles have been described so far that code for 1,005 different proteins. DQB1 and DPB1 still show 277 and 147 protein variants, respectively. With two DRA1, 32 DQA1 and 19 different DPA1 proteins, α-chains are less polymorphic (The EMBL-European Bioinformatics Institute, 2013). Nevertheless, certain alleles are more frequent among certain population groups. As HLA mismatches lead to an increased risk for severe GvHD, graft rejection and poor immune reconstitution, patient and donor usually have to be matched for both alleles of the HLA molecules -A/-B/-C/-DR/-DQ according to high resolution typing (Tiercy, 2002; Li & Sykes, 2012). Due to the close proximity of the HLA genes on chromosome 6, they are inherited together. Nevertheless, this does not apply for HLA-DP,

which is currently not taken into account when searching for a HSCT donor. As a recombination hotspot was found between HLA-DP and the remaining class II genes, high frequencies of mismatches in this particular locus can be found in 5 % of all sibling transplantations and in over 80 % of unrelated patient/donor pairs that are matched for the remaining ten HLA alleles (Begovich *et al.*, 1992; Santamaria *et al.*, 1994; Büchler *et al.*, 2002). Mismatches in the relatively non-polymorphic locus DPA1 were shown to have no influence on patient survival, whereas the role of DPB1 is still discussed controversially. There are studies reporting an enhanced severity of GvHD and reduced survival of patients with DPB1 mismatches (Varney *et al.*, 1999), whereas others claim that DP mismatches do not affect survival rates (Lee *et al.*, 2007). In another group an algorithm was developed to predict the immunogenicity and influence of DPB1 mismatches, categorising the mismatches into permissive and non-permissive disparities (Crocchiolo *et al.*, 2009).

2.5.5 Cellular therapies for leukaemia after HSCT

Various approaches have been investigated to reduce GvHD whilst maintaining the GvL effect and ensuring a rapid reconstitution of the T-cell repertoire. As an example, alloreactive T cells can be depleted from the transplant by use of the expression of activation markers. Nevertheless, GvHD could not be completely circumvented with this method and without GvHD also the GvL effect is often reduced, leading to an increased relapse risk (Solomon *et al.*, 2005; Barrett, 2008). Cells from the donor can also be administered to the patient after transplantation in a donor lymphocyte infusion (DLI) to induce a GvL effect. The potency of this approach was shown in CML patients, who relapsed after HSCT but went into long-term remission after DLI (Kolb *et al.*, 1990). *In vitro* generated or specifically selected T cells that target certain structures can be used for an improved DLI. Possible targets for cell-based leukaemia therapy are proteins that result from chromosomal translocations or mutations that lead to the generation of antigens that are specific for certain leukaemias (leukaemia-specific antigens, LSAs). A well-studied example for malignant fusion proteins is BCR-ABL, which is found in 95 % of all patients with CML, resulting from a translocation between chromosomes 9 and 22 (t(9;22)). BCR-ABL-specific CTLs have been found in CML patients as well as in individuals after HSCT (Brayer & Pinilla-Ibarz, 2013). In a phase II clinical trial of chronic-phase CML patients, they were shown to respond with BCR-ABL-specific T-cell proliferation after peptide vaccination (Cathcart *et al.*, 2004). Other antigens, which

are expressed by both, leukaemic and healthy cells, are called leukaemia-associated antigens (LAAs). Discrimination between normal and malignant cells can nevertheless be possible, as LAAs are often overexpressed on leukaemic blasts. Wilms' tumour protein 1 (WT1) for example, is overexpressed on AML blasts of most patients but is also weekly present in healthy tissue like kidney and the haematopoietic system. In a therapeutic mouse model WT1-specific T cells showed an elimination of AML blasts. Also the human telomerase reverse transcriptase (hTERT), which plays a role in promoting cell growth and survival, is overexpressed in a broad range of tumours but absent in normal, finally differentiated cells and can therefore be used for antigen-specific immunotherapy (Stauss *et al.*, 2008; Anguille *et al.*, 2012). The haematopoiesis restricted mHags HA-1 and HA-2 can also induce T-cell responses and complete remission in patients. Marjit and colleagues showed the presence of HA-1- and HA-2-specific CTLs in CML patients after DLI, which persisted and formed an immunological memory (Marijt *et al.*, 2003). In another study, they could also show that infusion of leukaemia-specific T cells can lead to complete remission in CML patients (Marijt *et al.*, 2007). The potent GvL effect of adoptively transferred T cells was also shown in patients with acute leukaemia after HSCT, that received infusions of *ex vivo* expanded mHag-specific $CD8^+$ T cells, which led to a transient remission of disease and persisted in the patients for up to three weeks (Warren *et al.*, 2010). Another option in contrast to the isolation and *in vitro* expansion of mHag-specific T cells is the engineering of donor T cells by introducing vectors encoding for the mHag-specific TCR, which was isolated from a different T-cell clone (Bleakley & Riddell, 2011). Chimeric antigen receptors (CARs), which combine the specificity of an antibody with the intracellular signalling of a TCR, are also investigated in cancer immunotherapy. Cytokine-induced killer cells that were transduced with retroviral vectors encoding CARs specific for CD123, the IL-3 receptor α-chain, which is over expressed in AML blasts and leukaemic stem cells, showed a strong lytic activity towards primary AML blasts (Tettamanti *et al.*, 2013). Re-infusion of autologous T cells engineered with CD19-specific CARs was also shown to lead to partial and sustained complete responses in patients with relapsed lymphoma and refractory CLL, respectively (Kochenderfer *et al.*, 2010; Porter *et al.*, 2011). For preventing latent virus reactivation, approaches to expand EBV-specific $CD8^+$ T cells *in vitro* have been investigated. CTLs were transferred into patients after HSCT and restored immunity to EBV (Heslop *et al.*, 2010).

2.5.6 $CD4^+$ T cells in cancer immunotherapy

Most research in terms of GvL/GvHD separation focuses on the role of transferred $CD8^+$ T cells. Nevertheless, as HLA class II expression is restricted to haematopoietic cells and thymic epithelial cells under non-inflammatory conditions, donor-derived $CD4^+$ T cells might additionally prove valuable in terms of generating specific cellular immune responses after HSCT to induce a GvL effect without causing severe GvHD (Klein & Sato, 2000). Furthermore, tumours frequently develop immune escape mechanisms like MHC class I downregulation or defects in antigen processing, leading to a loss of antigen presentation to $CD8^+$ T cells (Muranski & Restifo, 2009). A comparative study for assessing the potency of tumour-specific $CD8^+$ versus $CD4^+$ T-cell clones even showed that $CD4^+$ T cells were superior in tumour eradication (Perez-Diez *et al.*, 2007). In other groups it was found that from $CD8^+$/$CD4^+$ mixed cultures leukaemia-specific reactivity mostly arose from $CD4^+$ T cells (Thomas-Kaskel *et al.*, 2007; Zhong *et al.*, 2008). Nevertheless, the first autosomal HLA class II restricted mHags were not discovered until 2008. By the use of cDNA libraries, the HLA-DQ restricted antigen LBPI4K2B-1S, which is encoded by the phosphatidylinositol 4-kinase type II β gene, could be identified and a $CD4^+$ T-cell response against it was observed in a CML patient (Griffioen *et al.*, 2008). Important for targeting B-cell cancers was the discovery of the HLA-DQ restricted mHag that is encoded by the B-cell specific CD19 gene, which shows a single-nucleotide polymorphism (SNP) (Spaapen *et al.*, 2008). In a patient suffering from CML, $CD4^+$ T cells were discovered that recognised the four different HLA-DR restricted mHags (LB-MR1-1R, LB-PTK2B-1T, LB-LY75-1K and LB-MTHFD1-1Q). After DLI, the patient was in complete remission and showed only mild signs of GvHD, as the T cells were specific for haematopoietic cells but did not recognise non-haematopoietic cells like fibroblasts. LB-LY75-1K, which functions as scavenger receptor, shows a SNP and is predominantly expressed in haematopoietic cells. PTK2B, a tyrosine kinase, which is also mainly expressed by cells of the haematopoietic system, is of particular interest as it was reported to be involved in the dysregulation of several processed in malignant cells. The minor antigens MR1 and MTHFD1 however, are ubiquitously expressed. The fact that MR1- and MTHFD1-specific $CD4^+$ T cells spare epithelial targets might be explained with altered protein translation, which was described for CML progenitors (Stumpf *et al.*, 2009; Bleakley & Riddell, 2011). Additionally, cytotoxic $CD4^+$ T cells specific for the AML-associated fusion protein DEK-CAN have been described (Ohminami *et al.*, 1999). Furthermore, once potent

HLA class II-restricted mHag are identified, specificity of a certain $CD4^+$ T-cell clone can, like shown for $CD8^+$ T cells, be passed on to other cells by TCR transfer (Spaapen *et al.*, 2007).

Due to the high mismatch frequency regarding HLA-DP alleles, mismatch-reactive $CD4^+$ T cells have also been investigated by several groups. AML blasts were shown to be lysed directly by HLA-DP-specific T helper cells (Ibisch *et al.*, 1999). Rutten and colleagues found that in a patient suffering from chronic B-cell leukaemia $CD4^+$ and $CD8^+$ T cells could be isolated after DLI, but only $CD4^+$ HLA-DP mismatch-reactive clones showed lytic activity. In this patient a strong GvL response and long-term complete remission was observed with only minimal GvHD (Rutten *et al.*, 2008; Rutten *et al.*, 2013).

With regard to the predominant $CD4^+$ subsets, both T_H1 and T_H2 cells showed anti-tumour activity in therapeutic models. Nevertheless, the T_H1 compartment appeared to be superior in forming an immunological memory with long-term persistence (Xie *et al.*, 2010; Nishimura *et al.*, 2000). Others reported that T_H2 cells induced GvL but prevented lethal GvHD in a B-cell leukaemia model. Novel strategies might include the administration of T_H1 for mediating a GvL effect with a delayed infusion of T_H2 cells for the treatment of GvHD (Fowler, 2006). Regarding the differentiation state of T helper cells, it was shown that the effector memory compartment, characterised by the absence of CD62L expression, mediated strong anti-tumour activity with a reduced induction of GvHD when compared to unfractioned naive cells due to a reduced alloreactive potential (Anderson *et al.*, 2003; Chen *et al.*, 2004).

2.6 Aim and motivation of this study

Progress in leukaemia therapy has been made over the last couple of years. Nevertheless, patients suffering from high-risk acute myeloid leukaemia (AML) can only be cured by allogeneic haematopoietic stem cell transplantation (allo-HSCT). This therapeutic approach is unfortunately often associated with graft-versus-host disease (GvHD) which leads to high morbidity and mortality rates in patients after allo-HSCT. However, GvH-reactivity is frequently accompanied by graft-versus-leukaemia (GvL) responses, which facilitate prevention of disease relapse. Allo-HSCT and donor lymphocyte infusion (DLI) are constantly revised and improved, but mediating a satisfactory GvL effect without inducing severe GvHD still remains a challenging task. Previous research has mainly focused on $CD8^+$ cytotoxic T cells as GvL effectors. Nonetheless, T helper cells can not only stimulate and recruit other

cells of the immune system but were also shown to exhibit direct cytolytic activity towards malignant haematopoietic cells. Moreover, frequent tumour evasion strategies like major histocompatibility complex (MHC) class I downregulation can be avoided using $CD4^+$ T cells (Knutson & Disis, 2005). In clinical studies it was previously shown that CD8 depleted DLI after HSCT led to an accelerated immune reconstitution with a low risk of inducing severe GvHD (Meyer *et al.*, 2007) and in a therapeutic acute lymphoid leukaemia mouse model the potential of cytolytic leukaemia-specific $CD4^+$ T cells could already be shown (Stevanović *et al.*, 2012). As $CD4^+$ T cells recognise peptides presented by human leucocyte antigen (HLA) class II molecules that are not expressed on non-hematopoietic cells under non-inflammatory conditions, they might have a more favourable safety profile compared to $CD8^+$ T cells in terms of avoiding GvHD (Knutson & Disis, 2005). Furthermore, leukaemia-reactive $CD4^+$ T cells have been isolated from allo-HSCT patients upon GvL responses, and were successfully used to identify HLA class II-restricted minor histocompatibility antigens (mHag). $CD4^+$ T cells can either recognise tumour antigens through presentation by antigen presenting cells or directly by HLA class II expressing tumours. Most tumours do not express HLA class II molecules, whereas malignant cells of the haematopoietic system usually do, even under non-inflammatory conditions (Knutson & Disis, 2005; Klein & Sato, 2000). This suggests that $CD4^+$ T cells do not necessarily need to be directed against differences in minor but can also directly recognise major histocompatibility antigens to selectively mediate GvL effects. Due to the high mismatch probability in the HLA-DP locus of over 80 % in unrelated transplantations, $CD4^+$ T cells specific for the mismatched alleles might play a major role in GvL effects, simultaneously avoiding HLA class II negative GvHD targets (Falkenburg & Warren, 2011).

The aim of this study was to develop a reliable protocol for the *in vitro* generation of monoclonal AML-reactive $CD4^+$ T cells. Therefore, the recently published method for the generation of AML-reactive $CD8^+$ T cells was adapted (Albrecht *et al.*, 2011). Naive $CD4^+$ T cells that contain the most diverse T-cell receptor repertoire (Nikolich-Zugich *et al.*, 2004) and therefore most AML- and alloreactive precursors were isolated from healthy donors and were stimulated against patients' AML blasts. After expansion, T cells were analysed for their recognition of fibroblasts, which represent potential GvHD targets. To analyse the target structure that was recognised by the $CD4^+$ T cells, the HLA status of patient and stem cell donor had to be investigated. They were usually matched for HLA-/-B/-C/-DR/-DQ but

mismatched for HLA-DP, following common clinical rules of donor selection in allogeneic HSCT. However, there are controversial reports about mismatches in the HLA-DP alleles. It is believed that there are permissive and non-permissive alleles that either reduce the relapse risk or increase mortality rates and the likelihood of GvHD, respectively (Fleischhauer *et al.*, 2012). Therefore, mismatched HLA-DP alleles were cloned and investigated as target structures of the aforementioned $CD4^+$ T cells. In addition to the activation of the T cells, which was assessed by their cytokine secretion profile, their potential to lyse leukaemic blasts was tested, which would be necessary for mediating a potent GvL effect.

To determine the therapeutic potential of leukaemia-reactive $CD4^+$ T cells, they were used for adoptive T-cell transfer into NOD-scid IL2Rγ^{null} (NSG) mice engrafted with human AML blasts. During these experiments, the ability of $CD4^+$ T cells to migrate into lymphoid tissue, which is associated with the expression of homing molecules (Appay *et al.*, 2008), and their ability to persist within the mice was analysed. To assess the *in vivo* effect mediated by the transferred $CD4^+$ T cells, their capacity to reduce the level of engrafted AML blasts was determined.

Patient-specific $CD4^+$ T cells generated *in vitro* from healthy stem cell donors by stimulation with patient primary AML blasts could serve as a valuable tool for adoptive T-cell therapy of leukaemia, mediating GvL effects without causing severe GvHD. In the future, they might be clinically applied in allogeneic HSCT patients at the time of minimal residual leukaemia disease, when immunotherapy was shown to be most effective (Slavin, 2005).

3 Methods

3.1 Cell culture techniques and immunological methods

3.1.1 General materials for cell culture

For a detailed list of manufacturers see chapter 3.4.

Instruments

Centrifuge	Megafuge 1.0R	Her
	Megafuge 1S-R	Her
Cryobank Espace 331 Gaz		AL
Incubator Hera cell 240		Her
Microscope Axiovert 40 C, 25		Zei
Purelab classic (for deionising water)		ELG
Sterile bench Hera safe HS 18		Her

Special Implements

Cell culture flasks	25 cm^2, 75 cm^2, 175 cm^2	Gre
Cell culture plates	6/24/48/96-F/96-U/96-V well	Gre
Cell strainer 100 µm		BD
Counting chamber Fuchs-Rosenthal		Mar
Cryo.s 1 ml tubes		Gre
Falcon tubes	15 ml, 50 ml	Gre
Freezing container		Nal
Petri dish	35 ml, 60 ml, 95 ml	Gre
Pipetboy acu		Int
Pipette	10 µl	Epp
	20 µl, 200 µl, 1000 µl	Sta
	Multichannel	Sta
	1ml, 2 ml, 5 ml, 10 ml, 25 ml, 50 ml	Gre
Pipette tips TipOne	10 µl, 200 µl, 1000 µl	Sta
Reaction tube	0.5 ml, 1.5 ml	Epp
Stericup Express plus 0.22 µm		Mip

Stericup Durapore 0.45 µm for filtration	Mip
Syringes and cannulas	Bra

Buffers, chemicals and media

2-Propanol	Rot
AIM-V medium	G/I
Bovine serum albumin (BSA)	SiA
Cyclosporin A (Sandimmun 50mg Konzentrat)	Nov
DMEM	G/I
Dimethylsulfoxide (DMSO)	Rot
DNase I	Roc
Ethylendiaminetetraacetic acid (EDTA)	SiA
Fetal bovine serum (FCS)	PAA
Ficoll lymphocyte separation medium LSM1077	PAA
Formaldehyde (37 %)	Mer
Heparin-natrium 25000	Rat
Human albumin	CSL
NaCl	Rot
Penicillin/Streptomycin (10000 U/ml Pen + 10000 µg/ml Strep)	G/I
Phosphate buffered saline (PBS) (for sterile use)	G/I
PBS dry chemical “instamed” (for unsterile use)	Bic
Phytohaemagglutinin (PHA)	Mur
RPMI 1640 + 1 % L-glutamine medium	G/I
Sodiumbutyrate	SiA
Trypsin-EDTA (0.05 %)	G/I
Trypan blue	Mer
Tween20	App

Human serum was provided by the blood transfusion centre of the University Medical Centre Mainz. Serum from at least 15 patients was pooled, sterile filtered, heat inactivated at 56 °C for 45 min and stored at -80 °C.

Solutions

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

Culture media

T-cell medium	AIM-V + 10 % human serum
Cell-culture medium	RPMI 1640 + 10 % FCS + 1 %Pen/Strep
Fibroblast medium	DMEM + 10 % FCS + 1 %Pen/Strep
Freezing medium	AIM-V + 8 % human albumin + 10 IU/ml heparin + 10 % DMSO
LP freezing medium	AIM-V + 8 % human albumin + 10 IU/ml heparin + 10 % DMSO + 0.1 mg/ml DNase I

Recombinant human cytokines

GM-CSF	San
IFN-γ	R&D
IL-2 (Proleukin™)	Nov
IL-4	Mil
IL-7	R&D
IL-12	R&D
IL-15	R&D
IL-21	BiM
IL-27	R&D
TNF-α	PrK
TNF-β	SiA
SCF	R&D

3.1.2 General cell culture conditions

All eukaryotic cells, cell lines as well as primary cells were handled under sterile conditions and were cultivated at 37 °C in an incubator with a humidity of 95 % and a 5 % CO_2 atmosphere. For the counting of cells, a dead-live staining using trypan blue solution was performed.

Cells were stored in a cryo bank with liquid nitrogen. Therefore, cells were harvested by centrifugation (470 x g, 5 min, RT, break turned on), resuspended in freezing medium and stored in aliquots of 1 ml in cryo tubes that were gradually frozen down to -80 °C in a cryo box filled with isopropanol. The cryo tubes were then transferred into the cryo bank at -180 °C. Portion sizes varied from 2-15x10^6 for T cells, from 1-20x10^7 for PBMC and AML blasts and from 0.4-3x10^6 for fibroblasts. For thawing, the cells were defrosted in AIM-V or RPMI 1640. DMSO was removed by centrifugation, and cells were resuspended in the appropriate medium for counting and further use.

The cell line K562 that were originally derived from a patient suffering from chronic myeloid leukaemia (Lozzio & Lozzio, 1975) was used as NK-cell target due to its low expression levels of HLA molecules (Drew *et al.*, 1977; Ziegler *et al.*, 1981). They were adjusted twice a week to 4x10^5 cells in 75 cm^2 cell culture flasks in cell-culture medium.

3.1.3 Isolation of human peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated from whole blood samples of healthy donors, bone marrow aspirates or leukapheresis products by ficoll gradient density centrifugation. Therefore, the buffy coat was diluted with PBS and 15 ml separation medium were carefully overlaid with 30 ml of this diluted mixture in 50 ml falcon tubes. After centrifugation at 836 x g for 20 min with the break turned off to separate the leucocytes from the erythrocytes, the leucocytes in the interphase were collected, washed with cold PBS and centrifuged afterwards at 677 x g for 10 min. The cells were then pooled and washed two more times with cold PBS, each washing step followed by a centrifugation step of 470 x g for 5 min. Cells were counted, resuspended in freezing medium or LP freezing medium and were then gradually frozen for storage at -180 °C with a concentration of 5-20x10^7 cells/ml.

Alternatively, leukapheresis products could be frozen without prior ficoll separation in LP freezing medium.

3.1.4 Primary cell material

AML blasts were isolated from leukapheresis products, bone marrow aspirates or peripheral blood of patients. Patient samples as well as buffy coats of healthy donors were provided by the Centre for Blood Transfusion of the University Medical Centre of the Johannes Gutenberg-University Mainz. The study was approved by the local ethics committee.

Informed consent was obtained from participants in accordance with the Declaration of Helsinki.

For a genomic high-resolution typing of HLA-alleles, samples were sent to the Institute of Immunology and Genetics in Kaiserslautern, Germany.

3.1.5 Pre-incubation of AML blasts

To obtain a suitable antigen-presenting cell (APC) phenotype, primary AML blasts were thawed and pre-incubated at ≤3x10^6 cells/ml in T-cell medium prior to the stimulation. Various cytokines were added for different time spans (Table 3.1). From these different variants, the one leading to an ideal expression of co-stimulatory and HLA class II molecules on the AML blast surface in combination with a sufficient rate of cell survival was determined for each patient sample individually for further experiments.

Table 3.1: Cytokine combinations used for AML maturation. To obtain an APC phenotype of AML blasts, they were pre-incubated under five different cytokine conditions for a certain time span.

variant No	cytokine combinations	duration of incubation	citation
1	w/o cytokines	1 day	(Brouwer *et al.*, 2000a)
2	1 mM sodiumbutyrate 500 U/ml IFN-γ	2 days	(Maeda *et al.*, 2000)
3	1 ng/ml TNF-β 500 U/ml IFN-γ	3 days	(Eljaafari *et al.*, 2006)
4	1 ng/ml TNF-β 500 U/ml IFN-γ	4 days	(Eljaafari *et al.*, 2006)
5	1000 U/ml IL-4 10 ng/ml TNF-α 5 ng/ml SCF 10 ng/ml GM-CSF	4 days	(Brouwer *et al.*, 2000b)

3.1.6 Cell separation by magnetic beads isolation

Materials

CD4 MicroBeads	Mil
CD8 MicroBeads	Mil
CD45RO MicroBeads	Mil
Columns (LD, LS, MS)	Mil
MACS Multistand	Mil
MiniMACS and MidiMACS Separator	Mil
Naive $CD4^+$ T Cell Isolation Kit II	Mil

Naive $CD8^+$ T Cell Isolation Kit	Mil
Separation filter	Mil
MACS buffer	PBS + 5 g/l BSA + 2mM EDTA

Procedure

In a magnetic activated cell sorting (MACS), different cell populations can be separated from one another. They can be labelled with antibodies according to their expression of surface antigens. The antibodies are either directly coupled to magnetic beads or in a second incubation step. When the cells then pass through a column that is placed in a magnetic field, labelled cells are retained and can be eluted by removing the column from the magnet. With this method, cells can either be positively selected by binding them to an LS- or MS-column or negatively selected by depleting them on an LD-column.

Naive $CD4^+$ T cells were isolated from donor PBMCs using the Naive $CD4^+$ T Cell Isolation Kit II according to the manufacturer's protocol. In this kit, untouched naive $CD4^+$ T cells ware isolated in an indirect labelling system using LS- or MS-columns. To improve purity of the desired fraction, an additional $CD8^+$ depletion step could be added. Therefore, cells were subsequently incubated with CD8 MicroBeads according to the manufacturer's protocol. The depletion was performed on an LD-column.

Alternatively, whole $CD4^+$ T cells could be isolated using CD4 MicroBeads and LS- or MS-columns. To obtain naive $CD4^+$ T cells, a $CD45RO^+$ and $CD8^+$ depletion could alternatively be performed previously. Therefore, the incubation with CD45RO MicroBeads was followed by one using CD8 MicroBeads, each using LD-columns for the depletion (see the manufacturer's protocol).

For comparison, also naive $CD8^+$ T cells could be isolated, either using the Naive $CD8^+$ T Cell Isolation Kit (LD- and MS-column) or a $CD45RO^+$ depletion (LD-column) followed by a $CD8^+$ Isolation (LS-column).

Purity of the isolated T cells was assessed, using flow cytometric analysis (see chapter 3.1.11) by staining the cells for the surface markers CD3, CD4, CD8, CD16, CD27, CD28, CD31, CD45RO, CD45RA, CD56, CD57, CD62L, CD95, CD122, CD127, CCR7 and CXCR4.

The flowthrough was also collected in all approaches and used as feeder cells for T-cell stimulation (see chapter 3.1.7).

3.1.7 Stimulation of mini-mixed lymphocyte/leukaemia cultures (mini-MLLCs)

Instruments

Gamma-irradiator Gammacell 2000 (^{137}Cs) Mol

Patient data

For the generation of leukaemia-reactive $CD4^+$ T cells, eight patient/donor pairs were used. Unless indicated otherwise, the donors mentioned also donated stem cells and DLI for the actual transplantation of the patients. AML blasts from each patient were obtained either from leukapheresis products, peripheral blood or bone marrow aspirates.

Patient MZ237 was first diagnosed with M4eo AML in September 2009 with an initial white blood count (WBC) of $1.1x10^5$/µl. She was transplanted 16 months later from an unrelated, 10/10-matched donor (Don662). Typing of the HLA-DP alleles revealed a mismatch in one HLA-DP allele. Two months after HSCT, the patient died. For in vitro experiments, an additional donor was found (Don256) that actually donated for another patient (see Table 3.2 for all patient/donor pairs). AML blasts from patient MZ237 were obtained at initial diagnosis.

First diagnosis of patient MZ285 with AML M4 occurred in November 2008. She showed a WBC of $5.8x10^4$/µl. After chemotherapy, the AML relapsed in 2010 and the patient was transplanted in November of the same year from an unrelated donor (Don205) that showed a mismatch in one HLA-C and one –DP allele. Afterwards, she suffered from acute GvHD of the skin (grade III) and intestine (grade I) and died in May 2011. Patient material was obtained from peripheral blood in 2009.

Patient MZ369 was first diagnosed in November 2005 with a WBC of $1.5x10^5$/µl. She was transplanted from her 10/10-matched brother (Sib167) 15 months later but relapsed twice (May and July 2006). After a donor lymphocyte infusion (DLI) in August 2006, she relapsed again and died in November of the same year. AML blasts used for experiments were obtained at the time of relapse.

First diagnosis of patient MZ667 occurred in May 2007. After reconstitution, she was transplanted from an unrelated 10/10-matched donor and died one day later. Patient material for experiments was obtained at first diagnosis.

Patient MZ683 was hospitalised in August 2005 with a severe leucocytosis (>2x10^5 leucocytes/µl) and a therapeutic leukapheresis was performed on two consecutive days. After conditioning, she received an allogeneic transplantation from a donor (Don595) that was mismatched in both HLA-DP alleles but matched in the remaining alleles in January 2006. Shortly afterwards, she suffered from grade II GvHD of the skin, relapsed in March 2006 and died three months later.

First diagnosis of patient MZ770 with AML M5a occurred in June 2009, when she was hospitalised with a WBC of 8.05x10^4/µl. She was transplanted from an unrelated donor matched for HLA class I, -DR and -DQ alleles in October 2010. After a first CD8-depleted DLI in January 2010 she went into remission but relapsed only one month later. She received a second DLI in April, relapsed again in May and died in September 2010. AML blasts from leukapheresis products at initial diagnosis were used for experiments.

AML blasts from patient MZ747 were obtained in May 2007 when he was admitted to the hospital with a WBC of 1x10^5/µl. The 10/10-matched transplant (Don974) he received in September 2007 was derived from an unrelated donor. The patient suffered GvHD of the skin in October 2009 and died two months later.

Patient MZ849 was diagnosed in November 2010 as high risk patient with 6.8x10^4 leucocytes/µl. He showed a complete remission after induction and was transplanted in February 2011 from his HLA-identical brother (Don691). Three months later, he received a CD8-depleted DLI. Even though he suffered from chronic GvHD of the skin, he is still alive. AML blasts used for *in vitro* experiments were obtained in 2010.

In the case of patient MZ921, AML was first diagnosed in March 2012 and she was classified as high risk patient. She showed a very high number of leucocytes (1.8x10^5/µl) and was transplanted in June 2012 by an unrelated donor (Don671) that was matched except for both HLA-DP alleles. In same year's November, she received DLI but relapsed two weeks afterwards. After a second DLI and transplantation, she died in March 2013. This patient's blasts, which were obtained at initial diagnosis, were not only used for the stimulation of naive $CD4^+$ but also $CD8^+$ T cells (HLA-matched for A*01:01/30:01, B*08:01/58:01, C*07:01).

Table 3.2: AML blasts used for the generation of CD4$^+$ T cells. AML patients are listed with their disease characteristics (FAB classification, karyotype and molecular markers). (mut. = mutated, inv = inversion)

name	FAB class.	year of birth	karyotype	molecular markers
MZ237-AML	M4eo	1963	46, XX	inv (16 p13.1q22) [24]
MZ285-AML	M4	1940	46, XX	none
MZ369-AML	M4	1976	46, XX	none
MZ667-AML	M1	1953	46,XX	FLT3-ITD mut. NPM1 mut.
MZ683-AML	M2	1951	46, XX	FLT3-ITD mut.
MZ770-AML	M5a	1947	46, XX	FLT3-ITD mut.
MZ747-AML	M2	1955	46, XY	FLT3-ITD mut. NPM1 mut.
MZ849-AML	M1	1952	46, XY	FLT3-ITD mut. NPM1 mut.
MZ921-AML	M5	1963	46, XX	FLT3-ITD mut. NPM1 mut.

Procedure

For the stimulation of AML-reactive CD4$^+$ T cells, a mini-MLLC approach was used. Therefore, naive T cells that were isolated from donor PBMCs by FACS sorting (see chapter 3.1.11) or MACS (see chapter 3.1.6) were co-cultured with pre-incubated patient AML blasts (see chapter 3.1.4). In matched settings, patients and donors were HLA-identical in their -A/-B/-C/-DR/-DQ alleles, but not necessarily in HLA-DP, according to high resolution typing. In mini-MLLCs, 1x10^4 irradiated primary AML blasts (35 Gray) were seeded per well in a 96-well U-bottom plate as stimulator cells. Non-naive T cells and remaining PBMCs from the same donor were used as feeder cells after an irradiation at 35 Gray at a concentration of 1x10^4 cells/well. To obtain clonal T-cell populations, various starting concentrations of naive T cells were compared in a limiting dilution approach (1x10^4/7.5x10^3/5x10^3/2.5x10^3/ 1x10^3 cells/well). Cells were cultured in a volume of 200 µl T-cell medium supplemented with 5 ng/ml IL-7, 1 ng/ml IL-12 and 5 ng/ml IL-15. To determine optimal cytokine conditions, approaches additionally using 10 ng/ml IL-27 (day 0-13), 10 ng/ml IL-21 (day 0-20) or without IL-12 were compared. Cells were restimulated weekly 1:1 with irradiated AML blasts (plus cytokines) and were split 1:2 in between with T-cell medium without cytokines if growing well. From day 14 on, IL-12 was replaced by 50 U/ml IL-2. On day 14+5, split-well IFN-γ ELISpot assays (2x10^4 effector cells plus 5x10^4 target cells/well) were performed, where each mini population was separately tested for its spontaneous IFN-γ release and its reactivity towards AML blasts. The mini populations identified as AML-reactive, were transferred on

day 21 into 48 well-plates in a total volume of 1 ml and later, according to growth, to 24 well-plates in a total volume of 2 ml. Cells were tested either on day 4 or 5 after restimulation and frozen on day 7. This protocol was adapted from published protocols established by Distler *et al.*, 2008 and Albrecht *et al.*, 2011.

In case of patient/donor pairs with multiple HLA mismatches, which were used to obtain control T cells ($CD4^+$ or $CD8^+$), split-well ELISpots could already be performed on day 12 and therefore, cells were transferred into bigger wells at an earlier time point.

3.1.8 Generation and cultivation of B-lymphoblastoid cell lines (LCLs)

LCLs were generated by incubating $1x10^7$ PBMCs with 500 µl of Epstein-Barr virus supernatant generated from B95.8 cells and 200 µl FCS over night at 37 °C. 4.3 ml cell-culture medium containing a total of 20 % FCS and 1 µg/ml cyclosporine A were then added, and the cells were transferred to a 25 cm^2 tissue culture flask. After 24 h of incubation, cyclosporine A was removed by centrifugation (470 x g, 5 min). The cells were then resuspended in 5 ml cell-culture medium supplemented with 20 % FCS for expansion. Once LCLs grew properly, they were transferred into normal cell-culture medium and adjusted to $4x10^5$ cells in a 75 cm^2 cell culture flask twice a week.

3.1.9 Enzyme-linked immuno spot (ELISpot) assay

Materials

3-amino-9-ethylcarbazole (AEC)	SiA
Acetic acid	Rot
Anti-hIFN-γ-antibody #1-D1K (capture antibody)	Mab
Anti-hIFN-γ-antibody #7-B6-1 (detection antibody)	Mab
BD ELISPOT AEC substrate set	BD
Ethanol (>99 %, EtOH)	Rot
Human TNF ELSPOT Set	BD
Hydrogen peroxide (30 %, H_2O_2)	SiA
N,N-dimethylformamid (DMF)	SiA
Multiscreen HST™ IP 96-well filtration plate	Mip
Sodium acetate	SiA
Reagent A + B Vectastain Elite Kit	Vec

Buffers and solutions

Avidin/HRP-complex solution	10 ml PBS + 0.01 % Tween20 + 1 drop reagent A + 1 drop reagent B
Acetate buffer	Aqua bidest + 2.9 g/l sodium acetate + 1.5 % acetic acid
AEC solution	10 AEC tablets dissolved in 25 ml DMF ad 500 ml acetate-buffer 0.5 µl/ml 30 % H_2O_2 prior to use
Wash buffer	PBS + 0.05 % Tween20

Monoclonal blocking antibodies

Monoclonal antibodies (mAb) from murine origin with anti-human blocking activity (Table 3.3) were purified from hybridoma supernatant and stored at -20 °C.

Table 3.3: Monoclonal blocking antibodies used in IFN-γ ELISpot assays. To determine the specificity of T ells, blocking antibodies that target different HLA molecules were used.

mAb name	target structure	concentration	citation
B7/21 (IgG3a)	HLA-DP	215 µg/ml	(Watson *et al.*, 1983)
Hb55 (IgG2a)	HLA-DR	90-110 µg/ml	(Lampson & Levy, 1980)
SPV-L3 (IgG2a)	HLA-DQ	440 µg/ml	(Spits *et al.*, 1983)
W6/32 (IgG2a)	HLA class I	83–150 µg/ml	(Barnstable *et al.*, 1978)

Instrument and software

CTL ELISpot reader S5 Versa Analyzer	CTL
ImmunoCapure software	CTL
ImmunoSpot software	CTL

Procedure

The frequency of cells that release a certain cytokine can be measured in ELISpot assays. Therefore, capture antibodies are bound to a membrane in 96-well ELISpot plates. Effector and target cells are then seeded into the well. After this co-incubation, cells are washed away and the released cytokines that are bound to the primary antibody bind a secondary antibody. This detection antibody can be coupled to an avidin/horse radish peroxidase (HRP)-complex. The enzyme can then convert a substrate (AEC) and induce coloured spots on the membrane surface, whereupon each spots corresponds to one activated, cytokine producing effector cell.

IFN-γ ELISpot assay

When getting in contact with target cells, e.g. AML blasts, T cells are activated and produce the cytokine IFN-γ that is secreted into the medium. In an IFN-γ ELISpot assay, plate membranes had to be pre-conditioned using a 35 % ethanol solution. After washing the plates three times with PBS the wells were coated with 60 µl capture antibody (10 µg/ml in PBS) and incubated over night at 4 °C. Unbound antibodies were washed away in three washing steps using PBS. For blocking of unspecific binding sites, the plates were then incubated with 100 µl T-cell medium per well. One hour later, T cells were seeded in duplicates with 0.4-2x10^4 cells/well in T-cell medium. T cells were tested 4 or 5 days after restimulation. Target cells were added in a concentration of 5x10^5 cells/well in T-cell medium, except for fibroblasts (1x10^5 cells/well). The total volume amounted to 100 µl/well. If HLA-blocking was included in the experiment, the target cells were pre-incubated with blocking antibodies (see Table 3.3) for 30 min at 37 °C. When finished, ELISpot plates were incubated at 37 °C for 18 to 20 h for blocking experiments and 20 to 22 h if no blocking antibodies were used.

For detection, plates were washed six times with wash buffer and then incubated with 60 µl detection antibody (2 µg/ml in PBS 0.5 % BSA) per well for 2 h at 37 °C. After six more washing steps using wash buffer, 100 µl avidin/HRP-complex solution, which was pre-incubated for 30 min, were added per well and incubated for 1 h (RT) in the dark. Plates were then washed three more times in wash buffer and subsequently three times in PBS. In a final step, 100 µl AEC solution were added per well and incubated for 10 min at RT. The colour reaction was stopped by washing the plate with tap water. After the membranes were dry, spots were counted on a CTL ELISpot reader.

TNF-α ELISpot assay

Activated T_H1 cells also produce TNF-α which can be detected in an ELISpot assay. Coating and blocking of the ELISpot plates was performed as described for IFN-γ. Nevertheless, TNF-α ELISpot incubation was prolonged to 40 h at 37 °C to achieve an optimal spot production. For the detection, the BD ELISPOT AEC substrate set was used which led to clearer, more distinguishable spots. All other steps were performed as described above for the IFN-γ ELISpot assay.

3.1.10 IL-4 Enzyme-linked immunosorbent assay (ELISA)

Materials

ELISA plate 96-well flat, high binding	Gre
H_2SO_4	Rot
IL-4 ELISA Ready-SET-Go!	EBS
TMB substrate	SiA

Instruments

ELISA Reader MRX Revelation and software	DyT

Procedure

Similar to ELISpot assays, a sandwich ELISA can be used to detect molecules that are secreted into the supernatant by activated cells. High binding ELISA plates are coated with a primary antibody directed against an epitope of the cytokine in question. After incubation with cel-culture supernatant, a secondary biotin-conjugated antibody can bind to a different epitope. An avidin-HRP can be added to induce a colour reaction of a substrate solution. The concentration of the detected cytokine can then be assessed by comparing its absorbents values to a serial dilution of a standard solution.

To distinguish between T_H1 and IL-4-producing T_H2 cells, an IL-4 sandwich ELISA was performed according to the manufacturer's protocol. Supernatant from the T-cell culture was taken on day 3 after restimulation and stored at -20 °C. For the assay 100 µl of supernatant were used per well. Values were measured in duplicates. In addition to the substrate solution contained in the kit, TMB substrate was used. The reaction was stopped with H_2SO_4. Extinction was measured at λ = 450 nm.

3.1.11 Flow cytometry (FACS)

Materials

Cytofix/Cytoperm	BD
FACS Clean Solution	BD
FACS Flow Sheath Fluid	BD
FACS Rinse Solution	BD
FACS Shutdown Solution	BD
Saponin, from quillaja bark	SiA

Buffers

FACS buffer	PBS + 1 g/l BSA
FACS FIX	PBS + 1 % formaldehyde
Sort buffer	PBS + 1 % human serum + 2 mM EDTA
Saponin buffer	PBS + 1 g/l BSA + 1 g/l saponin

Instruments and software

FACS Aria (Cell sorter)	BD
FACS Canto II (Flow cytometer)	BD
FACS Diva Software (for measuring)	BD
Expo32 Software (for re-analysis)	BC

Antibodies

All antibodies used were of murine origin directed against human target structures. They were stored at 4 °C and handled under sterile conditions.

Table 3.4: Fluorochrome-coupled monoclonal antibodies for FACS staining: Antibodies directed against a broad panel of intra- and extracellular molecules were used. For multicolour staining, they were coupled to different fluorochromes. In titration experiments, ideal antibody amounts were determined. Antibodies conjugated with Horizon V450 were diluted in PBS.

marker	fluoro-chrome	µl/tube	manufacturer
CD3	APC	3	BD
	FITC	3, 3	BC, BD
	Horizon V450	1:5, 2	BD
	PE	4, 2	BC, BD
CD4	APC	2	BD
	FITC	2, 3	BC, BD
	Horizon V450	1:5, 2	BD
	PE	2, 2	BC, BD
	PerCP	1	BD
CD8	APC	2, 2	BC, BD
	FITC	5, 5	BC, BD
	Horizon V450	1:10, 2	BD
	PE	3, 3	BC, BD
	PerCP	1	BD
CD16	FITC	5	BC
CD25	FITC	10	BD
CD28	PE	10, 10	BC, BC
CD31	APC	5	Mil
CD33	APC	5	BD
	PE	5	BD
CD40	PE	10	BD
CD45	APC	2	BD
	APC-H7	1	BD
	FITC	2, 2	BC, BD
	PE	2, 2	BC, BD
CD45RA	APC	3	BD
	PE	10	BC
CD45RO	PE	5, 5	BC, BD
CD54	PE	5	BD
CD56	PE	5	BC, BD
CD57	APC	3	Mil
CD58	FITC	5	BC
CD62L	PE	3, 3	BC, BD
CD80	FITC	20	BC
CD83	PE	5	Mil
CD86	PE	3	BC
CD90	PE	5	BC
CD95	PE	10, 10	BC, BD
CD127	PE	5, 2	BC, BD
CD184 (CXCR4)	APC	10	BD
CD197 (CCR7)	FITC	5	R&D

marker	fluoro-chrome	µl/tube	manufacturer
GranzymeA	PE	2	BD
GranzymeB	Alexa Fluor 674	2	BD
HLA-ABC	FITC	5	BD
HLA-DP	PE	1	Abc
HLA-DQ	FITC	3	BD
HLA-DR	PE	5	BC
IgG1	Alexa Fluor 674	/	BD
	APC	/	BD
	FITC	/	BC, BD
	Horizon V450	/	BD
	PE	/	BC, BD
	PerCP	/	BD
IgG2b	FITC	/	BD
Perforin	FITC	3	BD (Lot: 70068)
TCR Vβ1	FITC	5	BC
TCR Vβ2	PE	5	BC
TCR Vβ3	FITC	5	BC
TCR Vβ4	PE	5	BC
TCR Vβ5.1	PE	5	BC
TCR Vβ5.2	FITC	5	BC
TCR Vβ5.3	PE	5	BC
TCR Vβ7.1	FITC	5	BC
TCR Vβ7.2	PE	5	BC
TCR Vβ8	FITC	5	BC
TCR Vβ9	PE	5	BC
TCR Vβ11	FITC	5	BC
TCR Vβ12	PE	5	BC
TCR Vβ13.1	PE	5	BC
TCR Vβ13.2	PE	5	BC
TCR Vβ13.6	FITC	5	BC
TCR Vβ14	FITC	5	BC
TCR Vβ16	FITC	5	BC
TCR Vβ17	PE	5	BC
TCR Vβ18	PE	5	BC
TCR Vβ20	FITC	5	BC
TCR Vβ21.3	FITC	5	BC
TCR Vβ22	FITC	5	BC
TCR Vβ23	PE	5	BC

Procedure

In flow cytometry (short FACS for fluorescence activated cell sorting), cells can be characterised by their relative size (forward scatter), relative granularity (side scatter) and

phenotypic molecules. Cell surface structures or intracellular molecules are bound by fluorochrome-conjugated antibodies that can be excited by lasers. The low-energy light that is then emitted can be detected and cells can be classified due their phenotypic appearance.

Therefore, an average of $1x10^5$ cells were first washed with FACS buffer and were then incubated with the respective antibodies (see Table 3.4) for 10 min at room temperature in the dark. After washing the cells with FACS buffer to remove unbound antibodies they were resuspended in FACS FIX and stored at 4 °C in the dark until measured using the FACS Canto II. The relative fluorescence intensity was calculated from median fluorescence intensity (MFI) values divided by MFI values of respective IgG isotype control stainings.

For intracellular staining, the cells were incubated with Cytofix/Cytoperm at 4 °C for 20 min after the extracellular staining and washing step. They were then washed twice with saponin buffer and subsequently incubated with antibodies for the intracellular staining or isotype controls for 30 min at 4 °C. Afterwards, a washing step and fixation was performed as it was described for the extracellular staining.

Flow cytometry could also be used for the sorting of cells. To prevent the cells from clogging the instrument, they war thawed in 2 ml DNase I (1 mg/ml). Up to$1x\ 10^8$ cells were stained with the 10-fold amount of antibody that was used for a normal FACS staining, listed in Table 3.4. Antibody combinations used were CD3/CD4/CD45RA for sorting of naive $CD4^+$ T cells and CD3/CD4/TCR Vβ2 for the isolation of one clonal T cell out of an oligoclonal cell population. Staining was performed for 15 min at 4 °C. Unbound antibodies war removed by washing with sort buffer. Cells were then filtered through a separation filter and adjusted to a final concentration of $1\text{-}1.5x10^7$ cells/ml. FACS sorting was kindly performed by the Flow Cytometry Core Facility (University Medical Centre Mainz) using a FACS Aria cell sorter.

3.1.12 ^{51}Chromium-release assay

Materials

Concanamycin A	SaC
^{51}Chromium	Per
Cytotox tubes (0.6 ml)	Gre
EGTA	SiA
Triton X	SiA

Blocking antibodies

Anti-CD95-PE	Mil
IgG1-PE isotype control	BD

Instruments and Software

Gamma-counter Wizard 2	Per

Procedure

Chromium-release assays are used to determine the cytolytic activity of effector cells. Target cells are first incorporated with the radioactive ^{51}Cr isotope and then incubated with the effector cells that are to be tested. If the target cells are lysed by the effector cells, radioactive ^{51}Cr is released into the supernatant and can be detected.

For the labelling of target cells, a medium of $5x10^5$ cells were incubated with 100-150 µCi and 50-75 µl FCS for AML blasts and 50 µCi and 25 µl FCS for LCLs or K562. Chromium should not be older than day 14 after calibration to achieve a satisfying uptake into the target cells. Labelling took place at 37 °C for 2 h. Target cells were then washed three times with cell-culture medium and adjusted to their final working concentration in T-cell medium. $1.5x10^3$ target cells/well were seeded in a total volume of 80 µl in 96-well V-bottom plates. Effector T cells were added as duplicates in a serial dilution (E:T 1/2/7/20/60) in a final volume of 80 µl. Incubation took place at 37 °C for 5 h. The supernatant was then harvested by centrifugation at 282 x g for 5 min with the break turned off. 80 µl of the supernatant were transferred into cytotox tubes and measured in the Gamma-counter Wizard 2.

Controls for a minimum and maximum lysis of target cells were performed in at least four replicates. For minimum lysis (min), the labelled target cells were incubated with plain T-cell medium. For maximum lysis (max), the labelled target cells were incubated with PBS containing 1 % Triton X. Specific lysis was calculated according to the following formula (exp = experimental value):

$$\text{Specific lysis (\%)} = (\text{exp} - \text{min}) / (\text{max-min}) \times 100$$

In lysis blocking experiments, either T cells were seeded with 5 mM EGTA (no pre-incubation) and 100 nM concanamycin A (pre-incubation for 30 min at 37 °C), respectively, or AML blasts with anti-CD95 antibody and isotype control (each diluted 1:100, pre-incubated for 30 min at 37 °C), respectively.

3.2 Molecular Biology - Cloning of HLA-DP alleles

3.2.1 General materials for molecular biology

Instruments

Centrifuge 5415R	Epp
Dry bath system	Sta
Mastercycler gradient	Epp
NanoDrop ND-1000 spectrophotometer	TFS
Ultrospec 1000 UV visible spectrophotometer	Pha
Shaker	EdB
Incubator	WTB

Special Implements

RNase free pipette tips – SafeSeal-Tips® professional (10 µl)	BiZ
RNase free pipette tips – TipOne (20 µl, 200 µl, 1000 µl)	Sta
RNase free tubes – Safe lock tubes Biopur®	Epp
RNase ZAP™	SiA

Buffers and chemicals

Ampcillin	SiA
H_2O	Bra
Peptone	Rot
NaCl	Rot
Yeast extract	Rot

LB medium	10 g/l peptone + 5 g/l yeast extract + 10 g/l NaCl in dH_2O

3.2.2 Isolation of total RNA

Materials

Ethanol (≥99,5 %)	Rot
QIAshredder™ Spin Columns	QIA
RNeasy Mini Kit	QIA

Procedure

For the cloning of HLA-DP alleles, the total RNA was isolated from three million cells of the respective AML sample using the RNeasy Mini Kit according to the manufacturer's protocol. RNase free tubes and pipette tips were used to avoid RNA degradation, and surfaces were cleaned using RNase ZAP. RNA concentrations were determined photometrically at 260 nm using the NanoDrop ND-1000 spectrophotometer. Samples were stored at -80 °C.

3.2.3 Synthesis of cDNA

Materials

SuperScript™ First-Strand Synthesis System	Inv

Procedure

Total RNA isolated from leukaemic blasts was used to synthesise cDNA using the SuperScript™ First-Strand Synthesis System according to the manufacturer's protocol. cDNA concentrations were determined photometrically at 260 nm using the NanoDrop ND-1000 spectrophotometer. Samples were stored at -20 °C.

3.2.4 Polymerase chain reaction (PCR)

Materials

Advantage 2 PCR Enzyme System	Clo
Deoxynucleotide Solution Mix 10 mM each (dNTPs)	NEB
$MgSO_4$ (50 mM)	Inv
OneTaq Hot Start 2x MM w/Std Buffer	NEB
QIAquick® PCR Purification Kit	QIA

Primers

Primer synthesis was performed by eurofins mwg operon (Ebersberg, Germany).

XbaI-HLA-DPA1*01:03-for (36 bp, T_M = 72.9 °C)

5' CGC TAG TCT AGA ACC ATG CGC CCT GAA GAC AGA ATG 3'

XhoI-HLA-DPA1*01:03-rev (27 bp, T_M = 74.1 °C)

5' CCG ACC CTC GAG TCA CAG GGT CCC CTG 3'

XbaI-HLA-DPA1*02:01-for (34bp, T_M = 70.7 °C)

5' ATA GCT CTA GAG GAT GCG CCC TGA AGA CAG AAT G 3'

XhoI-HLA-DPA1*02:01-rev (26 bp, T_M >75 °C)

5' CGG CCT CGA GTC ACA GGG GCC CCT GG 3'

Mut-HLA-DPA1*02:01-for (22bp, T_M = 64.0 °C)

5' ATG GCT GTG CAA TGG GGA GCC A 3'

Mut-HLA-DPA1*02:01-rev (22bp, T_M = 64.0 °C)

5' TGG CTC CCC ATT GCA CAG CCA T 3'

XbaI-HLA-DPB1*13:01-for (29 bp, T_M = 66.7 °C)

5' GCG TCT AGA ATG ATG GTT CTG CAG GTT TC **3'**

XhoI-HLA-DPB1*13:01-rev (30 bp, T_M = 69.5 °C)

5' GCG CTC GAG TTA TGC AGA TCC TCG TTG AAC **3'**

Procedure

For the selective amplification of the desired DNA fragments, PCR was used. Therefore, different PCR-programs could be applied. DNA was purified after PCR using the QIAquick® PCR Purification Kit according to the manufacturer's protocol. After eluting the DNA in 50 µl H_2O, the concentration was determined photometrically at 260 nm using the NanoDrop ND-1000 spectrophotometer.

Amplification of cDNA in an Advantage-2 step-PCR

For the amplification of cDNA with the Advantage 2 PCR Enzyme System, the following reaction mix was prepared:

1 µl Forward Primer (10 pmol/µl)

1 µl Reverse Primer (10 pmol/µl)

1.5 µl dNTPs (10 mM)

1 µl $MgSO_4$ (50 mM)

2 µl cDNA (irrespective of the concentration)

5 µl 10x Advantage 2 PCR Puffer

1 µl 50x Advantage 2 Polymerase Mix

ad 50 µl H_2O

DNA amplification was performed using the following 2 step-PCR program:

5 min	95 °C	Pre-denaturation	
1 min	95 °C	Denaturation	12x
30 s	65 °C	Annealing	
1 min	68 °C	Extension	
1 min	95 °C	Denaturation	23x
30 s	63 °C	Annealing	
1 min	68 °C	Extension	
10 min	68 °C	Final extension	

Amplification of DNA in a Hot Start-Touchdown-PCR

For the amplification of DNA with the OneTaq Hot Start 2x MM, the following reaction mix was prepared:

0.5 µl Forward Primer (10 pmol/µl)

0.5 µl Reverse Primer (10 pmol/µl)

DNA (1 ng – 1 µg)

12.5 µl OneTaq Hot Start 2x MM w/Std Buffer

ad 25 µl H_2O

Amplification was achieved with a touchdown-PCR. In this program, the high initial annealing temperature is gradually reduced in each cycle. This procedure can significantly improve specificity of PCR reactions and reduce spurious annealing of the primers (Don *et al.*, 1991). The Hot Start-Touchdown-PCR program used was as follows:

30 s	94 °C	Pre-denaturation	
30 s	94 °C	Denaturation	Touchdown from 74 °C to 65 °C, 1 cycle each
1 min	74 °C	Annealing	
1 min	68 °C	Extension	
5 min	68 °C	Final extension	

3.2.5 Agarose gel electrophoresis

Materials

Acetic acid	SiA
Boric acid	Rot
EDTA	SiA
Ethidiumbromid	Rot
GelRed nucleic acid stain	Bio
Gel loding dye blue (6x)	NEB
QIAquick Gel Extraction Kit	QIA
Quick-load® 2-log DNA ladder (0.1 – 10.0 kb)	NEB
StarPure Agarose	Sta
Tris	Rot

Buffers

TBE buffer (5x)	54 g/l tris + 27.5 g/l boric acid + 0.02 % EDTA (0.5 M, pH 8.0) in dH_2O
TAE buffer (50x)	242 g/l tris + 44 g/l acetic acid (99 %) + 0.05 % EDTA (0.5 M, pH 8.3) in dH_2O

Instruments

Electrophoresis system	Neo

Procedure

To confirm the successful amplification of DNA, a horizontal agarose gel electrophoresis using a 1 % (w/v) agarose gel containing 1x GelRed in 0.5x TBE buffer was performed. The samples were mixed with 6x gel loading dye. To determine the size of the DNA fragments, a

2-log DNA ladder was loaded into a separate pocket. The separation was performed at 115 V for 1 h.

An ethidiumbromid gel was used, when DNA needed to be excised from the gel. Therefore, a 2 % (w/v) agarose gel containing 1 µg/ml ethidiumbromid in 1x TAE buffer was performed. Relevant DNA bands were excised under UV light and extracted from the gel using the QIAquick Gel Extraction Kit according to the manufacturer's protocol.

3.2.6 Restriction digestion

Materials

BSA (100x)	NEB
QIAquick® PCR Purification Kit	QIA
XbaI (20.000 U/ml) and buffer	NEB
XhoI (20.000 U/ml) and buffer	NEB

Procedure

The purified DNA that was obtained after PCR amplification was digested in a total restriction volume of 70 µl. Therefore, 60 U of the restriction enzymes *XbaI* and *XhoI* and their appropriate buffer 1x NEB4 together with 1x BSA were applied and incubated over night at 37 °C. Restriction enzymes and buffer were removed afterwards, using the QIAquick® PCR Purification Kit.

3.2.7 Ligation

Materials

T4 DNA ligase (400.000 U/ml) and buffer	NEB

Procedure

Ligation of vector DNA and insert was performed in a total volume of 10 µl using 400 U T4 DNA ligase and 1x ligase buffer. Insert and dephosphorylated vector were applied in a molar ratio of 1:3. Ligation took place at 16 °C over night or at room temperature for 1 h.

3.2.8 Production of chemically competent cells

Materials

$CaCl_2(x2H_2O)$	Rot
CH_3COOH	SiA
E.coli K12 JM109	NEB
Glycerine	Rot
KCl	Rot
$MnCl_2(x4H_2O)$	Rot
MOPS	SiA
NaOH	Mer

Buffers

TfbI buffer	30 mM CH_3COOH + 50 mM $MnCl_2(x4H_2O)$ + 100 mM KCl + 10 mM $CaCl_2$ + 15 % glycerine in H_2O, adjusted to pH 5.8 with CH_3COOH, sterile filtration
TfbII buffer	10 mM MOPS + 75 mM $CaCL_2(x2H_2O)$ + 10 mM KCl + 15 % glycerine in H_2O, sterile filtration
MOPS (100mM)	2.0926 g/l in H_2O adjusted to pH 7 with NaOH
SOB medium	20 g/l peptone + 5 g/l yeast extract + 0.5 g/l NaCl + 1 % KCl (250 mM) in dH_2O, adjust to pH 7 with NaOH and autoclave

Procedure

Chemically competent *E.coli* K12 JM109 cells were inoculated from a 50 % glycerol stock in an overnight culture in 25 ml LB medium. It was incubated at 37 °C on a shaker. On the next day, 100 ml SOB medium were inoculated with this overnight culture at an $OD_{550} = 0.05$, which was determined using the Ultrospec 1000 spectrophotometer. When an $OD_{550} = 0.5$ was reached, cells were centrifuged at 1300 x g for 8 min (4 °C). The cell pellet was resuspended in 60 ml TfbI buffer and incubated on ice for 30 min. After centrifugation at 840 x g for 6 min (4 °C), the pellet was resuspended in 4 ml TfbII buffer and cells were stored in aliquots at -80 °C.

3.2.9 Transformation

Materials

Agar	Rot

Plates

LB_{Amp} agar plates	LB medium + 15 g/l agar
	autoclave and add 100 µg/ml ampicillin

Procedure

10 µl of the ligation batch were transformed into 50 µl of chemically competent *E.coli* K12 JM109 that were defrosted on ice and mixed 1:1 with H_2O. Uptake of plasmid DNA was accomplished by heat shock procedure. Therefore, the cells and the plasmid were first incubated on ice for 30 min, heated to 42 °C for 90 s and then cooled down on ice for 5 min. Subsequently, 1 ml LB medium was added and the cells were incubated for 45 min at 37 °C on a shaker to allow the expression of the resistance gene. Afterwards, the cells were plated onto LB_{Am} plates and incubated over night at 37 °C.

3.2.10 Isolation of plasmid DNA (mini and maxi preparation)

Materials

2-Propanol	Rot
EndoFree® Plasmid Maxi Kit	QIA
Ethanol (≥99.5 %)	Rot
QIAprep® Spin Miniprep Kit	QIA

Procedure

For the preparation of plasmid DNA, an overnight culture (2 ml LB medium with 100 µg/ml ampicillin for mini preparation) was inoculated with a colony from an LB_{Am} plate and incubated on the shaker at 37 °C. On the following day, the cells were harvested by centrifugation at 3350 x g for 15 min at 4 °C and the plasmid DNA isolation was performed using the QIAprep® Spin Miniprep Kit according to the manufacturer's protocol. DNA was eluted in 50 µl H_2O.

For maxi preparation of plasmid DNA, a starter culture (2 ml LB medium with 100 µg/ml ampicillin) was inoculated with a colony from an LB_{Am} plate and incubated on the shaker at 37 °C for 8 h. 100 ml LB medium with 100 µg/ml ampicillin were then inoculated with 200 µl of this starter culture and were incubated on the shaker at 37 °C over night. The cells were harvested by centrifugation at 3350 x g for 15 min at 4 °C and the plasmid DNA isolation was performed using the EndoFree® Plasmid Maxi Kit according to the manufacturer's protocol. The isolated DNA was then air dried and dissolved in 200 µl TE buffer. DNA concentrations were measured, using the NanoDrop ND-1000 spectrophotometer, and samples were stored at -20 °C.

3.2.11 Sequence analysis

Software

GATC viewer – GATC Biotech, http://www.gatc-biotech.com/de/support/ support/ downloadlinks (12/06/2013)

BLAST – PubMed, http://blast.ncbi.nlm.nih.gov/Blast.cgi (12/06/2013)

Procedure

For sequence analysis, 20 µl of the DNA at a concentration of 100 ng/µl in H_2O were sent to GATC Biotech (Konstanz, G). Sequence alignment and analysis was performed using the BLAST algorithm and GATC Viewer. Reference sequences were obtained from http://hla.alleles.org/alleles/text_index.html (12/06/2013).

3.2.12 *In vitro* transcription of RNA

Materials

mMESSAGE mMACHINE® T7 Ultra Kit	Amb
RNeasy Mini Kit	QIA

Procedure

2.5 µg of maxi DNA were transcribed into RNA using the mMESSAGE mMACHINE T7 Ultra Kit according to the manufacturer's protocol. RNA was subsequently purified with the aid of the RNeasy Mini Kit according to the manufacturer's protocol. After determining the RNA concentration using the NanoDrop ND-1000 spectrophotometer, samples were stored in aliquots of 10 µg at -80 °C.

3.2.13 Electroporation of RNA

Materials

Fetal bovine serum (FCS)	PAA
OptiMEM® I (1x) - reduced serum medium	G/I
Penicillin/Streptomyocin (10000 U/ml Pen + 10000 µg/ml Strep)	G/I
RPMI 1640 (1x) without phenol red	G/I

Instruments and special implements

Gene pulser Xcell™	BiR
Electroporation cuvettes 4 mm	Peq

Procedure

For the electroporation of *in vitro* transcribed RNA, $1x10^6$ donor-derived LCLs were first washed with RMPI without phenol red and with OptiMEM afterwards (centrifugation at 210 x g for 8 min at 4 °C) and were then resuspended in 200 µl OptiMEM. 10 µg of RNA were pipetted into the 4 mm electroporation cuvette, the cells were added and immediately electroporated using 400 V at a square wave pulse for 5 ms. The electroporated cells were rapidly placed in 5 ml pre-heated cell-culture medium in a 6 well plate. To allow the expression of the electroporated genes, cells were tested 24 h after the electroporation.

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

3.3.1 Materials

Additional materials are listed in chapter 3.1.1.

NH_4Cl	Rot
$KHCO_3$	Rot

Instruments

Gamma-irradiator 0B58/905-2 (^{137}Cs)	Buc

Cytokines

IL-2 (human)	Nov
Fc-IL-7 (human)	Mer

Buffers

Lysis buffer	8.29 g/l NH_4Cl + 1 g/l $KHCO_3$ + 0.0372 g/l EDTA in dH_2O
	adjust to pH 7.29, sterile filtration
Prep medium	DMEM + 1 % FCS + 1 % P/S

NOD-scid IL2Rgamma^{null} (NSG)-mice

Six to twelve week old mice from the NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ strain, commonly known as NOD scid gamma or NSG, were used for *in vivo* experiments. These mice combine a non-obese diabetic NOD/ShiLtJ background with the severe combined immune deficiency mutation (scid) and an IL-2 receptor gamma chain deficiency, which leads to a lack of mature T cells, B cells, or functional NK cells, and a deficiency in cytokine signalling. NSG mice were furthermore reported to show high engraftment of human haematopoietic stem cells (Shultz *et al.*, 2005) and leukaemic blasts (Agliano *et al.*, 2008). Mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and were bred by the Animal Facility of the University Medical Centre Mainz.

Mice were kept under specific pathogen free conditions and were provided with autoclaved water supplemented with 0.08 mg/ml Borgal (sulafadoxinum, trimethoprimum). Animal studies were approved by the German state authorities and were performed according to guidelines of the European Union and Germany.

3.3.2 AML engraftment in NSG mice

For AML engraftment, NSG mice were injected with human AML blasts. 24 h beforehand, they were sub-lethally irradiated with 1.5 Gray (Greiner *et al.*, 1998) to ensure enhanced engraftment. AML blasts prepared from leukapheresis products were defrosted and filtered through a 100 µm cell strainer. Every mouse received $5x10^6$ blasts in 200 µl PBS supplemented with 0.5 % FCS i.v. into the tail vein (adapted from Sanchez *et al.*, 2009). AML samples used showed FLT3 mutations, which were shown previously to correlate with an enhanced engraftment capacity in murine models (Rombouts *et al.*, 2000a; Sanchez *et al.*, 2009).

3.3.3 Adoptive transfer of AML-reactive T cells into NSG mice

Adoptive transfer of T cells generated in mini-MLLCs (see chapter 3.1.7) was performed on day three after restimulation of the T cells, to provide optimal conditions for T-cell growth and leukaemia reactivity in mice *in vivo*. Per mouse $5x10^6$ T cells were transferred by i.v. injection in 200 µl PBS supplemented with 0.5 % FCS, 1000 U IL-2 and 20 µg Fc-IL-7 to maintain proper conditions for T cells. On the day of transfer, T-cell reactivity was confirmed by chromium-release assays (see chapter 3.1.12) and their phenotype was determined using FACS analysis (see chapter 3.1.11).

3.3.4 Isolation of bone marrow, spleen and peripheral blood of NSG mice

To determine the AML level, analysis of bone marrow, spleen and peripheral blood of the treated mice was performed. After cervical dislocation, 50 µl blood were taken from the pericardium and diluted with 20 µl heparin to prevent coagulation. The spleen was excised and pressed through a 100 µm cell strainer that was washed with 5 ml prep medium. The femur was also excised and freed from surrounding tissue. It was cut open on both ends and the bone marrow was flushed out with prep medium. Bone marrow was also filtered through a 100 µm cell strainer. Spleen and bone marrow samples were centrifuged and cell pellets were then incubated with 1-3 ml lysis buffer for 2 min. Lysis was stopped by adding prep medium, and cells were centrifuged again. Subsequently, pellets were resuspended in FACS buffer (1 and 2 ml for bone marrow and spleen, respectively). 100 µl cell suspension and 30 µl blood were then stained for flow cytometry using CD3, CD4 and CD8, respectively and CD45 to identify human T cells and CD3, CD33 and CD45 to determine human AML blasts (15 min, 4 °C). For unstained control tubes, samples from one cohort were pooled. Blood samples were then incubated with 300 µl lysis buffer for 3 min. Afterwards, blood and organ samples were washed with FACS buffer and resuspended in 300 µl FACS FIX. Remaining cells were pooled per cohort and frozen. Statistical analysis of median values was performed using a Mann-Whitney test.

3.4 Index of manufacturers

Abc Abcam, Cambridge, UK

Amb Ambion, Austin, USA

AL Air Liquid, Düsseldorf, Germany

App AppliChem, Darmstadt, Germany

BC Beckmann Coulter, Karlsruhe, Germany

BD BD Pharmingen/Biosciences, Heidelberg, Germany

Bia BIAZOL, Eching, Germany

Bic Biochrom KG, Berlin

Bio Biotium, Hayward, USA

BiM Biomol, Hamburg, Germany

BiR Biorad, Munich, Germany

BiZ Biozym, Oldendorf, Germany

Bra Braun, Melsungen, Germany

Buc Buchler, GE Healthcare Europe, Braunschweig, Germany

Clo Clontech,Mountain View, USA

CSL CSL Behring, Marburg, Germany

CTL Cellular Technology Limited, Shaker Hights, USA

DyT Dynex Technologies, Chantilly, USA

EBS eBioscience, San Diego, USA

EdB Edmund Bähler GmbH, Hamburg, Germany

ELG ELGA LabWater, Celle

Epp Eppendorf, Hamburg, Germany

G/I Gibco Invitrogen, Karlsruhe, Germany

Gre Greiner bio-one, Frickenhausen, Germany

Her Heraeus, Hanau, Germany

Int Integra Biosciences, Fernwald

Inv Invitrogen, Carlsbad, USA

Mab Mabtech AB, Stockholm, Sweden

Mar Marienfeld, Lauda Königshofen, Germany

Mer Merck, Darmstadt, Germany

Mil Miltenyi Biotec, Bergisch-Gladbach, Germany

Mip Millipore, Eschborn, Germany

Mol Moolsgard Medical, Gansloe, Denmark

Mur Murex Biotech, Kent, UK

Nal Nalgene labware, Wiesbaden, Germany

NEB New England BioLabs Inc., Ipswitch, USA

Neo NeoLab, Heidelberg, Germany

Nov Novartis, Nürnberg, Germany

PAA PAA, Pasching, Austria

Peq Peqlab, Erlangen, Germany

Per Perkin-Elmer, Rodgau, Germany

Pha Pharmacia Biotech, Cambridge, UK

PrK PromoKine

QIA Qiagen, Hilden, Germany

Rat Ratiopharm, Ulm, Germany

R&D R&D Systems, Wiesbaden-Nordenstedt, Germany

Roc Roche Applied Science, Mannheim, Germany

Rot Carl Roth, Karlsruhe, Germany

SaC Santa Cruz

San Sandoz Biopharmaceuticals, Milano, Italy

SiA Sigma-Aldrich, Steinheim, Germany

Sta Starlab, Ahrensburg, Germany

TFS Thermo Fisher Scientific, Waltham, USA

Vec Vector laboratories, Burlingame, USA

WTB WTB Binder, Tuttlingen, Germany

Zei Zeiss, Oberkochem, Germany

4 Results

4.1 Culture conditions for AML blasts

The aim of this study was to generate a reliable protocol for the generation of AML-reactive $CD4^+$ T cells. Therefore, naive T cells were stimulated with patient-derived AML blasts (see chapter 3.1.7). This stimulation relies on the interaction of the TCR with its appropriate HLA molecule as well as on the binding of co-stimulatory molecules that enable and modulate T cell activation (Whiteway *et al.*, 2003). Therefore, when acute myeloid leukaemia (AML) blasts act as antigen-presenting cells (APCs), they need to exhibit a suitable antigen-presenting phenotype. For the assessment of this, several molecules and their expression patterns were taken into account. HLA class II molecules are needed to present peptides to $CD4^+$ T cells. Co-stimulatory molecules (CD40, CD80 and CD86) provide a secondary signal and ensure the activation of the T cells (Ranheim & Kipps, 1993; Matulonis *et al.*, 1995; Dubey *et al.*, 1995). Adhesion molecules, such as CD54 and CD58, also play a major role in target recognition (Boussiotis *et al.*, 1994; Shinde *et al.*, 1996).

To identify the ideal conditions for patient-derived AML blasts to exhibit an APC phenotype, they were thawed and cultured either for one day without cytokines (Brouwer *et al.*, 2000a), two days with 1 mM sodiumbutyrate (SB) and 500 U/ml IFN-γ (Maeda *et al.*, 2000), three or four days with 1 ng/ml TNF-β and 500 U/ml IFN-γ (Eljaafari *et al.*, 2006) or four days with 1000 U/ml IL-4, 10 ng/ml TNF-α, 10 ng/ml GM-CSF and 5 ng/ml SCF (Brouwer et al., 2000b). Surface marker expression was analysed by FACS. Exemplarily shown in Figure 4.1 are data from AML blasts of patient MZ747. After overnight incubation without additional cytokines the HLA class II molecules were only weekly expressed (relative mean fluorescence intensity (rMFIs) HLA-DR = 1.4, HLA-DP = 2.2, HLA-DQ = 2.1) and the co-stimulatory molecule CD40 was not detectable at all (Figure 4.1 a). After SB/IFN-γ pre-incubation, amounts of expressed proteins were slightly increased. HLA-DR was up regulated 1.4-fold, HLA-DP 1.1-fold, HLA-DQ 1.2-fold and CD40 showed a relative mean fluorescence intensity of 1.4. Also the expression of CD54, CD58, CD80, CD83 and CD86 was enhanced 1.1- to 1.4-fold (Figure 4.1 b). To obtain even stronger expression levels of co-stimulatory molecules the cytokine cocktail containing IL-4/TNF-α/GM-CSF/SCF was needed (Figure 4.1 e). It induced considerable increases of HLA-DR (8.5-fold), HLA-DP (4.1-fold), HLA-DQ (4.8-fold), CD54 (36.4-fold) and CD58 (2.4-fold) as well as of CD80 (2-fold) and CD83 (1.6-fold) compared to the overnight culture. Overall, incubation of MZ747-AML blasts with

IL-4/TNF-α/GM-CSF/SCF proofed to be the most effective method for obtaining an APC phenotype (Figure 4.1 c and d) that was sufficient for T-cell stimulation, whereas AML blasts treated with overnight incubation or IFN-γ/SB pre-incubation would fail to stimulate the proliferation of $CD4^+$ T cells. Therefore, the combination of the four cytokines for a cultivation of four days was chosen for this patient's leukaemic blasts.

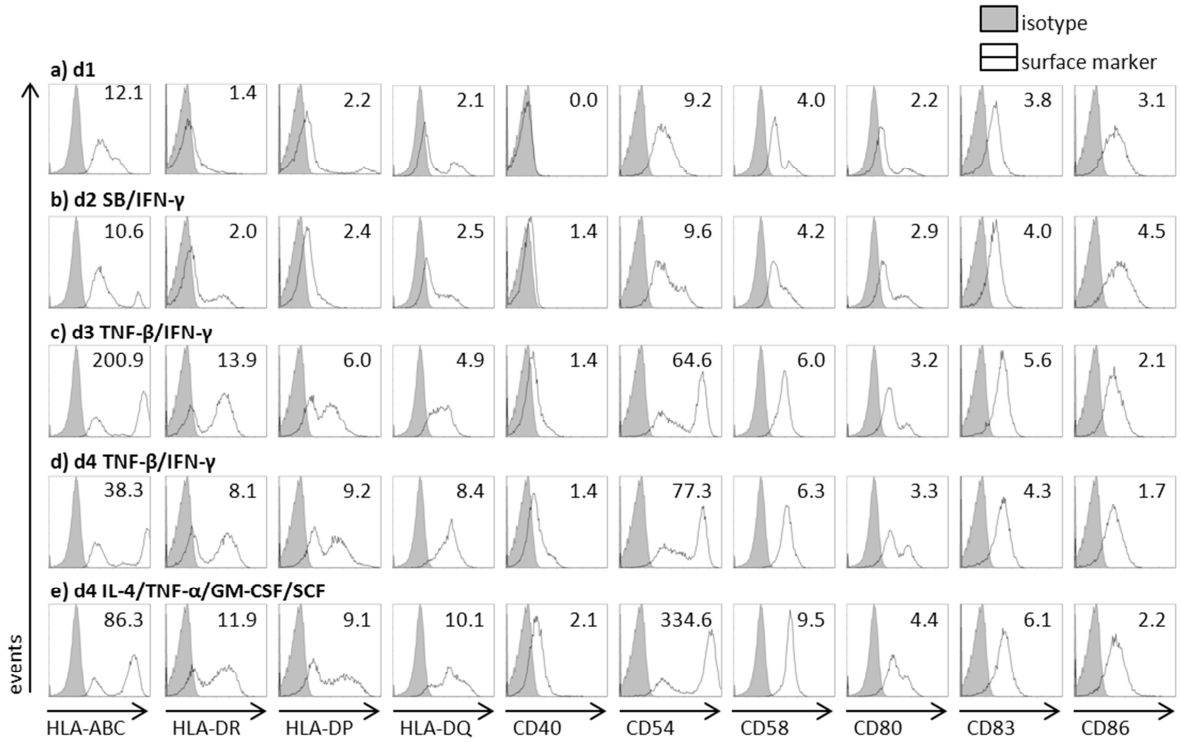

Figure 4.1: APC phenotype of AML blasts. The expression of HLA-, adhesion and co-stimulatory molecules on the surface of AML blasts from patient MZ747 was analysed by FACS after pre-incubation for a) one day without cytokines, b) two days with 1 mM sodiumbutyrate and 500 U/ml IFN-γ, c) three days with 1 ng/ml TNF-β and 500 U/ml IFN-γ, d) four days with 1 ng/ml TNF-β and 500 U/ml IFN-γ and e) four days with 1000 U/ml IL-4, 10 ng/ml TNF-α, 10 ng/ml GM-CSF and 5 ng/ml SCF, respectively. Cells shown were pre-gated on the myeloid marker CD33. Numbers indicate the relative mean fluorescence intensity (rMFI) of surface marker versus IgG isotype control.

For AML blasts from different patients, the conditions that resulted in a suitable APC phenotype varied (Table 4.1). Blasts from patient MZ921 for example showed a very strong expression of HLA class II and co-stimulatory molecules already on day one, whereas MZ237-AML blasts needed IFN-γ/TNF-β pre-incubation for three days. When at least a basic expression (+) of the APC relevant molecules was observed on the cell surface already on day one, this kind of cultivation was preferred due to higher numbers of viable cells at this early time point. Otherwise the cytokine pre-incubation procedure resulting in the strongest marker expression according to Table 4.1 was used for T-cell stimulation.

Table 4.1: Cultivation conditions for primary AML blasts. The expression of HLA-DP and several co-stimulatory molecules was categorised in four groups from negative (–), basal (+), strong (++) to very strong (+++) expression on the surface of the AML blasts tested. The AML was either incubated without cytokines (w/o) for one day, with 1 mM sodiumbutyrate (SB) and 500 U/ml IFN-γ for two days, 1 ng/ml TNF-β and 500 U/ml IFN-γ for three or four days, or with 1000 U/ml IL-4, 10 ng/ml TNF-α, 10 ng/ml GM-CSF and 5 ng/ml SCF for four days. Marker expression was determined by FACS analysis using IgG1 isotype controls as reference for the calculation of relative mean fluorescence values. The cultivation method that was subsequently chosen for the respective AMLs in MLLCs is indicated (*).

AML	d1	d2 IFN-γ/SB	d3 IFN-γ/TNF-β	d4 IFN-γ/TNF-β	d4 IL-4/ TNF-α/ GM-CSF/SCF
MZ237-AML	-	-	++*	++	+
MZ285-AML	+*	n.d.	n.d.	n.d.	++
MZ369-AML	-	-	+	+	++*
MZ683-AML	+*	++	n.d.	++	++
MZ747-AML	-	-	++	++	++*
MZ849-AML	-	-	+	+	++*
MZ921-AML	++*	+++	+++	+++	+++

4.2 Isolation of naive CD4$^+$ T cells from donor PBMCs

It was reported previously that CD8$^+$ T cells derived from the naive rather than the memory compartment show an enhanced anti-tumour reactivity (Hinrichs *et al.*, 2009). Also the potential of naive CD4$^+$ T cells to induce tumour-regression has been described (Xie *et al.*, 2010; Quezada *et al.*, 2010). To prove the superiority of naive-derived CD4$^+$ T cells in the context of leukaemia-reactivity, cells from the naive and memory compartment were compared in this study. They were isolated from healthy donor PBMCs and were then stimulated separately against AML blasts in mini-mixed lymphocyte/leukaemia cultures (mini-MLLCs). For obtaining these cell populations, different isolation methods were compared regarding the yield and purity of the enriched fractions.

First, FACS sorting according to the expression of CD3, CD4 and CD45RA was investigated. Since the isoform of the tyrosine phosphatase CD45 represents a marker that is strongly expressed on naive T cells and downregulated upon antigen contact, it was used to define T cell subsets (Appay *et al.*, 2008). Naive enriched (CD3$^+$/CD4$^+$/CD45RA$^+$) T helper cells were collected as well as memory cells (CD3$^+$/CD4$^+$/CD45RA$^-$). CD4$^-$ cells were also collected and used as feeder cells in mini-MLLCs (Figure 4.2).

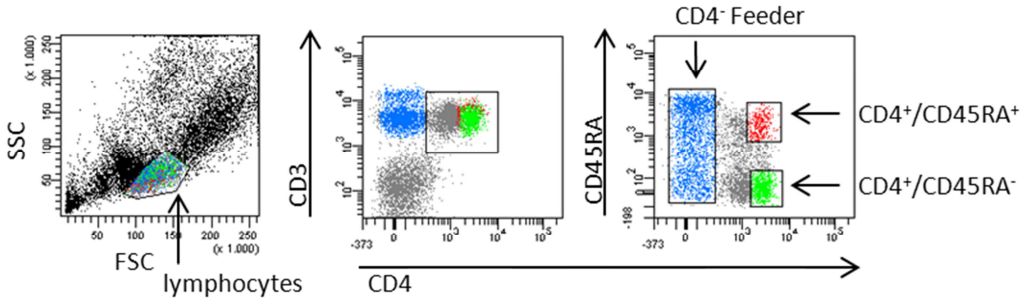

Figure 4.2: Setting for the isolation of naive CD4$^+$ T cells by FACS sorting. The gating strategy for the isolation of naive CD4$^+$ T cells is shown exemplarily for PBMCs from BC260. Lymphocytes were pre-gated in FSC/SSC and gated further on the co-expression of CD3 and CD4. CD4$^+$/CD45RA$^+$ naive enriched T cells and CD4$^+$/CD45RA$^-$ memory T cells were collected separately. Additionally, CD4$^-$ lymphocytes were collected to be used as feeder cells for mini-MLLC stimulation.

As an alternative to a FACS-based strategy, MACS MicroBeads were used. In the commercially available Naive CD4$^+$ T-cell Isolation Kit II, CD8$^+$ and NK cells, monocytes, neutrophils and cells with activation or memory markers like CD25 and CD45RO are specifically depleted in a magnetic field using a cocktail of antibodies coupled to microbeads. Since the resulting fraction was often not highly pure, an additional CD8$^+$ depletion was performed (used in the models MZ683-AML/Don595, MZ849-AML/Don691, MZ921-AML/Don671). An alternative way of isolating naive CD4$^+$ T cells was the subsequent depletion of CD8$^+$ and CD45RO$^+$ cells, followed by a positive selection of CD4$^+$ cells.

For different donor PBMCs several different isolation approaches and their efficacies were compared. FACS analysis was used to control the purity of the collected fractions. T cells isolated by either MACS or FACS sorting showed a high purity directly after the isolation process, which is shown for BC260 exemplarily in Figure 4.3. Nevertheless, after MACS sorting 2.4 % of the lymphocytes were CD4$^-$, resulting in an overall purity of 97.6 % CD4$^+$ T cells compared to 99.4 % after FACS sorting. Cells were stained for the expression patterns of differentiation markers (CD45RA, CD45RO, CD57 and CD31), co-stimulatory molecules (CD27, CD28), molecules responsible for cytokine responsiveness (CD127) and homing (CCR7, CXCR4, CD62L). MACS as well as FACS sorted CD4$^+$ T cells showed a naive phenotype according to the high expression of CCR7, CD27, CD28, CD31, CD62L, CD127 and CXCR4. The memory markers CD45RO and CD57 were expressed only weekly.

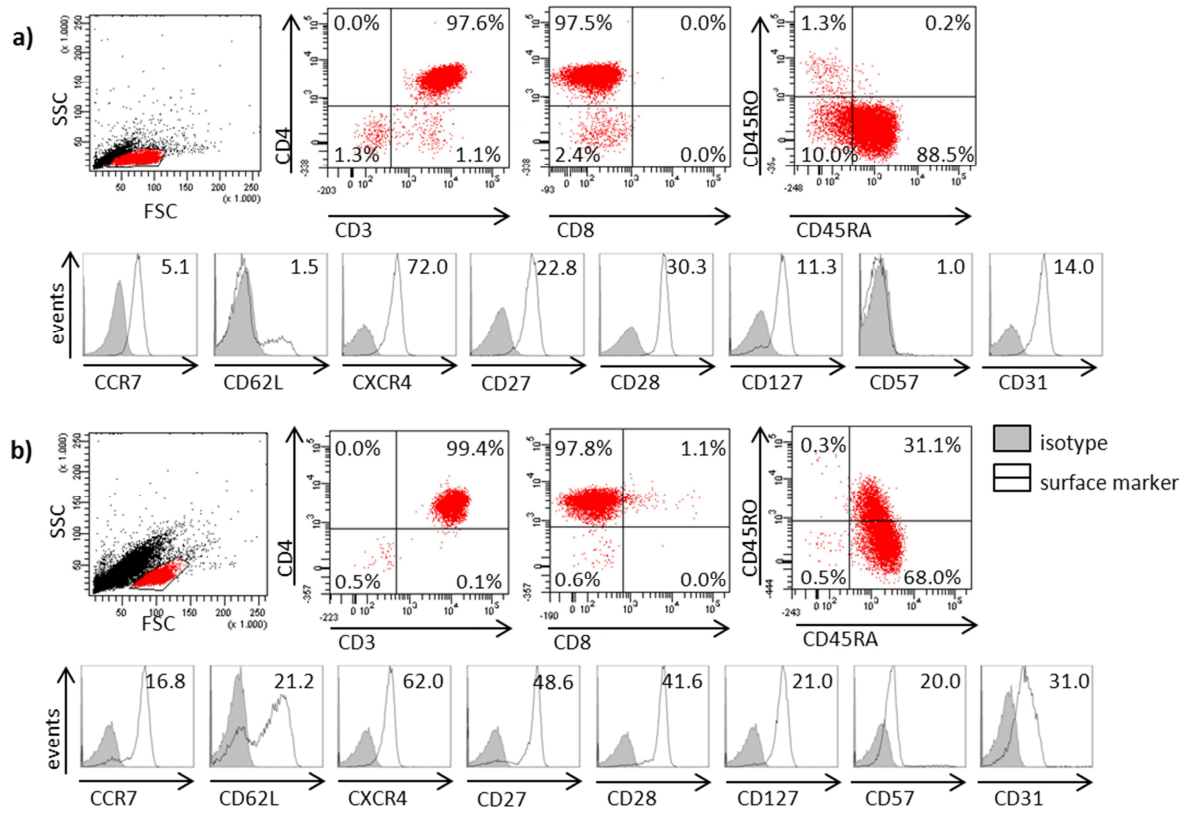

Figure 4.3: Phenotypic characterisation of freshly isolated naive CD4$^+$ T cells. T cells were isolated by MACS and FACS sorting, respectively. As these methods differ in the antigens that are targeted for the isolation process (MACS naive CD4$^+$ T Cell Isolation Kit: CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR and CD235a; FACS sorting: CD3/CD4/CD45RA), the resulting T cells can slightly vary in their surface marker expression. Naive enriched CD4$^+$ T cells are shown isolated from BC260 by a) MACS and b) FACS sorting. Cells shown in histogram blots were pre-gated on the expression of CD3 and CD4. Numbers indicate percentage of positive cells or relative mean fluorescence intensity (rMFI) of surface marker versus IgG isotype control.

While MACS isolation methods appeared to be sufficiently pure directly after the isolation process (>95 % of naive CD4$^+$ T cells), contaminating CD8$^+$ or NK cells tended to grow out after three to four rounds of restimulation (Table 4.2). The contamination frequency was very high (95 % of the mini populations tested) if only the naive CD4$^+$ T Cell Isolation Kit II was used, which can be seen e.g. in the MZ369-AML/Sib167 model, where all four populations tested contained CD8$^+$ T cells. Contaminating populations could be reduced to an average of 24.8 % if an additional CD8$^+$ depletion was performed. The alternative approach using CD45RO$^+$ depletion (MZ921-AML/Don671 model) resulted in very poor purities with contaminating CD8$^+$ T cells and NK cells in 95 % of the cultures tested. NK-cell growth was enabled due to the lacking expression of CD45RO on these cells' surfaces (Richards *et al.*, 1990) meaning that they were not targeted by the magnetic beads. FACS sorting turned out to be the most reliable method for the isolation of naive enriched CD4$^+$

T cells. Only 12.5 % of cultures showed contaminations with 95.5 % of them containing less than 5 % NK or $CD8^+$ T cells after at least four rounds of restimulation. Therefore, FACS sorting was used preferentially whenever possible.

Table 4.2: Methods for the isolation of naive $CD4^+$ T cells. Three different isolation processes for the collection of naive $CD4^+$ T cells were compared. Donor PBMCs were either separated by FACS sorting according to the expression of CD3/CD4/CD45RA or by MACS technology using the naive $CD4^+$ T cell isolation kit II, in some models combined with a CD8-depletion. Alternatively, a CD45RO-depletion and the isolation of whole $CD4^+$ cells were compared.

model	isolation method	contaminated mini-cultures/tested cultures	nature of the contaminations
MZ237-AML/Don662	FACS sort ($CD3^+/CD4^+/CD45RA^{+/-}$)	1/18	few $CD8^+$ T cells
MZ237AML/Don256	FACS sort ($CD3^+/CD4^+/CD45RA^{+/-}$)	4/25	few NK cells
MZ285-AML/Don205	FACS sort ($CD3^+/CD4^+/CD45RA^{+/-}$)	0/4	/
MZ369-AML/Sib167	MACS (naive $CD4^+$)	4/4	$CD8^+$ T cells
MZ683-AML/Don595	FACS sort ($CD3^+/CD4^+/CD45RA^{+/-}$)	1/13	$CD8^+$ T cells
	MACS ($CD8^+$ depletion/naive $CD4^+$)	2/17	$CD8^+$ T cells
MZ747-AML/Don974	FACS sort ($CD3^+/CD4^+/CD45RA^{+/-}$)	2/6	NK & $CD8^+$ T cells
	MACS ($CD4^+$)	5/23	NK cells
MZ849-AML/Sib691	MACS (naive $CD4^+$)	9/10	NK & $CD8^+$ T cells
	MACS ($CD8^+$ depletion/naive $CD4^+$)	15/24	$CD8^+$ T cells
MZ921-AML/Don671	MACS ($CD45RO^+$ depletion/$CD8^+$ depletion/$CD4^+$)	19/20	NK & $CD8^+$ T cells
	MACS ($CD8^+$ depletion/naive $CD4^+$)	18/18	NK & $CD8^+$ T cells

4.3 The generation of leukaemia-reactive mini-MLLCs from naive $CD4^+$ T cells

4.3.1 Patient/donor pairs and their characteristics

For the generation of AML-reactive $CD4^+$ T cells, eight patient/donor pairs were used (HLA-DR/-DQ-matched pairs in Table 4.3). Unless indicated otherwise, the donors mentioned were used for the actual haematopoietic stem cell transplantation (HSCT) of the patients. HLA class II molecules were determined in a high resolution typing by the Institute of Immunology and Genetics in Kaiserslautern and were matched in all cases for HLA-DR and -DQ except for the MZ667-AML/BC780 combination. Reflecting the most common situation in clinical HSCTs, HLA-DP was not matched except for the patient/donor pair MZ849-AML/Don691. In this case the transplant originated from the patient's brother. Control $CD4^+$ T cells for *in vivo* experiments were generated from a buffy coat (BC780) with a complete HLA mismatch towards blasts from patient MZ667.

Table 4.3: Patient/donor pairs for the generation of CD4$^+$ T cells. AML patients and their respective donors are listed. The patients' and donors' HLA class II alleles were determined in a high resolution typing. Mismatch alleles are indicated in red. (# = not used for the actual transplantation of the patient. n.d. = not determined)

	HLA-DRB1*	HLA-DQB1*	HLA-DPB1*	HLA-DPA1*
MZ237-AML	12:01/15:01	03:01/06:02	02:01/04:01	01:03
Donor662	12:01/15:01	03:01/06:02	04:01	01:03
Donor256#	12:01/15:01	03:01/06:02	04:01/20:01	01:03
MZ285-AML	04:04/08:01	03:02/04:02	03:01/04:02	01:03
Donor205	04:04/08:01	03:02/04:02	03:01/06:01	01:03
MZ369-AML	11:04/13:01	03:01/06:03	n.d.	n.d.
Sibling167	11:04/13:01	03:01/06:03	n.d.	n.d.
MZ667-AML	11:01/12:01	03:01	04:01/04:02	01:03
BC780#	04:04/15:01	03:02/06:02	02:01/04:02	n.d.
MZ683-AML	04:01/11:01	03:01/03:02	04:01/13:01	01:03/02:01
Donor595	04:01/11:01	03:01/03:02	02:01/03:01	01:03
MZ747-AML	11:04/15:01	03:01/06:02	01:01/06:01	01:03/02:01
Donor974	11:04/15:01	03:01/06:02	04:01/04:02	01:03
MZ849-AML	11:01	03:01	02:01/04:01	n.d.
Sibling691	11:01	03:01	02:01/04:01	n.d.
MZ921-AML	01:02/15:01	05:01/06:02	14:01/19:01	02:01
Donor671	01:02/15:01	05:01/06:02	04:01/19:01	01:03/02:01

4.3.2 The mini-MLLC approach

The aim of this study was to establish a reliable protocol for the generation of leukaemia-reactive CD4$^+$ T cells that are derived from the naive compartment of healthy donor PBMCs. Therefore, the mini-mixed lymphocyte/leukaemia culture (mini-MLLC) approach described previously was used (Distler *et al.*, 2008; Albrecht *et al.*, 2011). The naive T cells ($1x10^3$ to $1x10^4$) were stimulated with $1x10^4$ irradiated AML blasts at the respective optimal culture conditions (see Table 4.1) in 96-well plates. To generate a beneficial cytokine milieu for T-cell growth, IL-7/12/15 and irradiated feeder cells, by-products from the cell sorting, were added (overview in Figure 4.5). Low numbers of naive T cells were used in this method for the initial stimulation. Therefore, the approach of mini-MLLCs led to a high probability of expanding monoclonal populations and avoiding the outgrowth of non-specific T cells that show unwanted reactivity, for example against viruses. For determining the cell number for the initial stimulation, the precursor frequency of alloreactive donor anti-recipient CD4$^+$ T cells in 10/10-matched (both alleles of HLA-A/-B/-C/-DR and –DQ matched) transplantation pairs was taken into account, which is estimated to range between $3x10^{-4}$ to $2x10^{-6}$ (Schwarer *et al.*, 1993; Potolicchio *et al.*, 1996). If mini-MLLCs were initiated with $1x10^4$ naive T cells/well monoclonal populations were rare, which led to the decision to further reduce the starting cell number. Several clonal CD4$^+$ T cells were obtained by using a starting concentration of

$7.5x10^3$ naive T cells/well, and also lowering the cell number to $1x10^3$/well resulted in a rapid expansion of reactive T cells (Figure 4.4 b).

To investigate the anti-leukaemic effect of naive versus memory enriched T_H cells, both fractions were collected in FACS sortings (see Figure 4.2) and were stimulated identically in mini-MLLCs. The resulting populations were screened in IFN-γ split-well ELISpot assays on day 14+5 of culture, which showed that using naive enriched $CD4^+$ T cells for the initiation of cultures led to a 1.3 to 2.5-fold increase in the numbers of AML-reactive populations (Figure 4.4 a). These populations also showed a stronger IFN-γ production/well, thus confirming the higher anti-leukaemia potential within the naive enriched subset.

4.3.3 The influence of the cytokines IL-12, IL-21 and IL-27 on mini-MLLCs

In addition to the established cytokine cocktail, the influence of IL-21, a member of the common IL-2 receptor γ chain family, was analysed in IFN-γ split-well ELISpot assays on day 14+5. IL-21 was shown previously to be advantageous for proliferation and reactivity of $CD8^+$ T cells (Albrecht *et al.*, 2011) and also to enhance $CD4^+$ T-cell proliferation and IFN-γ production (Peluso *et al.*, 2007; Ferrari-Lacraz *et al.*, 2008). To investigate its influence, 10 ng/ml IL-21 were added to the mini cultures during the first three weeks of restimulation. It was observed in three patient/donor pairs that IL-21 had no or even a negative effect on the resulting number of AML-reactive $CD4^+$ T cells (Figure 4.4 c). Reactive mini populations were reduced by 27-62 %. Neither an enhanced IFN-γ production nor proliferation could be observed (data not shown).

As IL-12 is not available in GMP grade in contrast to the remaining cytokines, its influence on mini-MLLCs was investigated. Previously, IL-12 was reported to be important for T_H1 differentiation and the production of IFN-γ (Hsieh *et al.*, 1993; Afkarian *et al.*, 2002). It could be shown that omitting IL-12 not necessarily leads to a decreased number of AML-reactive $CD4^+$ T cells. In the patient/donor pair MZ237-AML/Don256 the number of reactive populations was reduced by 51 % (Figure 4.4 d). In the MZ921-AML/Don671 model however, no effect could be seen for $CD4^+$ T cells in contrast to $CD8^+$ T cells that were stimulated simultaneously, where reactive populations were reduced by 57 % without IL-12 (data not shown).

Furthermore, the influence of IL-27 was investigated. It was shown previously to control the IL-12 responsiveness, especially of naive $CD4^+$ T cells, and therefore to enforce

T_H1 commitment of the cells and to enhance their clonal expansion (Pflanz *et al.*, 2002; Lucas *et al.*, 2003). Nevertheless, these effects could not be confirmed. Results showed that the addition of IL-27 did not make any difference in terms of growth and AML-reactivity (Figure 4.4 e).

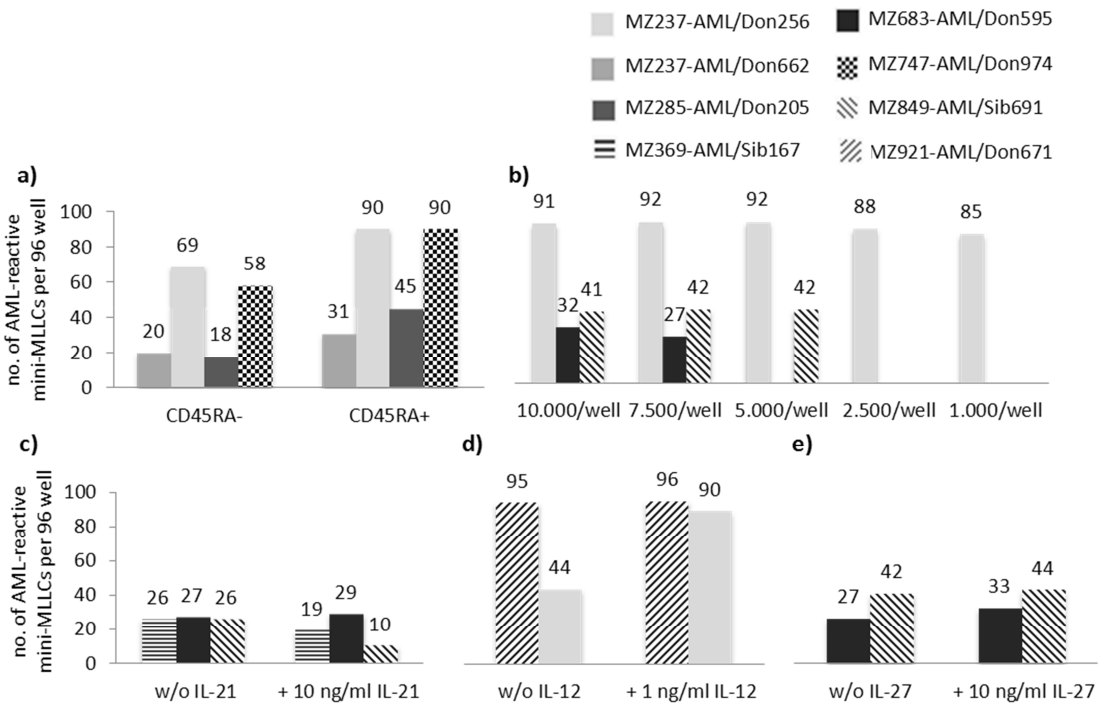

Figure 4.4: Number of reactive mini-MLLCs assessed in split-well IFN-γ ELISpot assays on day 14+5. To screen the 96-well plates for reactive mini populations in eight different patient/donor pairs, $2x10^4$ T cells from each well were analysed regarding their IFN-γ production when co-incubated with $5x10^4$ of the respective AML blasts. The number of AML-reactive cultures per plate is displayed. The difference between a) naive and memory $CD4^+$ T cells was analysed as well as b) the influence of various starting cell numbers on the yield of reactive mini-MLLCs. Also the influence of c) IL-21, d) IL-12 and e) IL-27 during the first two weeks of culture was investigated.

4.4 Functional *in vitro* characterisation of $CD4^+$ mini-MLLCs

4.4.1 Testing scheme for mini-MLLCs

After the screening for AML-reactive mini populations on day 14+5, they were transferred into 48-well plates and expanded further in weekly restimulations. When stable cultures were established, the T cells were characterised regarding their phenotype and purity in FACS, reactivity towards various targets including EBV-B transformed lymphoblastoid cell lines (LCLs) and fibroblasts in IFN-γ and TNF-α ELISpot assays, IL-4 ELISA, ^{51}Cr-release assay and *in vivo* activity. An overview is shown in Figure 4.5.

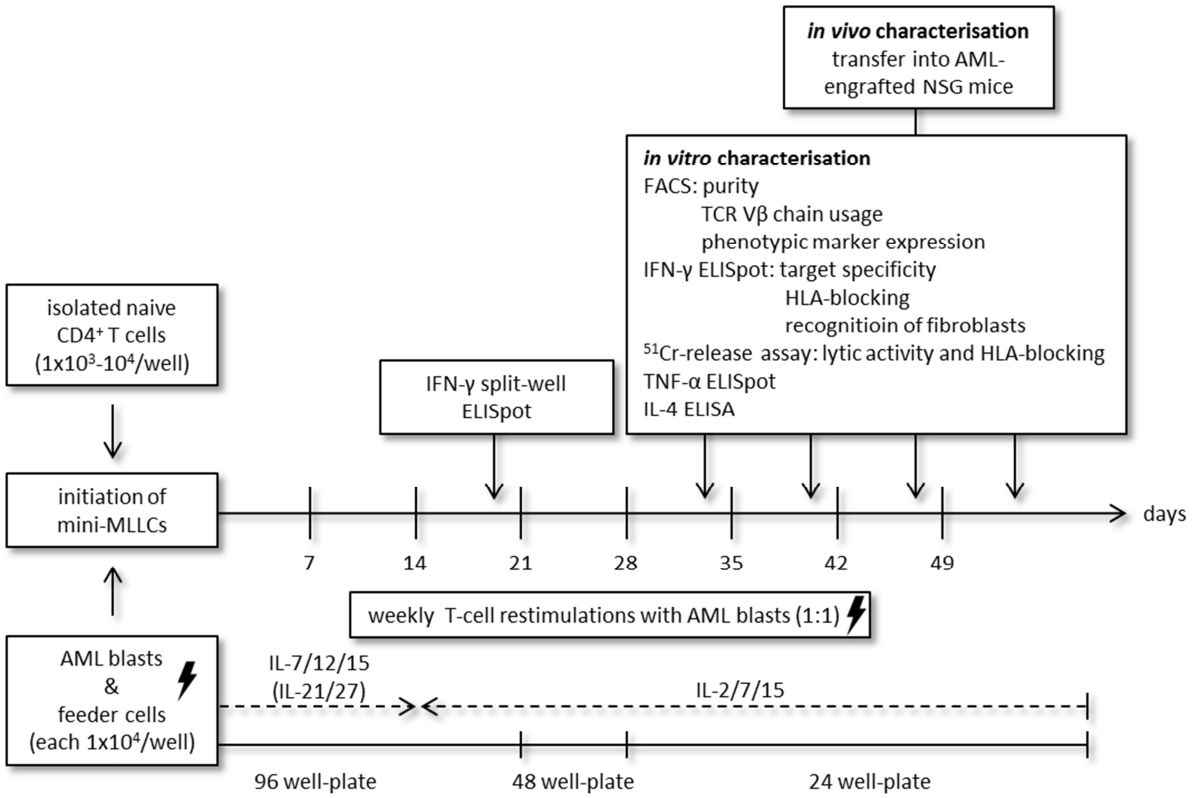

Figure 4.5: Scheme of the generation, expansion and functional testing of mini-MLLCs. After the initial stimulation of $CD4^+$ T cells against irradiated AML blasts, they were expanded for about eight weeks. Reactive mini populations were determined in an INF-γ split-well ELISpot after two weeks, were then transferred into 48 and later 24 well-plates according to their growth. *In vitro* characterisation in FACS analysis, ^{51}Cr-release assays and ELISpot assays was preferentially performed on day four or five after stimulation, IL-4 ELISAs on day three. Transfer into AML-engrafted NSG mice was performed on day 42+3 or 42+4.

4.4.2 General characteristics of $CD4^+$ mini-MLLCs

Numerous T-cell populations that were initiated in mini-MLLCs from naive $CD4^+$ T cells were restimulated weekly, expanded and analysed between week four and eight of culture for their functional features *in vitro*. The 37 most reactive and well growing $CD4^+$ T cells out of five patient/donor pairs that showed a high purity (>95 %) were fully characterised, regarding cytokine production, clonality, restriction elements and cytolytic activity (Table 4.4). Shown in detail are the representative minis 7B5, 2E2 and 2G10 from three different patient/donor models, namely MZ921-AML/Don671, MZ683-AML/Don595 and MZ747-AML/Don974, respectively. In ELISpot assays it was observed that they could specifically produce IFN-γ in response to patient's AML blasts used for primary stimulation (Figure 4.6 a). This recognition could only be blocked with antibodies targeting HLA-DP or CD4, indicating a CD4-mediated recognition of either the HLA-DP allele itself or the antigen it presented. They also showed IFN-γ production towards a patient-derived but not donor-

derived LCL line, indicating a patient-specific recognition and ruling out the possibility of EBV- and auto-reactivity. K562 cells, which represent an NK-cell target as they only express minimal amounts of HLA molecules, were not recognised. This reactivity pattern could be shown for all mini populations with the exception of 4 out of 37 populations tested (10.8 %) not recognising the patient's LCL.

Additionally, the three $CD4^+$ T cells shown were able to lyse patients' AML blasts (29-65 %, E:T ratio 20) as well as patients' LCLs (39-65 %, E:T ratio 20) in ^{51}Cr-release assays (Figure 4.6 b). Donor-LCLs and NK cells were not lysed. Overall, lysis ranged from 0% to 65 % (E:T ratio 20) for AML blasts and from 0 % to 79 % for patient-derived LCLs (Table 4.4). Nevertheless, IFN-γ production did not necessarily correlate with lytic activity as not all IFN-γ producing populations showed target cell lysis (e.g. mini cultures from the model MZ285-AML/Don205 listed in Table 4.4). In addition, T cells responded to antigen contact with a clonal expansion. By weekly restimulations, they could be readily expanded up to very high cell numbers ($1x10^8$-$1x10^9$) within seven weeks (Figure 4.6 c).

To identify clonal $CD4^+$ T cells, populations were also analysed for their TCR Vβ chain expression. They were defined as monoclonal, if >95 % of the cells expressed the same TCR Vβ chain. This could be shown for 7B5 and 2E2 among others (Figure 4.6 d), indicating that the mini-MLLC approach using $1x10^3$ to $1x10^4$ naive T cells/well for the initial stimulation, could effectively be used to generate clonal $CD4^+$ T cells. Due to a limited number of TCR Vβ chain antibodies commercially available, the complete repertoire could not be defined for all T cells tested, like shown for the $CD4^+$ T cell 2G10. On the whole, 12 out of the 37 $CD4^+$ T-cell populations shown in Table 4.4 were of clonal origin. Only 3 out of 37 populations expressed four or more different Vβ chains and were therefore of polyclonal origin. Therefore, 92 % of all T cells tested were derived from mono- or oligoclonal populations.

In addition to IFN-γ, $CD4^+$ T cells were also shown to produce the T_H1 type cytokine TNF-α (Table 4.4). Only 4 out of 37 populations also produced the T_H2 type cytokine IL-4. TCR Vβ chain analysis confirmed the oligoclonal origin of these cultures, which were therefore composed of T_H1 and T_H2 cells. On the whole, growth and reactivity could not be correlated with the different cytokine conditions used for $CD4^+$ T-cell stimulation. Reactive populations could be expanded from either approach, whereas the main difference among the cytokine combinations tested was reflected by the number of AML-reactive T-cell cultures.

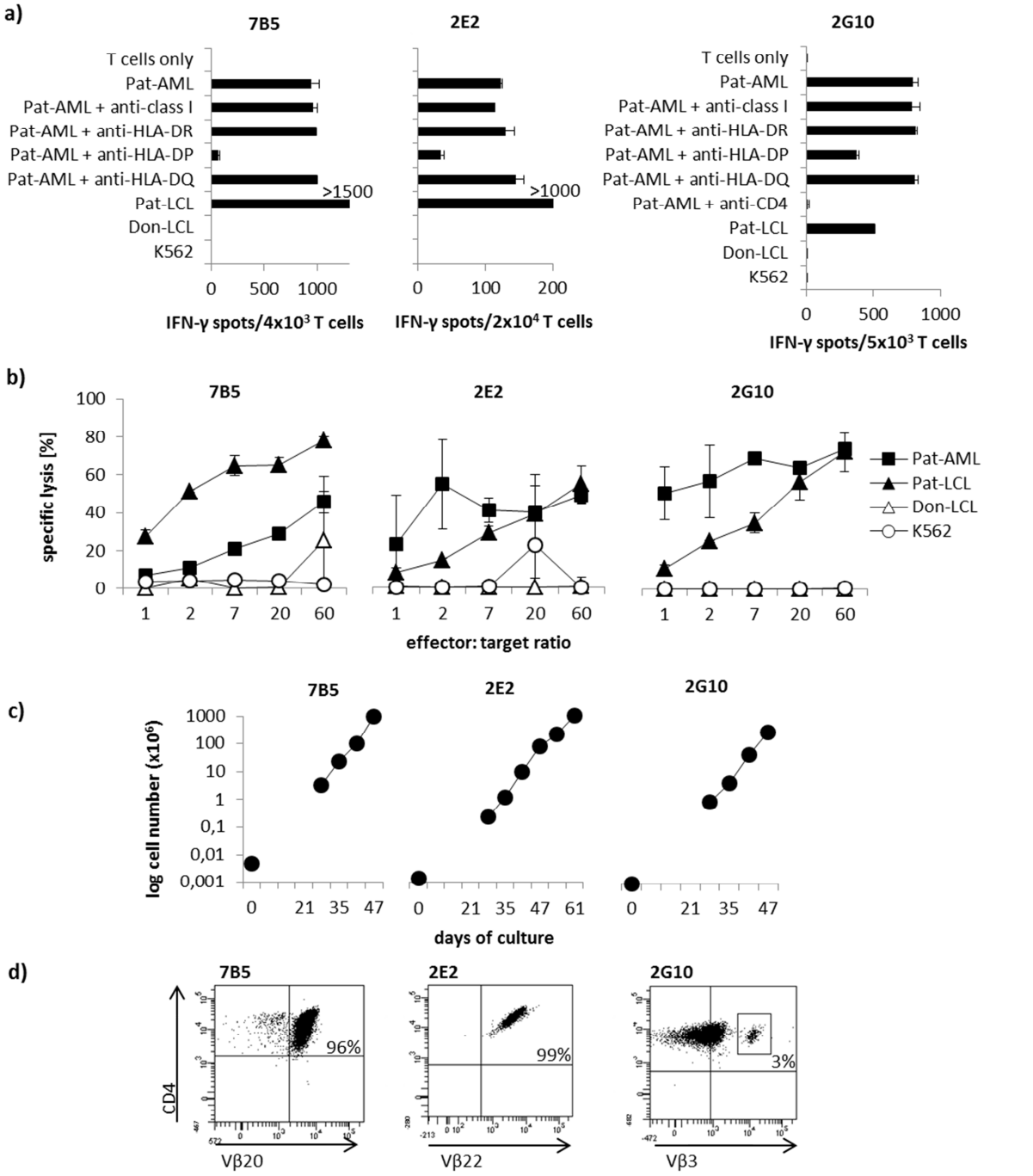

Figure 4.6: Functional characterisation of CD4$^+$ mini-MLLCs four to six weeks after the initial stimulation. Weekly restimulated T-cell populations were characterised regarding their functional properties five days after stimulation. Mini populations 7B5 (MZ921-AML/Don671), 2E2 (MZ683-AML/Don595) and 2G10 (MZ747-AML/Don974) were analysed regarding a) their IFN-γ spot production in ELISpot assays (5x10^4 target cells/well), b) lytic capacity in ^{51}Cr-release assays, c) potential to expand over time and d) clonality by looking at their TCR Vβ chain usage in FACS analysis. T-cell populations were defined as clonal if >95 % of the cells expressed the same TCR Vβ chain.

Table 4.4: Overview of CD4$^+$ T cells from five patient/donor pairs. Sufficiently pure (>95 % CD3$^+$/CD4$^+$) T cells were characterised for their cytokine production (TNF-α in ELISpot, IL-4 in ELISA) and lytic activity towards AML blasts and patient-derived LCLs. Clonality was assessed using TCR Vβ chain antibodies in FACS analysis. Their restriction to HLA-DP mismatch alleles was investigated in IFN-γ ELISpot assays using blocking antibodies and by assessing the cross-reactive recognition of AML blasts from different patients. (n.k.= not known - TCR detection not possible with antibody panel, n.d. = not determined)

model	CD4$^+$ T-cell popu-lation	TCR Vβ chain repertoire	TNF-α	IL-4	restriction to HLA-DPB1* mismatch	cytolytic activity of (at E:T 20)	
						Pat-AML	Pat-LCL
MZ237-AML/ Don662	1D4	Vβ13.1 (47 %), Vβ7.2 (37 %), Vβ5.2 (1%)	yes	no	02:01	0 %	0 %
	2B8	Vβ4 (71 %), Vβ2 (20 %), Vβ17 (6 %)	yes	no	02:01	0 %	0 %
	3H2	Vβ7.2 (53 %), Vβ5.3 (1 %)	yes	no	02:01	6 %	19 %
MZ285-AML/ Don205	2A9	Vβ8 (99 %)	yes	no	04:02	0 %	0 %
	2C2	Vβ17 (52 %), Vβ8 (37 %), Vβ5.1 (9 %), Vβ12 (1 %), Vβ13.6 (1 %)	yes	no	04:02	0 %	0 %
MZ683-AML/ Don595	1B4	Vβ13.6 (98 %)	yes	no	13:01	24 %	17 %
	2E2	Vβ22 (96 %)	yes	no	13:01	39 %	39 %
	2F3	Vβ22 (97 %)	yes	no	unclear	58 %	5 %
	2G2	Vβ13.6 (99 %)	yes	no	13:01	n.d.	n.d
	8F3	Vβ13.6 (95 %), Vβ2 (3 %)	yes	no	13:01	37 %	46 %
	10B1	Vβ2 (96 %)	yes	no	04:01	20 %	0 %
	10B2	Vβ5.1 (3 %), Vβ2 (2 %)	yes	no	04:01	20 %	20 %
	10C11	Vβ2 (87 %), Vβ8 (5 %)	yes	no	04:01	0 %	14 %
	11C12	Vβ13.2 (98 %)	yes	no	04:01	45 %	45 %
MZ747-AML/ Don974	1B5	Vβ2 (82 %), Vβ7.2 (16 %)	yes	no	Vβ7.2 → 01:01 Vβ2 → 06:01	39 %	48 %
	2A9	n.k.	yes	no	unclear	16 %	0 %
	2D7	n.k.	yes	yes	06:01	79 %	67 %
	2E7	Vβ7.1 (48 %), Vβ5.3 (31 %), Vβ17 (16 %)	yes	yes	01:01	18 %	8 %
	2G10	Vβ7.1 (3 %)	yes	yes	06:01	65 %	57 %
	2H10	Vβ17 (45 %), Vβ20 (45 %), Vβ13.2 (1 %)	yes	yes	01:01	31 %	45 %
	4C7	Vβ22 (19 %)	n.d.	no	01:01	16 %	53 %
MZ921-AML/ Don671	1A4	Vβ5.3 (55 %), Vβ23 (40 %)	yes	no	unclear	35 %	35 %
	2C4	Vβ13.6 (100 %)	yes	no	14:01	41 %	79 %
	4B8	Vβ20 (98 %)	yes	no	14:01	43 %	72 %
	5A8	Vβ13.1 (53 %)	yes	no	unclear	42 %	3 %
	5B7	Vβ13.1 (100 %)	yes	no	14:01	41 %	20 %
	5D6	Vβ22 (86 %), Vβ8 (4 %)	yes	no	unclear	23 %	58 %
	5G5	Vβ20 (99 %)	yes	no	unclear	19 %	7 %
	6E7	Vβ5.1 (99 %)	yes	no	14:01	29 %	84 %
	7B5	Vβ20 (96 %)	yes	no	14:01	29 %	65 %
	11B9	Vβ5.1 (93 %), Vβ2 (6 %)	yes	no	unclear	37 %	76 %
	11H5	Vβ13.1 (44 %), Vβ2 (7 %), Vβ5.1 (5 %)Vβ13.6 (3 %)	yes	no	unclear	30 %	48 %
	13B7	Vβ8 (69 %), Vβ20 (28 %)	yes	no	unclear	20 %	62 %
	13F4	n.k.	yes	no	unclear	24 %	29 %
	15G2	Vβ21.3 (51 %)	yes	no	14:01	20 %	64 %
	17D1	Vβ2 (100 %)	yes	no	unclear	24 %	75 %

4.4.3 The mechanism of target cell lysis

$CD4^+$ T cells were originally thought to exhibit only helper functions and therefore enhance the activity of $CD8^+$ T cells, but it was shown that helper T cells can also induce tumour killing (Perez-Diez *et al.*, 2007; Stevanović *et al.*, 2012). This cytotoxicity can be mediated by the release of the effector molecules perforin and granzyme A and B or in a Fas/FasL dependant manner (reviewed in Barry & Bleackley, 2002).

To investigate this cytotoxic mechanism in more detail, lytic pathways were blocked in a ^{51}Cr-release assay. For comparison, a $CD4^+$ T-cell clone (2C4) was tested and a $CD8^+$ T-cell population (21G3), which were both generated in the same patient/donor model MZ921-AML/Don671. $CD8^+$ contaminations in 2C4 T cells could be excluded by FACS analysis. Both T cells showed an intermediate lysis of AML blasts (48 % for 2C4 and 54 % for 21G3, respectively, at E:T 60). The $CD4^+$ T-cell population also lysed the patient-derived LCL (72 % at E:T 60). Both spared the donor-derived LCL and K562 cells, which were tested as representative NK-cell targets (Figure 4.7 a). When the cells were either pre-incubated with the calcium chelator EGTA or concanamycin A (CMA), both inhibiting perforin/granzyme-mediated lysis, lytic activity was completely blocked. By blocking Fas, lysis was not impaired (Figure 4.7 b). Also no effect was seen after blocking this pathway with an anti-FasL antibody (data not shown), indicating that lysis was not mediated via Fas/FasL interaction. For confirmation, cells were analysed in FACS for the amount of perforin and granzyme stored intracellularly. $CD4^+$ as well as $CD8^+$ T cells showed a strong expression of perforin (rMFI = 3.9 and 7.1, respectively), granzyme A (rMFI = 34.4 and 43.9, respectively) and granzyme B (rMFI = 13.9 and 11.4, respectively), confirming data from blocking experiments (Figure 4.7 c).

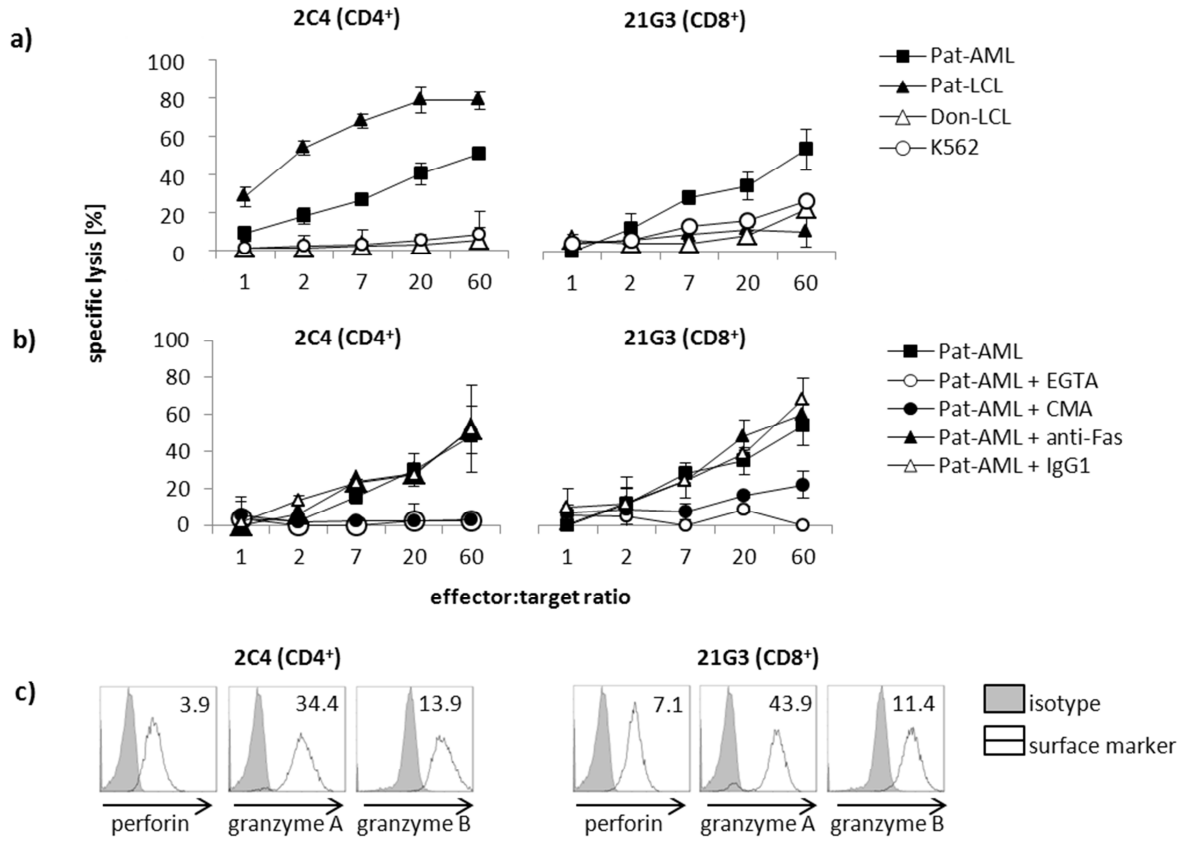

Figure 4.7: Assessment of the lytic activity and its mechanism of CD4$^+$ compared to CD8$^+$ mini-MLLCs *in vitro*. Weekly restimulated mini populations 2C4 (CD4$^+$, day 42+4) and 21G3 (CD8$^+$, day 35+4) from the model MZ921-AML/Don671 were analysed regarding their lytic capacity in 5 h ^{51}Cr-release assays. Cytotoxicity towards a) patient and donor-derived LCLs and K562 cells is shown. Different lytic pathways were blocked using b) 5 mM EGTA, 100 nM concanamycin A (CMA) and an anti-Fas antibody, respectively. IgG1 was used as isotype control. c) In an intracellular FACS staining the presence of perforin, granzyme A and B was shown. Numbers indicate the relative mean fluorescence intensity (rMFI) of surface marker versus IgG isotype control.

4.5 Target specificity of CD4$^+$ T cells

Target recognition of CD4$^+$ T cells is usually restricted to HLA class II molecules that present peptides. In IFN-γ ELISpot assays it was shown that the reactivity of the vast majority of CD4$^+$ T cells from all patient/donor pairs that were characterised (Figure 4.6 a and Table 4.4) could be blocked with antibodies targeting HLA-DP and CD4. As the DP alleles were not matched between patient and donor, this indicated alloreactivity either towards the mismatched HLA class II alleles alone or in combination with the peptides they presented. To prove this hypothesis, AML blasts from other patients expressing different HLA-DP alleles were tested. Furthermore, the mismatched DP alleles were cloned (see chapters 3.2 and 4.5.1) and tested for their recognition when their encoding RNA was electroporated into donor-derived LCLs. The two mini-populations 8F3 and 10C11 from MZ683-AML/Don595 were analysed for the

recognition of mismatch alleles expressed on the respective AML blasts. Between patient and donor, there was a mismatch in both HLA-DP alleles. The donor expressed DPB1*02:01/03:01 whereas the patient expressed DPB1*04:01/13:01, leaving two possible target structures for the T cells. HLA typing further revealed the expression of the α-chains DPA1*01:03/02:01 in the patient's cells.

4.5.1 Strategy for the cloning of HLA-DPB1 and A1 alleles

For the cloning of α- and β-chains of the mismatched HLA-DP alleles, different strategies were used, involving various AML-blasts, primers and PCR methods. HLA-DPA1*02:01 was cloned out of MZ683-AML blasts (HLA-DPB1*04:01/13:01, -A1*01:03/02:01). Due to completely identical sequences at the 5' and 3' end of both HLA-DPA1 alleles present, the amplification had to be performed in two steps. Complementary primers annealing within the sequence (Mut-HLA-DPA1*02:01-for and Mut-HLA-DPA1*02:01-rev) were designed in addition to those annealing at the start and stop codon (*XbaI*-HLA-DPA1*02:01-for and *XhoI*-HLA-DPA1*02:01-rev) (see Figure 4.8). In a first and second PCR, product 1 and 2 were amplified in Hot Star-Touchdown-PCRs, using cDNA as template. Both products were fused together in a third PCR step, using the same program.

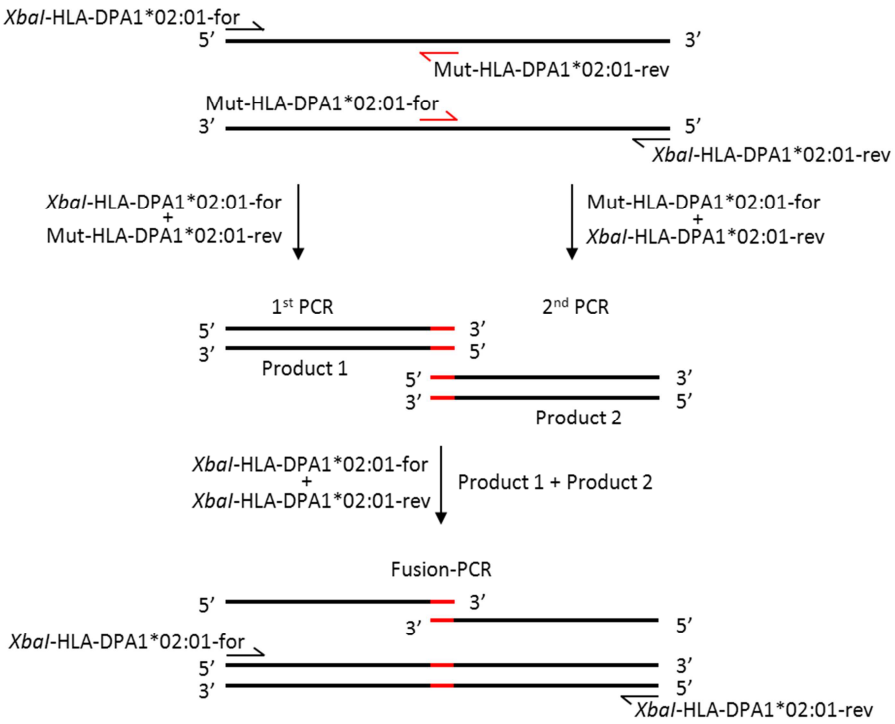

Figure 4.8: Schematic representation of a fusion-PCR. To specifically amplify an HLA-DP allele that shows a high homology to the second allele expressed in the cells, fusion-PCR needed to be performed. Specific complementary primers that bind within the sequence lead to the separate amplification of both halves that can be fused together in a third PCR reaction.

HLA-DPA1*01:03 was cloned out of MZ558-AML blasts (HLA-DPB1*04:01/13:01, -A1*01:03/02:01). Amplification of cDNA was performed in an Advantage-2 step-PCR using the primers *XbaI*-HLA-DPA1*01:03-for and *XhoI*-HLA-DPA1*01:03-rev.

The *in vitro* transcribed RNA encoding the HLA-DPB1*04:01 allele originated from MZ470-AML blasts (HLA-DPB1*04:01). Due to sequence homology to the HLA-DPB1*13:01 allele, the primers *XbaI*-HLA-DPB1*13:01-for and *XhoI*-HLA-DPB1*13:01-rev were used in an Advantage-2 step-PCR. HLA-DPB1*13:01 was cloned out of MZ558-AML (HLA-DPB1*04:01/13:01, -A1*01:03/02:01) by amplifying the cDNA in an Advantage-2 step-PCR, also using the primers *XbaI*-HLA-DPB1*13:01-for and *XhoI*-HLA-DPB1*13:01-rev.

4.5.2 Specific recognition of HLA-DP mismatch alleles

Mini populations 8F3 and 10C11 derived from the patient/donor pair MZ683-AML/Don595, which was matched for HLA-A/-B/-C/-DR/-DQ but not for the HLA-DP alleles, were first screened for their IFN-γ production when incubated with AML blasts from different patients in a cross-reactivity testing (Figure 4.9 a), where they showed two distinct recognition patterns. Mini 10C11 recognised 10 out of 22 AML blasts that all shared the expression of DPB1*04:01 (depicted in red) that was also present on the AML blasts that the T cells were stimulated against. Nevertheless, not all blasts expressing this mismatch allele were recognised by 10C11. MZ458-AML and MZ294-AML for instance also expressed DPB1*04:01. The reason for this lack of recognition might either be low HLA-DP expression levels which could be overcome by cytokine pre-incubation of the AML blasts to induce HLA-class II upregulation (similar to Figure 4.1), or T cells might be directed against recipient-specific allo-peptides not expressed in these AML blasts.

In contrast, mini 8F3 only recognised AML-blasts from one other patient (MZ558-AML), the only one, apart from MZ683-AML, expressing DP*13:01 (depicted in green). This indicated that the T-cell populations 8F3 and 10C11 were directed against the two different mismatches. To prove this idea, donor-derived LCLs that originally were not recognised by the T cells (Figure 4.9 b) were electroporated with HLA-DP-encoding RNA. It was described that certain β-chains exclusively pair with one distinct α-chain (Hollenbach *et al.*, 2012), leading to the HLA-DP alleles of patient MZ683 being built of β04:01/α01:03 and β13:01/α02:01. Therefore, either one of the β-chains was electroporated together with its

corresponding α-chain, which resulted in a transient expression of the HLA alleles. 12 to 24 h after electroporation, when surface expression reached a maximum, the LCLs expressing the mismatch alleles were used as target cells in an IFN-γ ELISpot assay. It could be proven that mini 8F3 only recognised the donor-LCL now expressing β13:01/α02:01 whereas mini 10C11 showed recognition of the donor-LCL electroporated with β04:01/α01:03. Therefore, the mini populations tested that were stimulated in the clinical 10/10-matched setting directly recognised HLA-DP mismatches, proven by the fact, that donor-LCLs were recognised when electroporated with the mismatch alleles. Reactivity towards tumour-associated antigens could be ruled out as those were most likely not expressed by donor-derived LCLs.

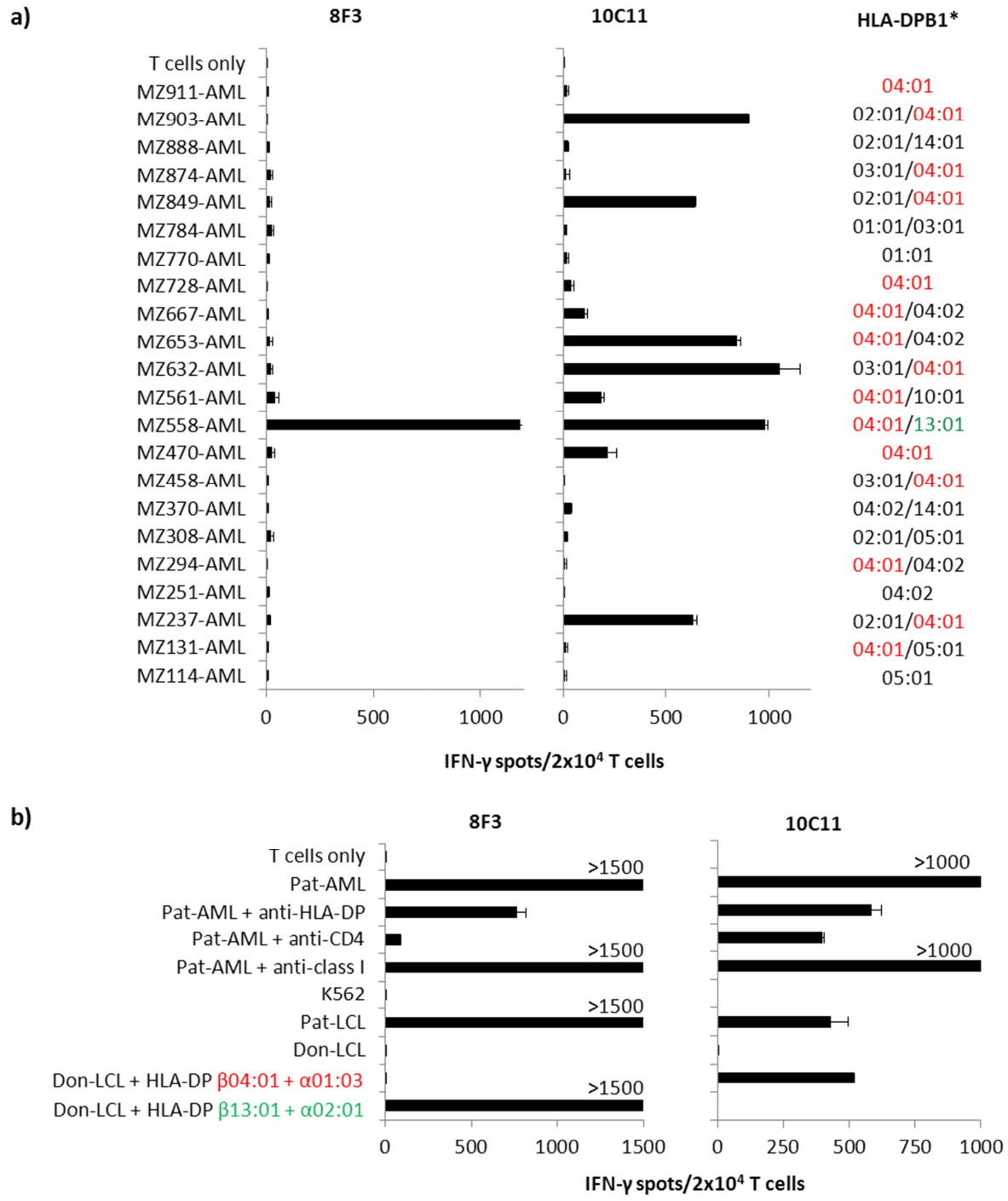

Figure 4.9: **Specificity analysis of CD4$^+$ T cells of the MZ683-AML/Don595 model.** To determine their specificity, mini-MLLC populations 8F3 and 10C11 were screened in IFN-γ ELISpot assays for a) their recognition of AML blasts of various patients expressing shared HLA-DPB1 alleles displayed on the right and b) autologous Donor-LCLs electroporated with the mismatch HLA-DP α and β chains (5x10^4/well target cells each). DP alleles displayed in red (HLA-DPB1*04:01) and green (HLA-DPB1*13:01) indicate the ones shared with the patients AML blasts, that could be targeted by the T cells.

Only one patient/donor pair (MZ849-AML/Don691) with a complete match in all twelve HLA alleles could be tested. Logically, no reactivity towards mismatch DP alleles could be detected. Nevertheless, reactivity could be blocked with anti-CD4 and anti-HLA-DR or -DP

antibodies, indicating the recognition of peptides presented by the matched HLA molecules (Figure 4.10). T cells were also of a T_H1 type and 20 % of the populations tested recognised patient-derived LCLs, whereas no IFN-γ was produced in response to donor-derived LCLs and K562. Due to a very weak proliferative capacity of the T cells, they could not be characterised further.

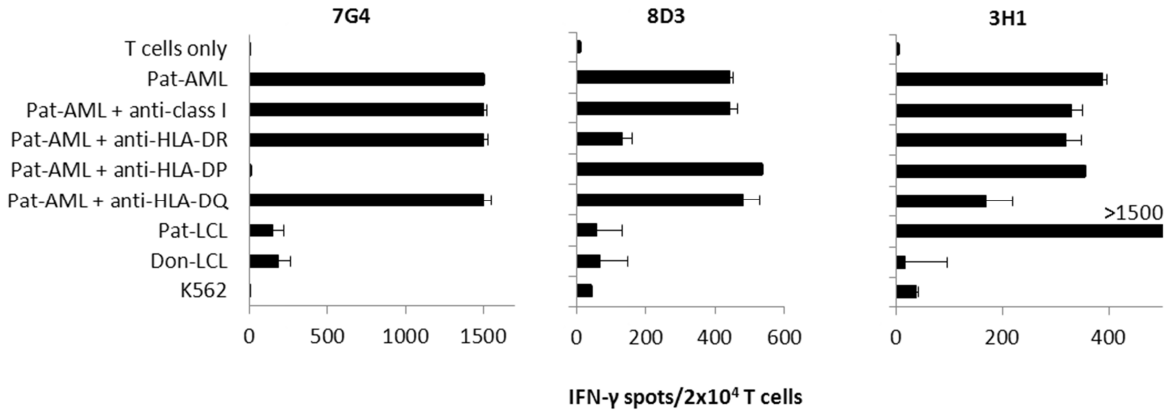

Figure 4.10: **Possible restriction elements in fully HLA-matched transplantation settings.** T cells were restimulated weekly and were tested in an IFN-γ ELISpot assay ($5x10^4$ target cells/well) on day five after stimulation. T-cell populations 7G4, 8D3 and 3H1 from the fully (i.e. 12/12) HLA-matched patient/donor pair MZ849-AML/Don691 were analysed regarding their restriction elements using antibodies specifically blocking different HLA alleles.

4.6 Cross-reactivity towards non-haematopoietic cells

One of the aims of this study was to separate the unwanted GvHD from the beneficial GvL effect. Therefore, T cells should be able to recognise haematopoietic cells in the patient but spare epithelial and mesenchymal cells. Primary fibroblasts (Fb) that were generated from foreskin served as a model for non-haematopoietic cells. They were used as target cells either without pre-incubation or after four days in fibroblast medium containing IFN-γ to enhance the expression of the HLA class II molecules on the surface and simulate inflammatory conditions. HLA-DP expression was monitored in FACS analysis and showed a 1.2- to 12.8-fold increase following IFN-γ incubation in four out of five fibroblast samples tested (Figure 4.11 b). These fibroblasts were then used to test their recognition by T cells in an IFN-γ ELISpot assay (Figure 4.11 a). Five mini-populations from MZ683-AML/Don595 were shown to recognise the AML blasts that they were stimulated against. This reactivity could be blocked with an anti-HLA-DP and in four out five cases also with an anti-CD4 antibody. Fibroblasts without IFN-γ pre-incubation were not recognised by either of the T cells.

However, if they were pre-treated minis 2E2, 2G2, 10C11 and 11C12 showed strong recognition, and spot production was also slightly increased for mini 8F3. This indicated that under non-inflammatory conditions mesenchymal targets were not recognised by the T cells, which therefore suggested a reduced risk of causing GvHD in fibroblast-rich tissues such as skin. Mini 8F3, which showed the weakest IFN-γ production towards fibroblasts, might have an even lower GvHD potential. Nevertheless, the T cells did not recognise all pre-incubated fibroblasts. In fact, recognition was specific for the HLA-DP mismatch allele that was targeted. Mini populations 10C11 and 11C12 recognised all fibroblasts expressing HLA-DP*04:01, whereas minis 2E2, 2G2 and 8F3 only produced IFN-γ when they were co-cultured with HLA-DP*13:01 expressing fibroblasts (Fb #09), which again proved the hypothesis of mismatch reactivity. Cells expressing irrelevant DP alleles (Fb #10) were not recognised.

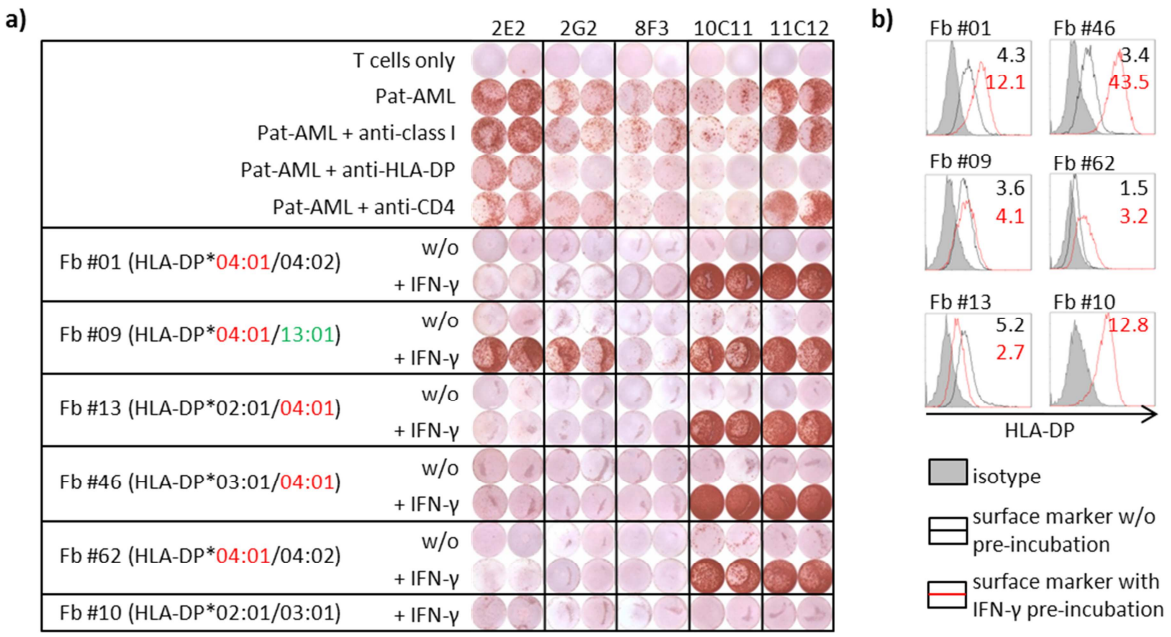

Figure 4.11: **Recognition of primary fibroblasts and their HLA-DP expression.** a) Various $CD4^+$ T cells ($2x10^4$/well) from MZ683-AML/Don595, which could be blocked with anti-HLA-DP antibodies, were tested for their recognition of primary fibroblasts (Fb) ($1x10^5$/well) without or with IFN-γ pre-incubation for four days (500 U/ml) in an IFN-γ ELISpot assay ($5x10^5$ AML blasts/well). HLA-DP alleles displayed in red (HLA-DPB1*04:01) and green (HLA-DPB1*13:01) indicate the mismatched alleles these fibroblasts share with the patient AML, that could be targeted by the T cells. b) The amount of HLA-DP expressed on the surface of fibroblasts with (red line) and without (black line) prior IFN-γ incubation is shown. Numbers indicate mean fluorescence intensity (rMFI) of surface marker versus IgG isotype control.

4.7 Functional *in vivo* characterisation of CD4$^+$ T cells

To assess the *in vivo* function of leukaemia-reactive T-cell populations, they were adoptively transferred into AML-engrafted NSG mice. Briefly, the immunodeficient mice were sub-lethally irradiated to improve engraftment of 5x10^6 primary AML blasts that were injected i.v. into the tail vain. Prior to T-cell transfer, a leukaemic burden of 1-3 % in the bone marrow had to be reached to resemble a minimal residual disease (MRD) in a patient, when immunotherapy was shown to be most effective (reviewed by Slavin, 2005). Engraftment was controlled by FACS analysis using the surface markers CD3/CD33/CD45 to identify human leukaemic blasts. AML blasts with FLT3-ITD mutations were previously shown to lead to the most reliable engraftment in NSG mice (Rombouts *et al.*, 2000b; Rombouts *et al.*, 2000a; Brunk, 2011). Therefore, FLT3-ITD$^+$ blasts from MZ921-AML and MZ770-AML were used for *in vivo* experiments.

4.7.1 Engraftment kinetics of AML blasts in NSG mice

For the definition of the time point of MRD, mice were sacrificed two, three and four weeks after AML injection. Bone marrow samples were stained with FACS antibodies and human AML blasts were identified by their simultaneous expression of the myeloid marker CD33 and the human haematopoietic marker CD45 as well as the absence of the T-cell marker CD3 (Figure 4.12 a). After the injection of 5x10^6 AML blasts/mouse, the kinetics of MZ921-AML showed a median engraftment of 1.2 % AML blasts in the bone marrow after 21 days, which was chosen for the following transfer experiments (Figure 4.12 b).

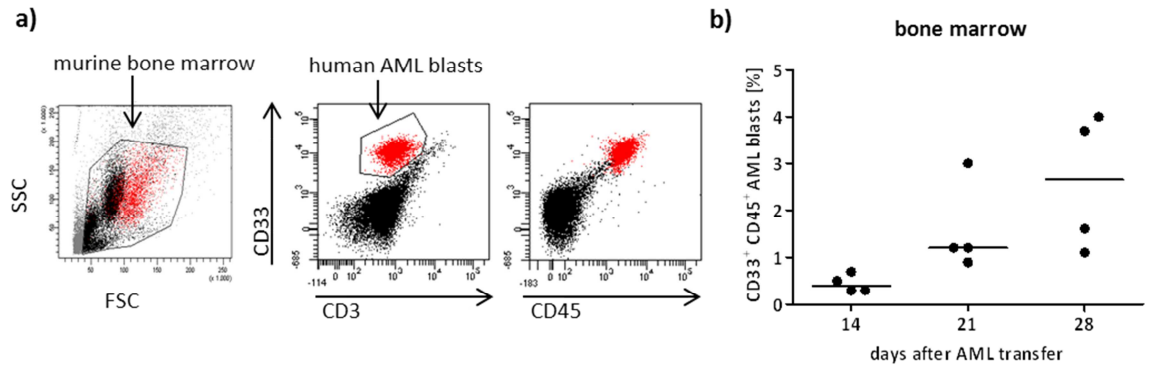

Figure 4.12: **Kinetics of AML engraftment.** a) For the assessment of the engraftment of MZ921-AML in the bone marrow of NSG mice, samples were stained in FACS analysis. AML blasts were identified by the expression of the myeloid marker CD33, the human haematopoietic marker CD45 and the absence of the T-cell marker CD3. b) The engraftment kinetics of AML blasts in the bone marrow was monitored two, three and four weeks after the injection of 5x10^6 AML blasts/mouse. Median values are shown.

AML blasts from patient MZ747-AML were shown to express only very low levels of HLA-DP (*01:01/06:01) on their surface. Without cytokine pre-incubation (see Figure 4.1), these blasts might potentially not be sufficiently recognised by HLA-DP-targeting T cells *in vivo* and were therefore not used for *in vivo* experiments. It should be remembered that the MZ747-AML/Don974-drived T cells were originally all generated with cytokine-treated MZ747-AML blasts. Blasts from patient MZ770 on the other hand, do express high amounts of the HLA-DP allele *01:01 on the surface even in the absence of promoting cytokines and thus appeared most suitable for the T cell transfer studies instead. Mini populations from MZ747-AML/Don974 that were directed against the DP*01:01 mismatch were shown to be cross-reactive towards blasts from patient MZ770. In engraftment kinetics, MZ770-AML blasts ($5x10^6$ AML blasts/mouse) took 21 days to show a median engraftment rate of 2.7 % in the bone marrow (data not shown). Therefore, T-cell transfer in this model was also performed three weeks after AML injection.

4.7.2 Design of *in vivo* experiments

Mice were sub-lethally irradiated and injected with $5x10^6$ AML blasts for engraftment. Three weeks afterwards, three mice were sacrificed and bone marrow was analysed to ensure engraftment of the AML blasts. All other mice received $5x10^6$ T cells by i.v. injection into the tail vain together with IL-2 and Fc-IL-7 to promote T-cell growth *in vivo*. Control mice were either injected with only cytokines or with irrelevant $CD4^+$ T cell that were generated against AML blasts expressing different HLA-DP alleles. They were characterised regarding their phenotype and lytic activity on the same day. Three and seven days after transfer, the mice were sacrificed and the levels of human $CD33^+/CD45^+$ AML blasts and human $CD45^+/CD3^+/CD4^+$ T cells in bone marrow, spleen and blood were analysed in FACS (Figure 4.13).

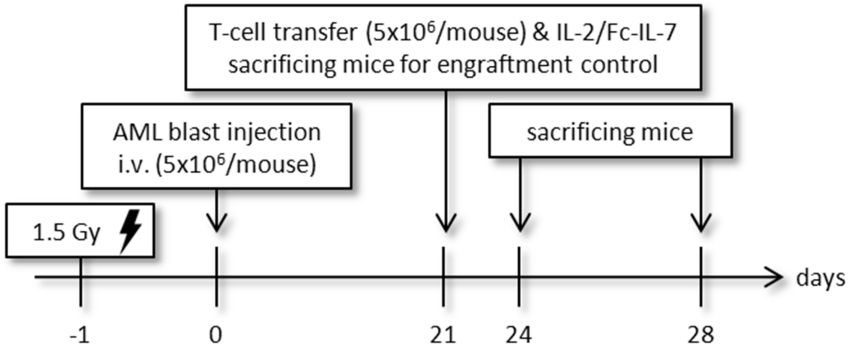

Figure 4.13: Scheme of the protocol to analyse leukaemia reactivity of CD4$^+$ T cells *in vivo*. After the sub-lethal irradiation of NSG mice, they were injected with 5x10^6 AML blasts/mouse that were allowed to engraft for three weeks. Then, 5x10^6 T cells were transferred per mouse together with 1000 U IL-2 and 20 µg Fc-IL-7. Mice were sacrificed either three or seven days after T-cell transfer and AML and T-cell infiltration were analysed in bone marrow, spleen and blood.

4.7.3 T cells on the day of transfer

For transfer experiments, T-cell populations from two different patient/donor pairs were used, namely CD4$^+$ 2C4 and 5B7 from MZ921-AML/Don671 for experiments in MZ921-AML engrafted mice and CD4$^+$ 4C7 from MZ747-AML/Don974 that also recognised blasts from patient MZ770 for MZ770-AML engrafted mice. T cells were thawed, restimulated twice and transferred on day 42+3 (5B7 and 4C7) or 49+3 (2C4) of culture to ensure proper growth and reactivity. On the day of transfer, they were characterised regarding their phenotype and lytic activity to assess their potential to home into lymphoid tissue and perform effector functions.

The clonal CD4$^+$ mini population 2C4 that was generated against AML blasts from patient MZ921 (HLA-DP*14:01/19:01) expressed the TCR chain Vβ13.6 to 100 % and was therefore of monoclonal origin, whereas Vβ13.1 could be detected on 5B7 from the same patient/donor, also indicating a monoclonal population. The reactivity of both populations towards the HLA-DP mismatch *14:01 (depicted in Table 4.4) could be blocked by anti-DP antibodies in an IFN-γ ELISpot assay. To ensure that the *in vivo* effect was specifically mediated by the HLA-DP-recognising T cells and not due to the presence of just any T cell, the polyclonal CD4$^+$ mini population 1H7 was generated as specificity control from the naive CD4$^+$ subset of BC780 and was stimulated against MZ667-AML expressing entirely different HLA class II alleles (HLA-DP*04:01/04:02). In FACS analysis all three T cells were shown to exhibit a memory phenotype by the expression of CD45RO and CD25 (Figure 4.14 a). The co-stimulatory molecule CD27, the IL-7R (CD127) and the naive markers CD45RA and CD31 were not detectable. The homing molecule CD62L was only present in low amounts on the

clone 2C4, whereas the chemokine receptor CCR7 could not be detected on either T cell. CXCR4 however, which also plays a role in T-cell homing, was strongly expressed on all T cells along with the co-stimulatory molecule CD28. In a cytotoxicity assay the mini populations 2C4 and 5B7 showed lysis of the relevant AML blasts (MZ921-AML) up to 57 % and 61 % (E:T 60), respectively, whereas the $CD4^+$ specificity control did not (Figure 4.14 b). Nevertheless, control cells were functional as they lysed the AML blasts that they were generated against up to 46 % (E:T 60) and the corresponding LCL to 82 % (E:T 60). Donor-derived LCLs and K562 were not lysed by either of the $CD4^+$ T cells.

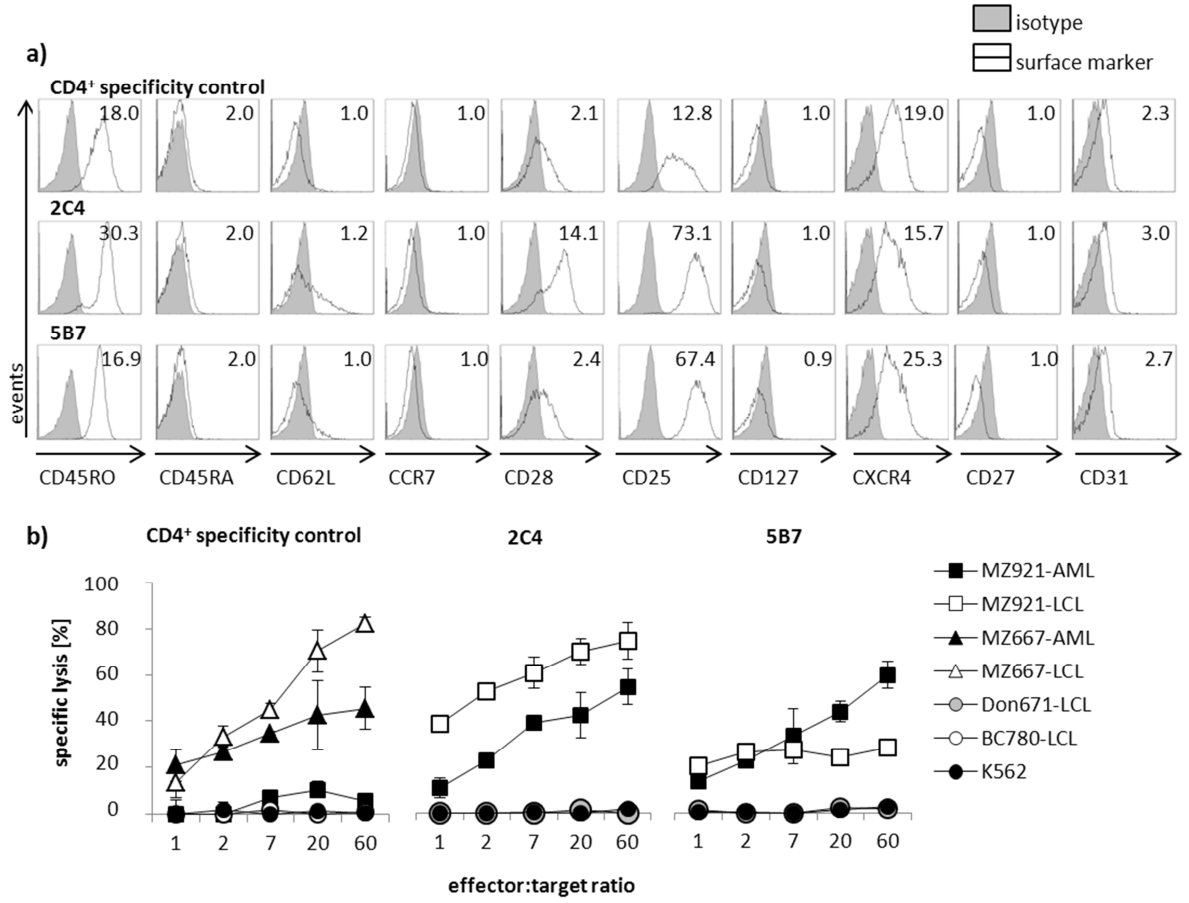

Figure 4.14: Phenotype and lytic activity of $CD4^+$ T cells on the day of transfer into MZ921-AML engrafted NSG mice. Mini-MLLCs used as $CD4^+$ specificity control (1H7 from MZ667-AML/BC780, day 42+3 of culture) and clonal $CD4^+$ 2C4 and 5B7 (MZ921-AML/Don671, day 49+3 and 42+3 of culture, respectively) were analysed on the day of transfer into MZ921-AML engrafted NSG mice regarding a) phenotypic markers in FACS analysis and b) lytic activity towards AML blasts, LCLs and K562 in a 5 h ^{51}Cr-release assay. Numbers indicate the relative mean fluorescence intensity (rMFI) of surface marker versus IgG isotype control.

The T-cell population 4C7 from MZ747-AML/Don974 (HLA-DP*01:01/06:01) was of oligoclonal origin as only the expression of the TCR chain Vβ22 could be identified on 19 % of

the cells (details in Table 4.4). The further Vβ chains expressed could not be detected with the commercially available antibody panel. Reactivity could be blocked with an anti-DP antibody in an IFN-γ ELISpot assay and was directed against the DP*01:01 mismatch, the same allele that was also expressed on MZ770-AML. The T-cell clone 6E7 from MZ921-AML/Don671 was used as specificity control as it was generated against a DP*14:01 mismatch and did not recognize MZ770-AML blasts. Both T cells showed a memory phenotype in FACS analysis as the naive markers CD45RA, CD27 and CD31 were downregulated and the activation markers CD25 and CD45RO were strongly expressed (Figure 4.15 a). The co-stimulatory molecule CD28 was expressed on 4C7 as well as the control T cell. The chemokine receptor CXCR4 was expressed on both T cells. The homing molecules CD62L and CCR7 were also analysed. L-selectin (CD62L) was present on 4C7, whereas the chemokine receptor CCR7 could not be detected. In ^{51}Cr-release assays 4C7 showed an intermediate level of lysis of patient-derived LCLs from MZ747-AML and of AML blasts and LCLs from patient MZ770. Surprisingly, blasts from MZ747-AML were not lysed, which was probably due to a low HLA-DP expression (Figure 4.15 b). $CD4^+$ cells of the specificity control 6E7 only showed cytotoxicity against AML blasts and LCLs from patient MZ921, which they were generated against. MZ770-AML blasts that were engrafted into the NSG mice were not recognised. Summed up, memory T cells that expressed the necessary homing molecules and showed specific cytolytic reactivity towards engrafted AML blasts *in vitro* were transferred into NSG mice.

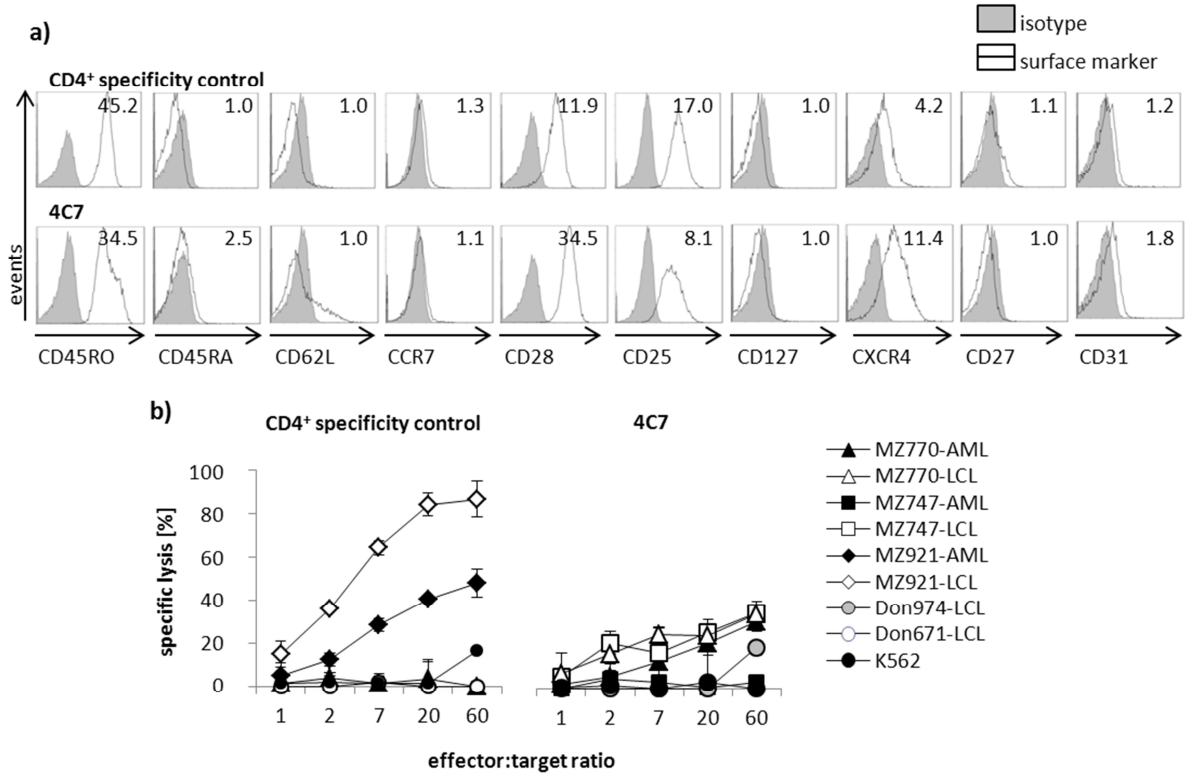

Figure 4.15: Phenotype and lytic activity of T cells on the day of transfer into MZ770-AML engrafted NSG mice. Mini-MLLCs used as $CD4^+$ specificity control (6E7 from MZ921-AML/Don671) and $CD4^+$ 4C7 (MZ747-AML/Don974) were analysed on day 42+3 of culture when they were transferred into MZ770-AML engrafted NSG mice regarding a) phenotypic markers in FACS analysis and b) lytic activity towards several targets including the cross-reactive recognition of MZ770-AML in a 5 h ^{51}Cr-release assay. Numbers indicate the relative mean fluorescence intensity (rMFI) of surface marker versus IgG isotype control.

4.7.4 T-cell persistence in NSG mice

Three and seven days after T-cell transfer, NSG mice were sacrificed and analysed for the persistence level of T cells. Therefore, organs were stained for FACS analysis for the co-expression of the haematopoietic marker CD45 and the T-cell markers CD3 and CD4 as it is shown exemplarily for the bone marrow (Figure 4.16 a). T cells in MZ921-AML engrafted mice were able to reach lymphoid organs as they could be found in bone marrow, spleen (Figure 4.16 b) and in very low levels also in peripheral blood (data not shown). Three days after transfer, a median of 0.1 % of 2C4 and 0.2 % of 5B7 could be detected in the bone marrow and 0.3 % of 2C4 and 0.45 % of 5B7 in the spleen. In the blood only 0.1 % of 5B7 persisted. The $CD4^+$ specificity control was only detectable in the spleen to 0.1 %. One week after transfer, T-cell levels were more pronounced, as a median of 0.2 % of the $CD4^+$ specificity control, 2.6 % of the clone 2C4 and 1.7 % of the clone 5B7 were detectable in the bone marrow (Figure 4.16 c). 2C4 also homed to the spleen, where 0.4 % were detected,

whereas the specificity control and 5B7 could only be found in individual mice. No potential T-cell contaminants grew out of the leukaemic graft, as none could be detected in the control group that only received cytokines and no $CD4^+$ T cells. These results indicated an efficient homing of the donor-derived T cells into the lymphoid tissue. Furthermore, their ability to proliferate within the NSG mice could be assumed as T-cell levels increased over time. Four weeks after T-cell transfer the clonal population 2C4 could still be found to 5.1 % in bone marrow, 0.2 % in spleen and 0.9 % in blood (data not shown). In addition, mice engrafted with MZ921-AML showed no signs of xenogeneic GvHD caused by the transferred T cells, which was assessed by the intact fur status and overall vitality of the mice.

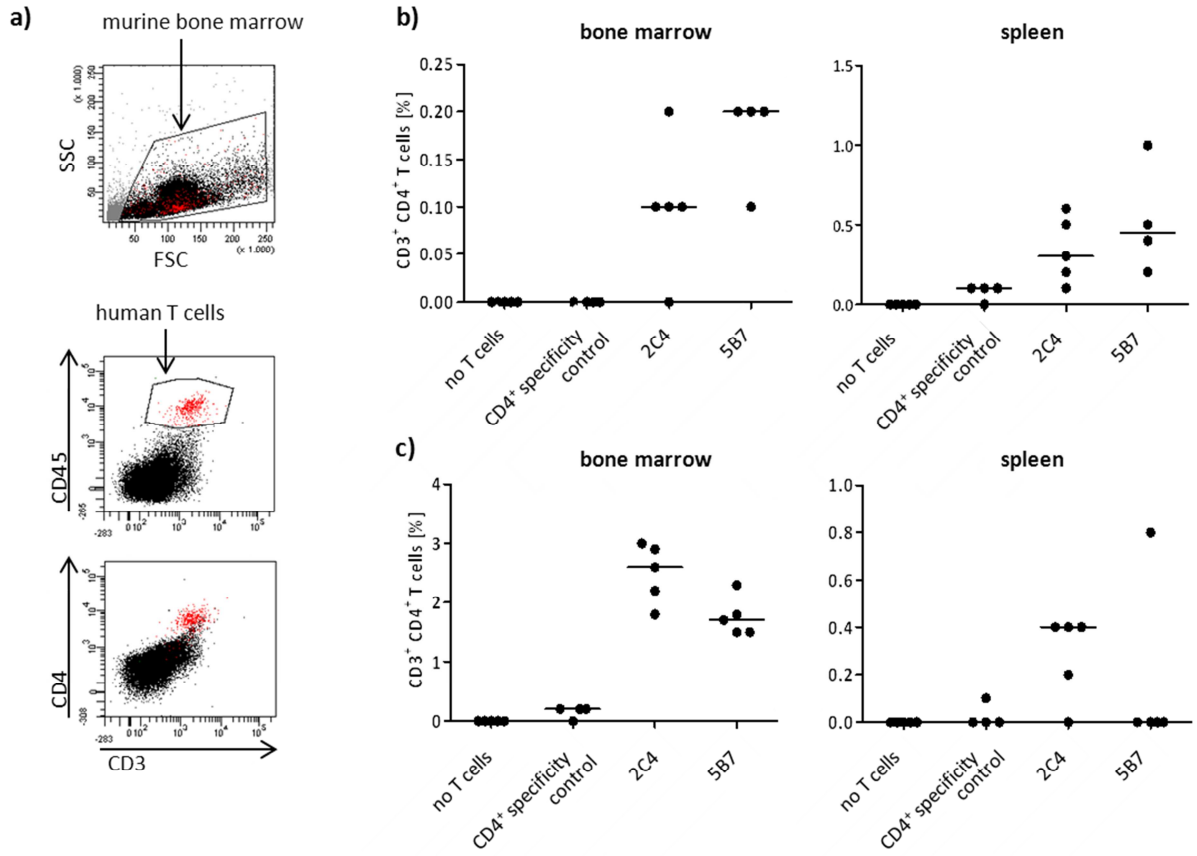

Figure 4.16: T-cell persistence in MZ921-AML engrafted NSG mice three and seven days after adoptive transfer. MZ921-AML blasts were engrafted in NSG mice for three weeks before T cells were transferred. Mice either received cytokines (Fc-IL-7 and IL-2) alone, a $CD4^+$ specificity control (1H7 from MZ667-AML/BC780) or the $CD4^+$ clones 2C4 and 5B7 (both from MZ921-AML/Don671), respectively. a) In FACS analysis, T-cell gating was performed according to the expression of human T-cell markers CD45, CD3 and CD4. T-cell levels were analysed b) three and c) seven days after adoptive transfer in bone marrow and spleen. Median values are shown.

T-cell levels in MZ770-AML engrafted mice could not be reliably determined. Due to T cells that grew out of the AML samples, it could not be distinguished between donor- and patient-derived $CD3^+$ cells. No significant increase in $CD3^+/CD4^+$ cells could be determined in mice that were adoptively transferred.

4.7.5 AML reactivity in NSG mice

To determine the anti-leukaemic reactivity of the transferred T cells *in vivo*, the sacrificed mice were analysed regarding infiltration levels of AML blasts in bone marrow, spleen and blood. Leukaemic blasts were detected in FACS analysis using the human haematopoietic marker CD45 and the human myeloid marker CD33. In mice that were engrafted with MZ921-AML both clonal T cells, 2C4 and 5B7, generated from the patient/donor pair MZ921-AML/Don671 significantly reduced the AML burden in the bone marrow after three days from 2.0 % down to 0.0 % ($p = 0.0119$) and 0.4 % ($p = 0.0195$), respectively (Figure 4.17 a). In the spleen no reduction could be observed, as AML levels were already as low as 0.1 % in mice that received cytokines alone. In the blood no AML engraftment could be detected at this early time point. The $CD4^+$ specificity control generated against the MZ667-AML carrying different HLA-DP alleles did neither reduce the AML levels in bone marrow (2.1 %) nor spleen. This effect was even more pronounced seven days after T-cell transfer (Figure 4.17 b). The AML level in the bone marrow was reduced from a median of 5.2 % in the control group that only received cytokines to 0.1 % in the groups transferred with 2C4 ($p = 0.0119$) and 5B7 ($p = 0.0119$), respectively. There were still only low levels of AML blasts (0.1 %) detectable in the spleen, so that no effect of the $CD4^+$ T-cell clones could be seen here. No significant influence of the $CD4^+$ specificity control on the AML burden could be measured, neither in spleen nor bone marrow. The same results were obtained by analysing peripheral blood, but AML levels were too low to draw definite conclusions.

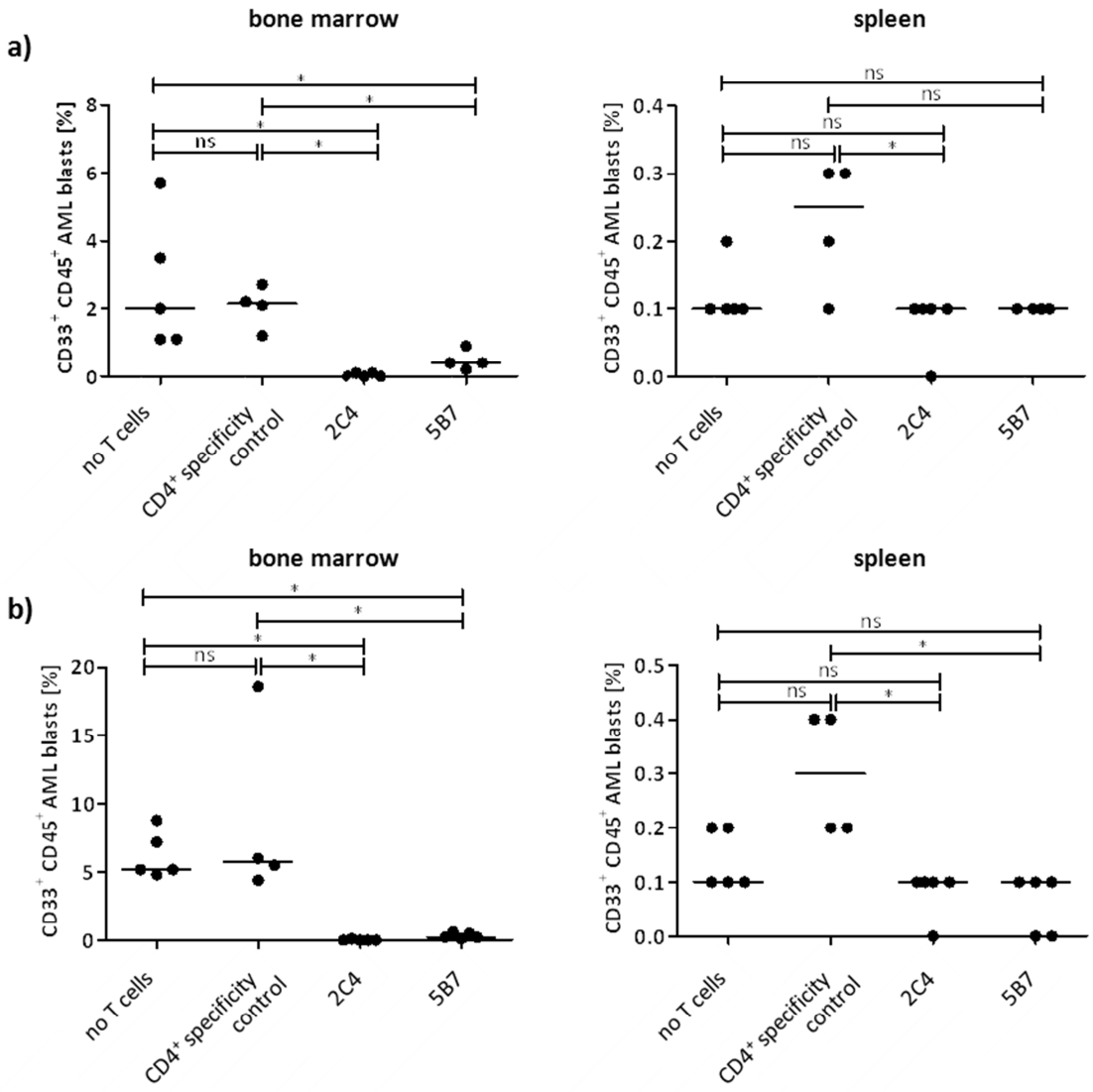

Figure 4.17: Level of AML-infiltration in NSG mice engrafted with MZ921-AML three and seven days after adoptive T-cell transfer. NSG mice were injected with AML blasts from patient MZ921. After an engraftment period of three weeks to ensure an engraftment rate of 1-3 % in the bone marrow, adoptive T-cell transfer was performed. Mice either received cytokines (Fc-IL-7 and IL-2) alone, a $CD4^+$ specificity control (1H7 from MZ667-AML/BC780) or the HLA-DPB1*14:01-specific $CD4^+$ T-cell clones 2C4 and 5B7 (both from MZ921-AML/Don671), respectively. Mice were sacrificed a) three and b) seven days after transfer and AML levels in bone marrow and spleen were analysed according to the expression of the human myeloid marker CD33 and the human haematopoietic marker CD45. Median values are shown and statistical analysis was performed using Mann-Whitney test. Asterisks indicate p-values <0.5 that were considered statistically significant.

Mice engrafted with MZ770-AML were analysed one week after T-cell transfer. Human AML blasts could be detected in control mice that only received cytokines without T cells. They showed a median engraftment rate of 1.65 % in the bone marrow and 0.25 % in the spleen (Figure 4.18). The AML burden could be significantly reduced down to 0.1 % ($p = 0.0286$) by the transferred $CD4^+$ T cell 4C7 in the bone marrow. The irrelevant control T cell did not lead to a significant reduction. In the spleen the injection of 4C7 resulted in a

decrease of leukaemia infiltration that was not significant. Median values were used for statistical analysis using the Mann-Whitney test. Taken together, these results showed that the adoptive transfer of HLA-DPB1-specific $CD4^+$ T cells could significantly reduce the leukaemic burden in the bone marrow of AML-engrafted NSG mice.

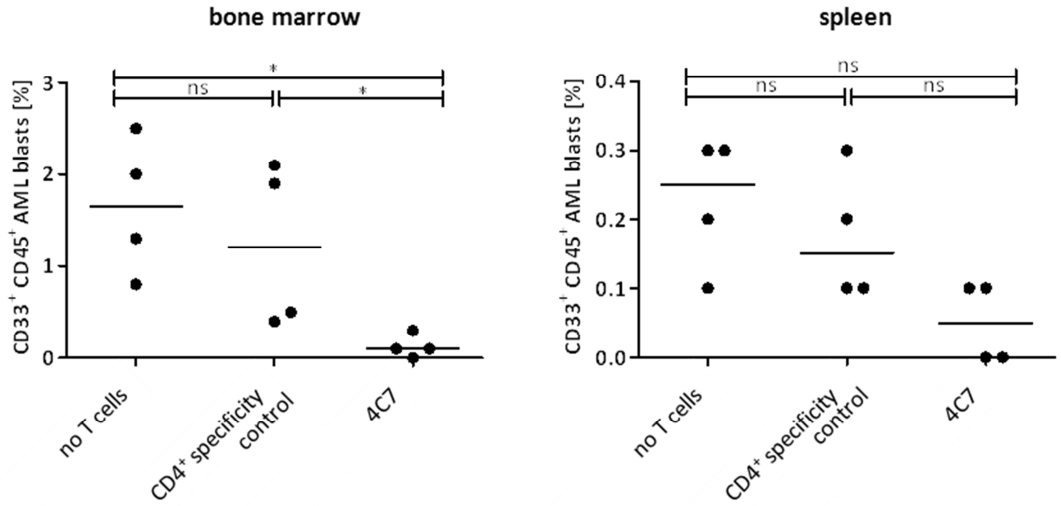

Figure 4.18: Level of AML-infiltration in NSG mice engrafted with MZ770-AML one week after adoptive T-cell transfer. NSG mice were injected with AML blasts from patient MZ770. After an engraftment period of three weeks to ensure an engraftment rate of 1-3 % in the bone marrow, adoptive T-cell transfer was performed. Mice either received cytokines (Fc-IL-7 and IL-2) alone, a $CD4^+$ specificity control (6E7 from MZ921-AML/Don671) or the $CD4^+$ T cell 4C7 (MZ747-AML/Don974) that showed cross-reactive allo-recognition of MZ770-AML via HLA-DPB1*01:01. One week after transfer, mice were sacrificed and AML levels in bone marrow and spleen were analysed according to the expression of the human myeloid marker CD33 and the human haematopoietic marker CD45. Median values are shown and statistical analysis was performed using a Mann-Whitney test.

5 Discussion

For relapsing leukaemia patients classified as high risk the only curative treatment is allogeneic haematopoietic stem cell transplantation (HSCT) (Burnett *et al.*, 2011). However, even after transplantation, the relapse and mortality rates remain unacceptably high and demand the exploration of new therapeutic options (Kubal & Lancet, 2013). Most research in this field is performed on the adoptive transfer of donor-derived $CD8^+$ T cells (Schetelig *et al.*, 2005). Nevertheless, it was shown in previous studies that the stimulation of T cells against multiple antigens expressed by myeloid leukaemias resulted in strong $CD4^+$ and $CD8^+$ T cell expansion and cytokine production (Weber *et al.*, 2013). Furthermore, anti-leukaemia reactivity was observed in other experiments to mainly reside within the $CD4^+$ compartment (Thomas-Kaskel *et al.*, 2007). In murine tumour models $CD4^+$ T cells were reported to exhibit stronger tumour-reactivity as compared to $CD8^+$ T cells. In addition, they could successfully eliminate tumours that were resistant to $CD8^+$ treatment due to HLA class I downregulation (Perez-Diez *et al.*, 2007). Therefore, the aim of this study was to establish a reliable protocol for the *in vitro* generation of AML-reactive $CD4^+$ T cells from the naive compartment of healthy donors. With this stimulation procedure T_H1 type cells with memory phenotype could readily be expanded in eight out of eight patient/donor pairs. They showed lytic activity against patients' AML blasts and were directed to HLA-DP disparities in HLA-A/-B/-C/-DR/-DQ-matched patient/donor settings (10/10). In a therapeutic *in vivo* model, where NSG mice were engrafted with human AML blasts, adoptively transferred $CD4^+$ T cells homed to bone marrow and lymphoid tissue and reduced the leukaemic burden in bone marrow after a single injection.

5.1 The generation of AML-reactive $CD4^+$ T cells from naive precursors

5.1.1 AML blasts and their potential for T-cell stimulation

For the generation of AML-reactive T cells, naive $CD4^+$ cells were isolated from healthy donors and were stimulated against primary AML blasts from patients. These AML blasts, obtained from peripheral blood or bone marrow of patients, were irradiated to prevent their growth and were pre-incubated with various cytokines to ensure a proper antigen-presenting cell phenotype according to the expression of HLA class II and co-stimulatory molecules like CD40, CD80 and CD86. CD80 was shown to be essential for the recognition of

myeloid leukaemia cells by T cells (Matulonis *et al.*, 1995; Dubey *et al.*, 1995) and already low amounts of CD80 and CD86 were associated with prolongation of remission in patients with AML (Whiteway *et al.*, 2003). Also B-cell leukaemias were better in terms of stimulating $CD4^+$ T-cell responses, if CD40 was strongly expressed (Ranheim & Kipps, 1993). Therefore, the expression of these molecules was investigated on all eight AML patient samples used in this study. Only the blasts from patients MZ285, MZ683 and especially MZ921 expressed co-stimulatory molecules and also HLA class II in high amounts after 24 h of culturing without cytokines. This approach was used, as it was shown previously that the relevant molecules could be upregulated after one day in culture when compared to analysis performed directly after thawing of the cells in all FAB subtypes (Brouwer *et al.*, 2000a). The blasts from patients MZ237, MZ369, MZ747 and MZ894 needed cytokine pre-treatment to obtain the upregulation of the APC molecules of interest. As this incubation lasted for two, three or four days, cell viability strongly decreased in some cases, especially for MZ683-AML blasts. Incubation of AML blasts with IL-4/TNF-α/GM-CSF/SCF for four days led to an upregulation of the relevant molecules in six out of seven AMLs. This cytokine cocktail induced morphologic changes in AML blasts that are typical for DCs, the professional antigen-presenting cells, like the upregulation of CD40, CD54, CD80 and CD86 and they were hence more potent in T-cell stimulation (Brouwer *et al.*, 2000b). A stronger T-cell recognition of cytokine pre-treated AML blasts was observed in the patient/donor pair MZ747-AML/Don974, where T cells did not recognise untreated AML blasts but did so with pre-treated leukaemia blasts. Two-day incubation with IFN-γ/sodiumbutyrate (Maeda *et al.*, 2000) and three-day incubation with IFN-γ/TNF-β (Eljaafari *et al.*, 2006) induced the upregulation of co-stimulatory molecules to a slightly lower extent compared to the aforementioned cytokine cocktail. Altogether, no general conclusion could be drawn, but AML blasts from each patient had to be tested individually for the APC phenotype upon cytokine pre-treatment, also taking into account the cell viability at the various time points.

5.1.2 $CD4^+$ T cells isolated from the naive subset

$CD4^+$ T cells for the stimulation against AML blasts were isolated from the naive subset of those healthy donors, who also served as stem cell donors for the transplantation procedure of the AML patient, except for the HLA-DR/-DQ-matched model MZ237-AML/Don256. The naive T-cell subset was chosen as it was reported previously that the earlier the phenotype

the higher the T cells' proliferative capacity and the better their potential to establish immunologic memory and to mediate anti-tumour activity after adoptive transfer (Hinrichs *et al.*, 2009; Hinrichs *et al.*, 2011; Albrecht *et al.*, 2011; Gattinoni *et al.*, 2011). It was shown previously that minor histocompatibility antigens could be identified by the stimulation of naive $CD8^+$ T cells (Bleakley *et al.*, 2010). Nevertheless, starting populations for *in vitro* testings were so far only isolated from the naive $CD8^+$ compartment (Albrecht *et al.*, 2011; Jedema *et al.*, 2011) or bulk $CD4^+$ T cells after *ex vivo* isolation from patients following HSCT or DLI were used (Stevanović *et al.*, 2012; Stevanović *et al.*, 2013a; Rutten *et al.*, 2013). Naive $CD3^+$ bulk cultures were also successfully applied to generate $CD8^+$ and $CD4^+$ CTL populations with EBV-reactivity (Hanley *et al.*, 2009). Nonetheless, the naive $CD4^+$ T-cell compartment has not been used before for the stimulation of AML-reactive populations, to the best of our knowledge. To compare the leukaemia- and alloreactivity of naive to memory $CD4^+$ T cells, the weekly restimulated populations were tested in IFN-γ ELISpot assays for their recognition of AML blasts. It could be shown that T cells derived from the naive compartment gave rise to 1.3 to 2.5-fold more reactive populations, which also showed stronger IFN-γ production compared to memory T cells. One possible explanation could be TCR diversity, which is 100-fold broader among naive T cells, compared to memory cells and can therefore lead to stronger alloreactivity by more available TCRs that can respond (Nikolich-Zugich *et al.*, 2004; Foster *et al.*, 2004).

Naive T cells were enriched either by magnetic (MACS) or fluorescence activated cell sorting (FACS). Within the Naive $CD4^+$ T Cell Isolation Kit II a cocktail of antibodies is responsible for the depletion of memory, $CD8^+$ and NK cells, monocytes and neutrophils. Nevertheless, this depletion was not sufficiently pure with 95 % of the populations tested being contaminated especially with $CD8^+$ T cells and sometimes also NK cells, which overgrew the $CD4^+$ T cells over several weeks of *in vitro* culturing, indicating an insufficient elimination of these cell types. Also the subsequent depletion of $CD45RO^+$ and $CD8^+$ cells resulted in a contamination frequency of 95 % of the tested populations, which could be attributed to the lack of CD45RO expression on the surface of NK cells (Richards *et al.*, 1990). For future experiments, a comparison of this separation process with an additional depletion of $CD56^+$ NK cells might be necessary.

An alternative way of separating naive $CD4^+$ T cells was FACS sorting according to the expression of $CD3^+/CD4^+/CD45RA^+$. CD45RA was described previously as a potent marker for

the separation of naive T cells (Rosa *et al.*, 2001; Stemberger *et al.*, 2012). The approach used in this study resulted in high purity of the collected fraction and low contamination rates after several weeks of culture. The T cells that were obtained after sorting showed a naive phenotype as the expression levels of the homing molecules CCR7, CD62L and CXCR4 and the co-stimulatory molecules CD27 and CD28 were high (Seder & Ahmed, 2003; Appay *et al.*, 2008). Contaminations with low numbers of CD45RA-expressing late stage effector cells did not adversely influence the results. Therefore, FACS sorting proved to be the more reliable method for the isolation of naive $CD4^+$ T cells.

5.1.3 The mini-MLLC approach for the generation of leukaemia-reactive $CD4^+$ T cell clones

AML-reactive $CD4^+$ T cells were generated successfully in eight out of eight HLA-DR and -DQ matched patient/donor pairs. The mini-mixed lymphocyte/leukaemia cell culture (mini-MLLC) approach described previously (Distler *et al.*, 2008; Albrecht *et al.*, 2011) was used in this study. Starting concentrations of isolated naive $CD4^+$ T cells were chosen low with $1x10^3$ to $1x10^4$ T cells per well, as precursor frequencies of alloreactive donor anti-recipient $CD4^+$ T cells were estimated to range between $3x10^{-4}$ to $2x10^{-6}$ (Schwarer *et al.*, 1993; Potolicchio *et al.*, 1996). Stevanović and colleagues also used a comparable method by sorting of $CD4^+$ T cells taken from PBMCs of patients after HSCT into 96-well plates for stimulation, which resulted in clonal populations (Stevanović *et al.*, 2013b). Monoclonal populations were detected in FACS analysis according to the TCR Vβ chains expressed on the surface of the T cells. 32 % of the T cells tested were shown to be of monoclonal origin, 60 % were oligoclonal and only 8 % were polyclonal. This indicated that the starting concentrations were chosen adequately and suggested for future experiments that ideally $7.5x10^3$ naive T cells per well should be used for the initial stimulation. Responder populations started in bulk cultures of $>1x10^4$ T cells would give rise to numerous expanding T cells with various specificities. In contrast, the approach established herein reliably led to a significant fraction of monoclonal leukaemia-reactive $CD4^+$ T-cell populations that could be used for subsequent functional and phenotypic studies.

During the first two weeks of stimulation a cytokine cocktail containing IL-7, IL-12 and IL-15 was added to mini-MLLCs according to a similar protocol that was previously established for naive $CD8^+$ T cells (Albrecht *et al.*, 2011). IL-7 and IL-15 were reported to play

a role in homeostatic proliferation of naive T cells with IL-15 additionally favouring memory formation (Tan *et al.*, 2001; Surh & Sprent, 2008). IL-12 is needed for T_H1 differentiation and IFN-γ secretion (Wurster *et al.*, 2000; Hsieh *et al.*, 1993). When this cytokine combination was applied, leukaemia-reactive $CD4^+$ T-cell populations could reliably be generated in all eight patient/donor pairs.

Nevertheless, the importance of adding IL-12 was investigated. For further clinical applications, when the *in vitro* generated T cells would be given to patients together with HSCT or as DLI, all media and cytokines needed to be provided in GMP (good manufacturing practice) grade. Unfortunately, GMP IL-12 is currently not available. Omitting IL-12 led to a 2-fold reduction in the number of AML-reactive $CD4^+$ populations in one patient/donor pair, whereas no effect was seen in a second one, while the number of reactive $CD8^+$ populations, generated in parallel using the same conditions, was reduced 2-fold without IL-12. Therefore, the IL-12 effect seemed to be strongly patient-specific and at least stronger in $CD8^+$ compared to $CD4^+$ T cells.

As the effect mediated by IL-12 was not so clear, this might explain the lacking influence seen when IL-27 was applied to naive $CD4^+$ T-cell cultures in addition to the IL-7/12/15 cytokine cocktail. IL-27 was reported to act synergistically with IL-12 and to drive T_H1 commitment and to enhance clonal expansion (Pflanz *et al.*, 2002; Lucas *et al.*, 2003). Nevertheless, if the effect of IL-12 was not too pronounced, the T cells' responsiveness to IL-12 could not be enhanced by the addition of IL-27. Therefore, further experiments combining IL-12 and IL-27 are necessary to clarify this point.

Moreover, the addition of IL-21, shown previously to enhance proliferation and reactivity of $CD8^+$ T cells (Albrecht *et al.*, 2011) and also to amplify $CD4^+$ proliferation and IFN-γ production and to maintain $CD4^+$ T-cell subsets (Peluso *et al.*, 2007; Onoda *et al.*, 2007; Ferrari-Lacraz *et al.*, 2008), was tested. IL-21 led to a reduction of reactive $CD4^+$ populations by 27-62 % in two out of three patient/donor pairs, and no effect on IFN-γ production or proliferation could be observed. Therefore, the experiments performed in this study showed that IL-21, which is mainly produced by $CD4^+$ T_H1 cells themselves, does not seem to be essential for $CD4^+$ T-cell differentiation (Casey & Mescher, 2007) and the formation of leukaemia-reactive $CD4^+$ T cells (Kaka *et al.*, 2009).

In summary, a protocol for the reliable generation of leukaemia-reactive $CD4^+$ T_H1 cells, as determined by their cytokine secretion profile (IFN-γ and TNF-α but no or low IL-4

production), could successfully be established in this study. The $CD4^+$ T cells could readily be expanded to high cell numbers of up to $1x10^9$ per single population within eight weeks of *in vitro* culture.

5.2 The lytic activity of $CD4^+$ T cells

$CD4^+$ T cells that were stimulated against AML blasts did not only secrete cytokines but also successfully lysed target cells, which supports earlier findings (van de Berg *et al.*, 2008; Brown, 2010). When compared side-by-side to $CD8^+$ T cells in the MZ921-AML/Don671 model after comparable time of *in vitro* culture, the level of cytolysis was similar. Overall, lytic activities observed were as high as 65 % for AML blasts and 79 % for patient-derived LCLs at effector to target ratios of 1:20. Cytolytically active $CD4^+$ T cells have been described before. They could be isolated from healthy individuals (Appay *et al.*, 2002) and they were reported to successfully lyse AML blasts (Ibisch *et al.*, 1999). Nevertheless, lytic $CD4^+$ T cells were previously described to be $CD28^-$ (Appay *et al.*, 2002), which could not be observed in this study. The fact that the populations tested herein still expressed high amounts of the co-stimulatory molecule CD28 might render them more susceptible to secondary stimulatory signals and therefore more reactive and proliferative *in vitro* as well as *in vivo*.

To address the question of the underlying mechanism that enables this lytic activity, intracellular FACS stainings were performed and various pathways were blocked in cytotoxicity assays. Granzyme A and B were shown to be intracellularly stored within $CD4^+$ and $CD8^+$ T cells in comparable amounts. 1.8-fold more perforin was detected in $CD8^+$ as compared to $CD4^+$ T cells. To proof that these intracellular cytolytic molecules were actually released when T helper cells got in contact with their leukaemia targets, experiments were performed blocking Fas/FasL interaction and the release of cytotoxic granules using the Ca^{2+}-chelating reagents EGTA and concanamycin A. These experiments showed that $CD4^+$ T cell-mediated lysis was not induced by Fas but by granzymes and perforin instead, at least in a 5 h Chromium-release assay. This predominant role of granule exocytosis was shown previously in various settings (Appay *et al.*, 2002; Mitra-Kaushik *et al.*, 2007). Nevertheless, this might be due to the fact that T cells are already pre-loaded with cytotoxic granules, which assures a rapid killing, whereas the *de novo* synthesis of Fas would require more time (Barry & Bleackley, 2002; Vergelli *et al.*, 1997). On the whole, this study indicated that $CD4^+$ T cells can be as potent as $CD8^+$ T cells in the early *in vitro* killing of AML blasts.

5.3 Allorecognition of mismatched HLA-DP alleles

Adequate donors for HSCTs are usually selected on the basis of the HLA alleles they express and have to be matched for HLA class I, -DR and -DQ alleles according to high resolution typing in order to minimise GvHD and graft rejection (Tiercy, 2002). Due to a recombination hotspot between HLA-DP and the remaining HLA alleles (Begovich *et al.*, 1992), DP mismatches occur in 5 % of all sibling (Büchler *et al.*, 2002) and in over 80 % of unrelated transplantation settings (Santamaria *et al.*, 1994), respectively. This could also be shown in this study as six out of seven patient/donor pairs (86 %) were mismatched for one or two HLA-DPB1 alleles, whereas only one patient/sibling pair was fully matched in all 12 alleles.

When $CD4^+$ T cells generated from mismatched patient/donor pairs were tested in IFN-γ ELISpot assays, it was observed that their reactivity could be blocked with antibodies targeting CD4 and HLA-DP. This indicated the specific recognition of the DP mismatches in a CD4-dependent manner. To confirm this hypothesis, two $CD4^+$ T-cell populations 8F3 and 10C11 that were generated in the patient/donor setting MZ683-AML/Don595 with two HLA-DP mismatches (DPB1*04:01/13:01) were tested for their recognition of AML blasts from different patients and showed two distinct recognition patterns. 8F3 produced IFN-γ only when incubated with AML blasts that shared expression of DPB1*13:01 with the original simulator cells, indicating mismatch reactivity towards this DP allele. 10C11 however, recognised several other AML blasts, all of which expressed DPB1*04:01, suggesting that this $CD4^+$ population was directed against this second mismatched allele. The fact that not all AML blasts carrying DPB1*04:01 were recognised, was probably due to insufficient HLA-DP and co-stimulatory molecule expression without prior cytokine treatment. Alternatively, the $CD4^+$ T cells might be directed against recipient-specific peptide epitopes not expressed in all AML blasts under study. Comparable T cells with HLA-DP mismatch specificity could also be generated *in vitro* by Ibisch and colleagues who also demonstrated the recognition of DP-expressing AMLs (Ibisch *et al.*, 1999). To confirm this mismatch reactivity, the mismatched HLA-DP alleles were cloned and expressed in donor-derived LCLs. HLA-DP β-chains were shown previously to pair exclusively with certain α-chains (Hollenbach *et al.*, 2012) and therefore, their RNA was electroporated in the combinations β04:01/α01:03 and β13:01/α02:01 to assure the presentation of functional molecules. 8F3 showed again the specific recognition of LCLs electroporated with β13:01/α02:01, whilst 10C11 produced IFN-γ only when incubated with β04:01/α01:03 expressing LCLs. This confirmed that both

combinations of α- and β-chains resulted in the surface expression of functional HLA-DP molecules and that both of them were immunogenic enough to stimulate mismatch reactivity in $CD4^+$ T-cell population. The fact that donor-derived LCLs were recognised after DP electroporation further indicated either sole reactivity towards the mismatched HLA-DP molecules or a combined recognition of DP mismatches with presented peptides. It was reported previously that the HLA molecules themselves might play a major role as target structures in mismatched transplantations and could therefore lead to enhanced peptide promiscuity for alloreactive T cells (Shlomchik, 2007). Alloreactive responses can be mediated either in an HLA-dependant or peptide-dependant manner or similar to the normal HLA/TCR interaction with an involvement of both units (Felix & Allen, 2007). If peptide recognition was involved in the alloreactive responses observed in this study, these are not tumour-associated antigens, as they would not be present in donor-derived LCLs. Therefore, the epitopes involved in the T-cell recognition process appeared to be widely expressed HLA-DP-binding peptide molecules.

Single antigen mismatches in the HLA-A, -B, -C or -DRB1 locus have been associated in large retrospective clinical studies with lower survival rates, lower disease-free survival and GvHD when compared to complete 8/8 matched transplantations. Mismatches in HLA-DQ however, could not be correlated with an adverse outcome (Lee *et al.*, 2007). When looking at HLA-DP mismatches, there are highly controversial reports. These mismatches were reported to be associated with higher probability for GvHD and GvL reactions (Warren *et al.*, 2012) without influencing survival rates (Lee *et al.*, 2007), whereas others stated that there is no correlation between the DP status and GvHD (Petersdorf *et al.*, 1993). Shaw and colleagues analysed leukaemia patients after unrelated HSCT and found that there is a significantly increased likelihood of disease relapse if patient and donor are matched for DPB1 (Shaw *et al.*, 2006). From all donors used in this study, except for one case, stem-cell grafts were transplanted into AML patients. Therefore, all experiments described here represent clinically relevant patient/donor combinations. Donor and patient were matched for the relevant HLA-DR and -DQ alleles, whereas there was a single HLA-DP allele mismatch in five patient/donor pairs and a mismatch in both alleles in two pairs tested. These mismatches were categorised according to an algorithm based on the immunogenicity of the DPB1 alleles and their shared alloreactive T-cell epitopes (TCEs) developed by Fleischhauer and colleagues (Crocchiolo *et al.*, 2009). The authors state that non-permissive DPB1

mismatches correlate with increased mortality in 10/10 matched unrelated HSCTs compared to permissive disparities (Fleischhauer *et al.*, 2012). According to this mathematical model the patient/donor pairs used in this study could be grouped into non-permissive (MZ237-AML/Don662, MZ237-AML/Don256, MZ683-AML/Don595 and MZ921-AML/Don671) and permissive (MZ285-AML/Don205 and MZ747-AML/Don974) disparities. Nevertheless, all patients, irrespective of this grouping, died after HSCT. Furthermore, reactive $CD4^+$ T cells could be generated from each patient/donor pair, even though some HLA-DP mismatches were classified as of low immunogenicity (*01:01, *04:01, *04:04, *06:01, *13:01) according to the TCE groups, which might indicate contrary results. In summary, this model of permissive and non-permissive HLA-DP disparities did not predict clinical and immunological outcome parameters in our study.

A second rationale for the generation of AML-reactive $CD4^+$ T cells is the identification of mHags or leukaemia-associated antigens. Nevertheless, the isolation and expansion of peptide-specific T cells proved to be difficult in this work. Preferably patient/donor pairs that are fully matched for all 12 HLA alleles could be used for identifying AML-associated antigens either with the use of cDNA libraries (Griffioen *et al.*, 2012), biochemistry (Hunt *et al.*, 1992) or genetic linkage analysis (Warren *et al.*, 2002). In the study described, only one such setting was available (MZ849-AML/Don691). Antibody blocking experiments proved the existence of HLA-DP as well as -DR and -DQ restricted $CD4^+$ T-cell populations. Nevertheless, antigens could not be identified as the T cells did not grow well over the course of several weeks. This was probably due to poor stimulatory conditions. As patient and donor were siblings in this case, there might not have been enough mHags that the T cells could target in the absence of an HLA-DP mismatch and apparently no or only low immunogenic leukaemia-associated or -specific antigens. Stevanović and colleagues already showed that in 12/12 completely matched DLIs no leukaemia-reactive T cells could be isolated from NOD/scid mice. According to their findings at least single mismatches between HLA-DP alleles are necessary for allorecognition and the induction of a GvL effect (Stevanović *et al.*, 2012; Stevanović *et al.*, 2013b).

In most patient/donor pairs HLA-DP mismatches cannot be avoided following current donor selection rules, so that the mismatch settings analysed in detail in this study reflect the clinical situation and are therefore of high relevance. The fact that HLA-DP mismatch-reactive $CD4^+$ T cells could also be isolated from patients suffering from B-cell lymphoma

following HSCT and subsequent DLI (Rutten *et al.*, 2008) further supports our findings that HLA-DP-reactive T cells can be successfully generated *in vitro* and applied in clinically relevant patient-tailored xenograft models.

One exception to the commonly observed recognition patterns and response blockade using anti-DP antibodies was the $CD4^+$ T cell 11C12 from MZ683-AML/Don595. The strong reactivity of this monoclonal population could be blocked with anti-HLA-DP antibodies and was shown to be directed against the HLA-DP*04:01 mismatch. Nevertheless, anti-CD4 antibodies did not inhibit IFN-γ secretion, when the T-cell clone was incubated with AML blasts, which indicated a strong T-cell receptor (TCR) binding in a CD4-independent manner. CD4, which usually binds to the HLA class II molecule, is responsible for stabilising and enhancing the TCR/HLA interaction, but it was shown in certain clones to be dispensable (Granja *et al.*, 1994). Therefore, cloning of such a high-affinity TCR might be worthwhile. It could then be expressed in cells that show a more efficient killing, e.g. $CD8^+$ T cells or $CD4^+$ T cells with higher amounts of intracellular levels of granzymes and perforin.

5.4 Separating GvL from GvHD

The $CD4^+$ T cells generated in the 10/10 matched setting were in all patient/donor pairs directed against HLA-DP mismatches. As the class II molecules are not expressed on non-haematopoietic cells under non-inflammatory conditions (Klein & Sato, 2000), mismatch-reactive T cells might be a promising tool for the separation of GvL and GvHD. Furthermore, malignant haematopoietic cells in contrast to most other tumours were reported to express HLA class II even under non-inflammatory conditions (Knutson & Disis, 2005). Primary fibroblasts isolated from juvenile foreskin were used to simulate non-haematopoietic GvHD targets. Fibroblast target cells were either left untreated or pre-incubated with IFN-γ to demonstrate that the simulation of inflammatory conditions leads to a strong upregulation of HLA-DP on the surface of most fibroblasts as shown by flow cytometry. IFN-γ ELISpot assays confirmed that all five $CD4^+$ T-cell populations tested exemplarily did not recognise fibroblasts under non-inflammatory conditions and therefore may spare GvHD targets. Strong cytokine secretion in response to cultivation with fibroblasts could only be seen after IFN-γ pre-incubation in four out of five $CD4^+$ T cells. Even though no HLA-DP upregulation was seen for fibroblast #13, it was only recognised under inflammatory conditions, suggesting the importance not only of HLA molecules but also of co-stimulatory molecules

that were probably strongly expressed after IFN-γ treatment. These findings are in accordance with Rutten and colleagues, who showed previously that $CD4^+$ T cells isolated from a leukaemic lymphoplasmocytic lymphoma patient after DLI recognised fibroblasts only if they were pre-treated with IFN-γ, simulating inflammation (Rutten *et al.*, 2008).

Nevertheless, the T-cell population 8F3 from MZ683-AML/Don595 did not recognise IFN-γ pre-treated fibroblasts, indicating the lowest alloreactive potential of all populations tested. The difference here might lie in the mode of allorecognition. There are two extremes, where donor T cells can recognise allogeneic targets either solely in a peptide- or HLA-dependant manner (Felix & Allen, 2007). This would mean that the TCRs of the populations 2E2, 2G2, 10C11 and 11C12 (all from MZ683-AML/Don595) were directed to the recognition of the allogeneic HLA molecule itself, as it was recognised after electroporation into donor-derived LCLs and on fibroblasts, while 8F3 specifically recognised a peptide bound to the mismatched HLA-DP. As there was cytokine secretion when donor-derived LCLs were electroporated with the mismatched DP alleles but not when 8F3 was incubated with fibroblasts under inflammatory conditions, this might indicate the recognition of a haematopoiesis-restricted antigen. If so, 8F3 could spare GvHD targets even under inflammatory conditions and might be an ideal candidate for adoptive T-cell transfer. This generation of different specificities within one patient/donor pair is in line with reports from Casucci and colleagues who reported that in HLA haploidentical settings, T cells could be expanded that contained alloreactive populations directed against unshared HLA alleles as well as against LAAs presented by shared HLAs (Casucci *et al.*, 2013).

5.5 The therapeutic potential of adoptively transferred $CD4^+$ T cells in a murine model

To assess the *in vivo* function of the *in vitro* generated AML-reactive $CD4^+$ T lymphocytes, cells were adoptively transferred into immunodeficient mice engrafted with human AML blasts. If they could migrate to lymphoid tissue and bone marrow and execute their cytolytic activity towards the malignant cells, they might provide powerful effector cells for adoptive immunotherapy in patients. NSG mice were used for *in vivo* experiments, which combine a severe combined immunodeficiency (scid) with a defect in cytokine signalling pathways and do therefore not possess mature T cells, B cells and NK cells. These mice were shown previously to effectively engraft haematopoietic stem cells (Shultz *et al.*, 2005) and

leukaemic blasts (Agliano *et al.*, 2008). For engraftment studies AML blasts from patient MZ770 and MZ921 carrying FLT3-ITD mutations were used, as patient material with this mutation was shown to be most reliable regarding engraftment (Rombouts *et al.*, 2000a; Rombouts *et al.*, 2000b; Brunk, 2011). These findings could be confirmed, as AML blasts could be detected in all mice after administration. After injecting the AML blasts into NSG mice, they were engrafted for three weeks until a leukaemic burden of 1-3 % AML blasts in the murine bone marrow could be detected, simulating the clinical state of minimal residual disease in which immunotherapy was reported to be most effective (Slavin, 2005).

It was shown for $CD8^+$ T cells that their differentiation state inversely correlated with their effector functions *in vivo* due to the downregulation of homing and co-stimulatory molecules and impaired capacities to produce IL-2 and to proliferate (Gattinoni *et al.*, 2005). Therefore, the $CD4^+$ T cells used were transferred at the earliest time point possible, in this case day 45 and 52 of *in vitro* culture. At the day of transfer, the T cells showed an overall memory phenotype, which was among others determined by the expression of CD45RO and CD25 (IL-2R α-chain) in the absence of CD45RA. As they still expressed CXCR4 and the co-stimulatory molecule CD28 but not CCR7, they showed an intermediate phenotype between central and effector memory T cells (Appay *et al.*, 2008), indicating that there is a smooth transition from one differentiation state to the next. For mediating anti-leukaemia effects, the T cells needed to migrate to bone marrow and lymphoid organs like the spleen. Even though they had lost the expression of the lymph node homing molecule CCR7, a chemokine receptor, and most of their CD62L (L-selectin), they still expressed high amounts of CXCR4. This chemokine receptor binds CXCL12 that is strongly expressed in bone marrow and peripheral lymph nodes (Mora & Andrian, 2006). Due to the high sequence homology of >92 % in human and mouse (Shirozu *et al.*, 1995), it seemed to be sufficient for an effective homing of the $CD4^+$ T cells across species barriers, as human T cells could be detected in murine bone marrow and spleen after injection. In addition to a successful homing, the T cells also persisted in the NSG mice and could be detected three, seven and 28 days after transfer in increasing numbers in bone marrow and spleen indicating *in vivo* proliferation. In contrast to the $CD4^+$ T cells, other studies showed that mHag specific $CD8^+$ T cells that were transferred after HSCT peaked as early as three to five days after injection and were cleared from the patients already seven days after administration (Warren *et al.*, 2010). Also AML-reactive $CD8^+$ T cells that were generated and transferred in an identical experimental

setting in our group failed to persist over longer periods (Distler, manuscript in preparation), therefore indicating that $CD4^+$ T cells might be able to mediate more profound and long-lasting effects. Furthermore, transferring $CD62L^-$ effector memory $CD4^+$ T cells was shown to mediate anti-tumour effects in different *in vivo* models but failed to induce GvHD (Chen *et al.*, 2004; Zheng *et al.*, 2008). Therefore, the T cells transferred in this study might have a favourable safety profile, allowing to separate GvL effects from GvHD.

Together with the $CD4^+$ T cells, cytokines were injected into the mice to provide advantageous conditions for T cells to grow also in this foreign environment. Homeostatic proliferation and survival of memory $CD8^+$ T cells was reported to depend on IL-7 and IL-15, whereas IL-7 alone was shown to suffice for memory $CD4^+$ T cells (Schluns *et al.*, 2000; Goldrath *et al.*, 2002; Tan *et al.*, 2002). Therefore, only IL-2, necessary for both $CD8^+$ and $CD4^+$ T cells, and Fc-IL-7 were administered. Due to the Fc-part joined to the cytokine IL-7, longer retention times in the serum of mice could be assured most likely based on the endosomal recycling pathway mediated by the neonatal Fc-receptor (Nam *et al.*, 2010). Therefore, a single cytokine infusion appeared sufficient and was hence chosen in the experimental design.

Transfer experiments were performed with materials from two different patient/donor pairs. $CD4^+$ T-cell clones 2C4 and 5B7 from Donor671 were generated against blasts from patient MZ921, whereas the oligoclonal population 4C7 from Donor974 was stimulated against AML from patient MZ747 but also recognised cells from patient MZ770, which expressed the same HLA-DP allele. The $CD4^+$ T cells that all showed specific lytic activity towards their respective AML blasts at the day of transfer could significantly reduce the AML burden in MZ921-AML and MZ770-AML engrafted NSG mice, respectively. T-cell clones 2C4 and 5B7 reduced the level of AML in bone marrow by 100 % and 80 %, respectively, three days after transfer. Seven days after transfer, the effect was even more pronounced as the AML level was lowered by 98 % from 4.8 % to 0.1 % with both T-cell clones. This indicated that the anti-leukaemia effect of the $CD4^+$ T cells occurred very fast. The T cells successfully migrated to bone marrow and spleen, which were infiltrated by AML blasts, and killed their targets in less than 72 hours. Furthermore, the T cells persisted and obviously prevented reoccurrence of AML growth one week after transfer. Also the oligoclonal T-cell population 4C7 that showed slightly weaker lysis of MZ770-AML blasts *in vitro* (33 % at E:T ratio 60) eliminated leukaemic cells in the bone marrow, so that only 0.1 %

were left one week after transfer compared to 1.65 % in the group that did not receive any T cells. These effects were specific as the $CD4^+$ control populations generated against AML blasts expressing different HLA-DP alleles did not reduce the level of AML in the bone marrow. Due to low numbers of AML blasts in the spleen of control mice that did not receive T cells (0.1 % for MZ921-AML, 0.25 % for MZ770-AML) we were not able to measure definite anti-leukaemic effects of $CD4^+$ T cells in the spleen.

Similar results were obtained in previous studies by other groups, in which pure $CD4^+$ DLIs mediated strong immune responses against human acute lymphoblastic leukaemia (ALL) cells in NOD/scid mice in the absence of $CD8^+$ T-cell contaminations and were therefore the sole mediators of the GvL effect observed. Furthermore, these T cells derived from unrelated donors were shown to be alloreactive and to recognise HLA-DP mismatches. Additionally, $CD4^+$ T cells also exhibited helper functions as the ALL blasts acquired an APC phenotype *in vivo* after DLI and could therefore be more susceptible to killing by $CD4^+$ T cells (Stevanović *et al.*, 2012). The potent effect of leukaemia-reactive $CD4^+$ T cells could also be shown in a CML patient who received injections of *in vitro* expanded lytic T cells from the stem cell donor and showed complete tumour eradication (Falkenburg *et al.*, 1999). The downside of this approach is the fact that a strong HLA class II specific $CD4^+$ T-cell response, especially early after HSCT, when tissue damage can be caused by the conditioning regimen, might induce local inflammation, which then results in the upregulation of HLA molecules on non-haematopoietic target cells and therefore increases the risk of causing GvHD (Falkenburg & Warren, 2011). Also the frequent CMV-reactivation in immune-compromised patients might lead to the release of pro-inflammatory cytokines and HLA class II upregulation (Stevanović *et al.*, 2013b). Therefore, $CD4^+$ T-cell transfer might be ideal at a later time point as DLI after HSCT, when patients have already recovered from early post-transplantation infections and are free of active GvHD.

It should be stressed that the potent GvL effect mediated by human $CD4^+$ T cells in our NSG mouse model was not associated with the occurrence of xenoreactivity against mouse antigen. This is in accordance with previous publications, where xenoreactive T cells were not found to develop in HLA-DP mismatch settings, when these T cells were transferred to leukaemia-engrafted NOD/scid mice (Stevanović *et al.*, 2012; Stevanović *et al.*, 2013b).

Nevertheless, the protocol established in this thesis needs further improvement if clinical application of such leukaemia-reactive $CD4^+$ T cells is the next aim. In our study

relatively high numbers of $CD4^+$ T cells were transferred into NSG mice. Regarding the dose per kg body weight this would not be possible in humans due to the currently still limited capacity of these T cells to expand. However, the $CD4^+$ T cells exhibited a potent anti-leukaemic effect and maintained strong proliferative activity *in vitro* and *in vivo*, so that the number of transferred T cells could surely be reduced, which would render the adoptive T-cell transfer more feasible in humans. Furthermore, it is not clear whether $CD4^+$ T cells alone are able to mediate an ideal long-term GvL effect in humans, as it was shown that depleting either $CD4^+$ or $CD8^+$ T cells from the transplant impaired the GvL effect (Truitt & Atasoylu, 1991). As this result indicates that both T-cell subsets are needed for long-term leukaemia control, transferring a combination of *in vitro*-primed AML-reactive $CD4^+$ and $CD8^+$ T cells might be superior to the sole transfer of $CD4^+$ T cells and needs to be tested in future studies.

6 Summary and outlook

Curative treatment of haematopoietic malignancies including acute myeloid leukaemia (AML) is frequently only possible by allogeneic haematopoietic stem cell transplantation (HSCT), which is unfortunately associated with graft-versus-host disease (GvHD). Both the beneficial graft-versus-leukaemia (GvL) effect and the unwanted GvHD develop from adoptively transferred T and NK cells of donor origin. In order to separate GvL from GvHD, $CD4^+$ T cells can be used. Their target antigens, the HLA class II molecules, are under non-inflammatory conditions only expressed on haematopoietic cells, including the remaining leukaemia blasts, but not on epithelial cells, meaning GvHD target tissue. Particularly, HLA-DP antigens are attractive targets for donor $CD4^+$ T cells, as they are mismatched in up to 80 % of patient/donor combinations according to current donor selection procedures. The aim of this study was to establish a reliable *in vitro* protocol to generate AML-reactive $CD4^+$ T cells in clinically relevant patient/donor pairs. These T cells were isolated from PBMCs of six healthy stem cell donors and enriched according to their surface marker expression, including the subset marker CD4 and the naive marker CD45RA. After isolation, they were stimulated against irradiated primary AML blasts from HLA-A/-B/-C/-DR/-DQ-matched patients and expanded in weekly restimulations up to high numbers of $1x10^8$ to $2x10^9$ after eight weeks. During the first two weeks of *in vitro* culture, the cytokines IL-7, IL-12 and IL-15 proved to be beneficial for generating leukaemia-reactive $CD4^+$ T cells, while IL-21 and IL-27 did neither improve proliferation nor reactivity. Rapidly expanding $CD4^+$ T cells were of T_H1 type and expressed central and effector memory markers including CXCR4, which is important for T-cell homing into bone marrow and lymphoid organs. T cells were shown to specifically recognise mismatch HLA-DP alleles that they were initially stimulated against, also on AML blasts of other patients and after electroporation into donor-derived lymphoblastoid cell lines. Furthermore, they did not recognise GvHD targets like fibroblasts under non-inflammatory conditions. As expected, however, IFN-γ treatment of fibroblasts upregulated HLA-DP and rendered them susceptible to recognition by the $CD4^+$ T cells. Only the $CD4^+$ T-cell population 8F3 from MZ683-AML/Don595 did not recognise fibroblasts even after IFN-γ treatment simulating inflammation, suggesting that the target is a haematopoiesis-restricted antigen presented on the mismatched DP allele. This population would therefore show the best safety profile with regard to mediating GvL effects in the absence of GvHD. When leukaemia-reactive $CD4^+$ T cells, as shown for two different

patient/donor pairs in this study, were transferred into an *in vivo* model of NSG mice engrafted with patients' AML blasts, they migrated to bone marrow and lymphoid organs, effectively eliminated the leukaemic blasts in the bone marrow and persisted for at least four weeks.

Leukaemia-reactive $CD4^+$ T cells generated according to the herein described strategy might be promising tools for immunotherapy of leukaemia patients. In a patient-specific manner, donor-derived $CD4^+$ T cells could be expanded and characterised *in vitro* and lytic clones could then be pooled and transferred into patients free of infections and GvHD, thereby hopefully mediating selective GvL responses. To realise this new treatment option for HSCT patients, the current version of the protocol has to be further developed using entirely good manufacturing practice materials.

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8 Abbreviations

ALL	acute lymphoid leukaemia
AML	acute myeloid leukaemia
APC	antigen-presenting cell
BC	buffy coat
BM	bone marrow
CD	cluster of differentiation
CLL	chronic lymphoid leukaemia
CML	chronic myeloid leukaemia
^{51}Cr	chromium
CMA	concanamycin A
^{137}Cs	caesium
CTL	cytotoxic T cell
DC	dendritic cell
DLI	donor lymphocyte infusion
DMSO	dimethyl sulphoxide
Don	Donor
EBV	Eppstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immune spot
E:T	effector-to-target
FAB	French-American-British
FACS	fluorescence-activated cell sorting
Fb	fibroblast
FSC	forward scatter
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
GvHD	graft-versus-host disease
GvL	graft-versus-leukaemia
HLA	human leucocyte antigen
HRP	horse radish peroxidase
HSCT	haematopoietic stem cell transplantation
IFN	interferon
IL	interleukin
i.v.	intra venous
LAA	leukaemia-associated antigen
LCL	lymphoblastoid cell line
LP	leukapheresis product
LSA	leukaemia-specific antigen
MACS	magnetic-activated cell sorting
MFI	mean fluorescence intensity
mHag	minor histocompatibility antigen
MHC	major histocompatibility complex
MLLC	mixed lymphocyte/ leukaemia culture
NK	natural killer cell
NSG	NOD-scid IL2Rgamma^{null}
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
SB	sodiumbutyrate
SCF	stem cell factor
Sib	sibling
SSC	side scatter
TCR	T-cell receptor
T_{CM}	central memory T cell
T_{EFF}	effector T cell
T_{EM}	effector memory T cell
T_H	T helper cell
T_M	melting temperature
T_N	naive T cell
T_{reg}	regulatory T cell
T_{SCM}	memory stem cells
TNF	tumour necrosis factor
WBC	white blood count
w/o	without

9 Appendix

9.1 Acknowledgements

Acknowledgements were removed for reasons of data protection.

9.2 Curriculum Vitae

The Curriculum Vitae was removed for reasons of data protection.