HLA CLASS II MISMATCH ALLELES AS TARGETS OF THE ALLOREACTIVE CD4 T-CELL RESPONSE

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Andrea Blötz

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1 Abstract

In allogeneic hematopoietic stem cell transplantation (allo-HSCT), alloreactive T lymphocytes of donor origin mediate the beneficial graft-*versus*-leukemia effect but also induce graft-*versus*-host disease (GvHD). Since human leukocyte antigens (HLA) mismatch alleles represent major targets of alloreactive T lymphocytes, patient and donor are usually matched for the class I molecules A, B, C, and for the class II molecules DRB1 and DQB1, in order do reduce the risk of GvHD. The HLA-DPB1 locus, however, is still ignored in donor selection. Interestingly, clinical studies have demonstrated that disparities at HLA-DQB1 alleles as well as distinct HLA-DPB1 mismatch constellations do not adversely affect the outcome of allo-HSCT. It has also been shown that HLA class II is predominantly expressed on hematopoietic cells under non-inflammatory conditions. Furthermore, the antileukemic activity of CD4 T cells after CD8-depletion of the allograft appeared to be preserved in clinical studies. Therefore, this PhD thesis focused on the application of CD4 T cells in adoptive immunotherapy of leukemias.

In the first part of this thesis we developed a rapid screening approach to detect T-cell reactivity of donors to single HLA class II mismatch alleles. Allo-HLA reactivity was measured in naive, memory, and entire CD4 T cells isolated from PBMC of healthy donors by flow cytometric cell sorting according to expression of the differentiation markers CD45RA, CD45RO, CD62L, and CCR7. T-cell populations were defined by a single marker to facilitate translation into a clinical-grade allo-depletion procedure. Alloreactivity to single HLA-DR/-DQ mismatch alleles was analyzed in short-term mixed lymphocyte reactions (MLR) *in vitro*. As standard antigen-presenting cells, we used the HLA-deficient cell line K562 upon electroporation with single HLA-DR/-DQ allele mRNA. We observed in IFN-γ ELISpot assays that allo-HLA-reactivity preferentially derived from subsets enriched for naive compared to memory T cells in healthy donors, irrespective of the HLA mismatch allele. This separation was most efficient if CD62L (P=0.008) or CD45RA (P=0.011) were used as marker to sort naive and memory subsets. Median numbers of allo-HLA-reactive effector cells were 3.5-fold and 16.6-fold lower in $CD62L^{neg}$ and $CD45RA^{neg}$ memory CD4 T cells than in entire CD4 T cells, respectively. In allele-specific analysis, alloreactivity to single HLA-DR alleles clearly exceeded that to HLA-DQ alleles. In terms of alloproliferation no significant difference could be observed between individual CD4 T-cell subsets.

The second part of this thesis dealed with the generation of allo-HLA-DQ/-DP specific CD4 T cells. Naive $CD45RA^{pos}$ CD4 T cells isolated from healthy donor PBMC by flow cytometric cell sorting were stimulated in MLR against single allo-HLA-DQ/-DP alleles transfected into autologous mature monocyte-derived dendritic cells by mRNA electroporation. Rapidly expanding HLA-DQ/-DP mismatch reactive T cells significantly recognized and cytolysed primary acute myeloid leukemia (AML) blasts, fibroblasts (FB) and keratinocytes (KC) in IFN-γ ELISpot and ^{51}chromium release assays if

the targets carried the HLA-DQ/-DP allele used for T cell priming. While AML blasts were recognized independent of pre-incubating them with IFN-γ, recognition of FB and KC required IFN-γ pre-treatment. The level of reactivity to AML blasts exceeded that to FB and KC, respectively. We further investigated HLA class II expression on hematopoietic and non-hematopoietic cells by flow cytometry. HLA class II was not detected on primary FB, KC, and non-malignant kidney cells, but was expressed at significant levels on primary AML blasts and B-LCL. Expression levels followed the hierarchy: DP>DR>DQ. Up-regulation of HLA class II expression was observed on all cell types after pre-incubation with IFN-γ.

In summary, the novel K562-HLA based MLR approach revealed that naive-depleted CD4 T-cell subsets of healthy individuals contain decreased allo-HLA reactivity *in vitro*. We propose the application of $CD45RA^{neg}$ naive-depleted CD4 T cells as memory T cell therapy, which might be beneficial for HLA-mismatched patients at high-risk of GvHD and low-risk of leukemia relapse. Memory T cells might provide important post-transplant immune functions against infectious agents but they frequently lack considerable anti-leukemia responses. Therefore, preferred allografts are those which contain leukemia-reactive memory T cells (e.g. induced by previous donor vaccination against leukemia antigens). Alternatively, replenishment with leukemia-specific T cell clones isolated from naive subsets is desirable. Additionally, the screening approach could be employed as test system to detect donors which have low risks for the emergence of GvHD after allo-HSCT. In the second part of this thesis we developed a protocol for the generation of allo-HLA-DQ/-DP specific CD4 T cell lines, which could be applied in situations in which patient and donor are matched in all HLA alleles but one HLA-DQ/-DP allele with low GvHD potential. These T cells showed lytic activity to leukemia cells while presumably sparing non-hematopoietic tissues under non-inflammatory conditions. Therefore, they might be advantageous for allo-HSCT patients with advanced stage AML after reduced-intensity conditioning and T-cell depletion for the replenishment of anti-leukemic reactivity if the risk for disease relapse is high. Altogether, in this thesis we have developed two methodical protocols using alloreactive CD4 T cells, which might be of potential clinical use in the adoptive immunotherapy of leukemias.

2 Introduction

2.1 Immunological Background

The human immune system comprises very sophisticated and comprehensive defense mechanisms against infections, diseases and malignant disorders. It is able to recognize, repel and eradicate foreign pathogens like bacteria, viruses, fungi, parasites or foreign molecules and malignant cells, while it omits own healthy cells and tissues. The importance of the immune system falls into place if it is malfunctioning and as a consequence severe infections, tumors, allergies, or autoimmune diseases arise.

The immune system can be described as a network of lymphoid organs, tissues, cells, humoral factors, and cytokines. Immunity is divided into the adaptive and the innate system, which work together to propagate optimal protection of the host. The innate immune system is the first obstacle for pathogens including anatomical barriers, cellular components, and secretory molecules. First, physical, chemical, and microbiological barriers comprising epithelial surfaces and mucosal membranes shelter the organism from pathogen invasion. Once the pathogens penetrate into the tissue phagocytic cells like macrophages, monocytes, and neutrophils as well as natural killer (NK) cells are recruited and activated. Additionally, inflammatory mediators are released by macrophages as well as basophils, mast cells and, eosinophils. As molecular components the complement system, acute-phase proteins and cytokines play an important role. The innate immune response is very fast but non-specific. If it is not sufficient to free the organism from the pathogens the adaptive immune system takes over. This is antigen-specific and recognizes pathogens by T and B lymphocytes in two stages. First, the antigen is presented to and recognized by T or B cells leading to cell priming, activation, and differentiation, which occurs within the lymphoid tissues. Second, the effector response takes place, either due to activated T cells leaving the lymphoid tissue and homing to the site of disease causing cell-mediated immunity, or due to antibody release from activated plasma cells into blood and tissue fluids mediating humoral immunity. The development of specific immune responses takes several days or weeks, but it exhibits immunological memory leading to more vigorous and rapid responses after subsequent exposure to the pathogen (Alam and Gorska 2003, Delves and Roitt 2000a, Parkin and Cohen 2001).

2.1.1 The Role of T Lymphocytes in Adaptive Immunity

Cell-mediated immunity against viruses and bacteria is one part of the adaptive immune system accomplished by T lymphocytes. Moreover, T cells play a crucial role in anti-tumor and anti-leukemia immune responses but also charge for the rejection of allo-transplants or destructive autoimmune

diseases. How T cells are selected in the thymus, how they interact with antigen-presenting cells (APC) in order to be activated after specific peptide recognition, and how they mature to effector and memory cells and perform their effector functions is described in this chapter in detail. Further subjects of this paragraph are the molecules, which are essential in antigen presentation and their importance in allogeneic transplantations.

2.1.1.1 T-Lymphocyte Selection in the Thymus

During development of T lymphocytes from hematopoietic stem cells they are educated in the thymus to recognize "self" and "nonself" antigens through the expression of antigen-specific receptors – the T-cell receptors (TCR). Their generation is a complex recombination process that cuts, splices and modifies variable-region genes coding for the antigen-binding sites of the receptors. In this way an impressive repertoire of about 10^{15} different TCR is developed, which is adequate for the number of pathogens that encounter the host in his lifetime. Linear peptides, which are processed and presented in the antigen-binding groove of major histocompatibility complex (MHC) molecules, are recognized by TCR.

Since the TCR generation occurs randomly both TCR that are reactive against self-MHC molecules as well as TCR that do not recognize self-MHC are generated. Since T cells require signaling through the TCR for survival and proliferation only T cells bearing a TCR that recognizes self-MHC molecules in conjunction with self-peptides are positively selected. T cells, which are unable to recognize self-MHC or have a very low avidity TCR for self-MHC die because of the lack of the TCR signal. Among the positively selected T cells, those with strong avidity for self-peptides (autoreactive T cells) are killed in a negative selection step. Surviving and proliferating T cells have a moderate avidity to self-peptides. They are self-tolerant and recognize foreign antigen when presented with self-MHC.

During thymic selection of the TCR the cells simultaneously enter their fate as CD4 or CD8 T cells. Immature T cells are double-positive meaning that they express both CD4 and CD8 coreceptors. If the cells differentiate into CD4 or CD8 T cells is determined by the interaction of the TCR on their surface with distinct MHC molecules. CD4 T cells are selected through interaction with MHC class II molecules, whereas CD8 T cells are selected based upon their interaction with MHC class I molecules. CD4 and CD8 T cells remain restricted by the MHC molecule for their whole lifetime (Alam and Gorska 2003, Parkin and Cohen 2001).

2.1.1.2 Antigen Presentation by MHC Molecules

Antigens are presented to the TCR of T lymphocytes via MHC molecules on professional antigen-presenting cells like dendritic cells (DC), macrophages, or B lymphocytes. DC play a key role in

mediating immunity and tolerance since they are the messengers between the innate and adaptive immune response. Immature DC are located at body surfaces like the skin or mucosa where they encounter, recognize and internalize antigens by phagocytosis. In response to antigen incorporation the DC leave the peripheral tissues and migrate to lymphoid organs, where they differentiate into mature DC (mDC). mDC lose their capacity to internalize antigens. They synthesize cytokines like interleukine (IL) -12, which attract and activate further immune cells like phagocytes and T lymphocytes. The antigens are processed into peptides and presented to the T cells on MHC molecules via different processing pathways. Endogenous peptides derived from viruses, bacteria, tumors or degenerated/defective cells are processed and presented by the MHC class I pathway to CD8 T cells. Whereas antigens derived from intravesicular or exogenous pathogens run through the MHC class II pathway and are presented to CD4 T cells. Additionally, mDC simultaneously upregulate the expression of costimulatory molecules like CD45 or molecules of the B7 family i.e. CD80. Taken together, the described features turn mDC to the most prominent stimulators for T lymphocytes (Banchereau and Steinman 1998, Steinman and Banchereau 2007).

Human leukocyte antigens. The human MHC called the human leukocyte antigen (HLA) system is known to be the most polymorphic genetic system in humans. It is located on the short arm of chromosome six, spans about 3.600 kilobases of DNA thereby including over 200 genes, and is divided into three regions. The class I region contains the classical HLA-A, HLA-B, and HLA-C genes, which encode the heavy high polymorphic alpha chains of HLA class I molecules. Assembling of the varying HLA class I alpha chains with beta$_2$-microglobulin encoded on chromosome 15 leads to the expression of complete HLA class I proteins on the cell surface. The class II region consists of A and B genes coding for the less polymorphic alpha and high polymorphic beta chains of the HLA class II molecules HLA-DR, HLA-DQ, and HLA-DP. The combination of an alpha and a beta chain is necessary for the expression of functional HLA class II molecules. The peptide-binding groove of HLA class II molecules is composed by both the alpha and the beta chain, whereas the peptide-binding groove of HLA class I molecules is solely presented by its alpha chain. HLA class I proteins are expressed on all nucleated cells of the body at varying densities, whereas the expression of HLA class II genes is restricted largely to cells of the hematopoietic system although their expression can also be induced under inflammatory conditions e.g. by interferon gamma (IFN-γ) on various cell types like fibroblasts, mesenchymal cells, epithelial cells, and endothelial cells (Bland 1988, Geppert and Lipsky 1985, Romieu-Mourez*, et al* 2007). The MHC class III region encodes for proteins of the complement system and cytokines (e.g. tumor necrosis factor alpha (TNF-α)), which are important for innate immune reactions. Additionally, amongst others genes encoding for proteins that are involved in peptide loading onto the HLA molecules (i.a. HLA-DO or HLA-DM) or in transport mechanisms (i.a.transporter associated with antigen processing (TAP)) are encoded by the HLA gene locus (Choo

2007, Klein and Sato 2000). The WHO Nomenclatur Committee for Factors of the HLA System manages HLA gene and allele designation, which is consistently revised and updated (Marsh*, et al* 2010). Today, 8016 HLA alleles are known encoding more than 4550 class I and more than 1200 class II proteins (HLA-A 1448, HLA-B 1988, HLA-C 1119, HLA-DRA1 2, HLA-DRB1 901, HLA-DQA1 29, HLA-DQB1 126, HLA-DPA1 17, HLA-DPB1 134) (IMGT/HLA Database, 11-07-2012).

Since HLA genes are closely linked the whole MHC locus is inherited as an HLA haplotype from each parent. Therefore siblings have a chance of 25% of being genotypically identical in all HLA alleles. However, a special coincidence can be found in the heredity of the HLA-DP genes. HLA-DP is encoded centromeric to the loci of the other HLA genes. Since there is a recombination hotspot between those loci the genes encoding the HLA-DP alleles are in weak linkage disequilibrium with the genes encoding for all other HLA alleles (Begovich*, et al* 1992). For that reason about 5% of siblings and 80% of HLA-A/B/C/DR/DQ matched donors are mismatched for HLA-DPB1 (Buchler*, et al* 2002, Varney*, et al* 1999). Nevertheless, in some populations certain combinations of HLA alleles are in linkage disequilibrium and can be found more frequently than expected by chance as it is the case for HLA-A1, HLA-B8 and HLA-DR17 in caucasians (Choo 2007).

Malfunctions in the HLA system can lead to various diseases like autoimmune disorders, cancer, or rheumatoid arthritis. In addition, HLA class I and II molecules play a major role in allogeneic hematopoietic stem cell transplantation (allo-HSCT) concerning graft rejection, disease relapse and overall survival as it is described in the next paragraph. Therefore high-resolution HLA typing of patients and donors are routinely performed prior transplantation (Choo 2007, Klein and Sato 2000).

Alloreactivity. The recognition of peptide-allogeneic-MHC complexes that were not encountered during thymic development by T cells is termed alloreactivity. This phenomenon occurs clinically after allo-HSCT and results in graft rejection and graft-*versus*-host disease (GvHD) (chapter 2.3.1). The frequency of alloreactive T cells is 100-fold to 1,000-fold higher than the precursor frequency of T cells, which are specific for any single foreign-peptide-self-MHC complex. However, the evolutionary reason for alloreactivity is still not fully understood.

A lot of research is ongoing to investigate the molecular basis of alloreactivity. During thymic selection TCR are selected due to their interaction with self MHC molecules, whereas there is no process that selects for or against the ability of the TCR to interact with foreign MHC molecules. Therefore, it has been suggested that alloreactivity is a consequence of an inherent affinity of the TCR for the MHC surface, which is conserved among MHC alleles. While conventional recognition involves specific interactions with both the peptide and the self MHC molecule, it was first stated by several models that alloreactivity can be explained either by interactions between the TCR and only the allogeneic MHC molecule or by interactions with the bound peptide alone. Today, it is known that both the allogeneic MHC molecule and the bound peptide contribute to the peptide-MHC

surface exposed to the TCR, and allorecognition involves interactions with both. Thereby the alloreactive TCR can either be highly peptide specific or to a lesser extent MHC specific.

Allorecognition can either be direct or indirect. If donor-derived alloantigens are presented by allogeneic MHC molecules the mechanism is called direct while the presentation of donor-derived allogeneic peptides by self-MHC molecules is called indirect allorecognition. It occurs, if cellular and membrane fragments and dead or dying cells from the donor graft are phagocytosed, processed into peptides, and presented on self-MHC molecules by host APC. The recognition of indirectly presented peptides is a form of conventional recognition and contributes to allograft rejection (Felix and Allen 2007, Nagy 2012).

2.1.1.3 T-Lymphocyte Activation

After selection in the thymus naive T cells migrate through lymphoid organs (lymph nodes, tonsils, spleen, mucosas) in order to meet their specific antigen. The antigens are presented on activated APC via MHC molecules (chapter 2.1.1.2). Activation requires two signals. The first signal is mediated by specific binding of the TCR associated with its coreceptors CD3 and CD4 or CD8, respectively, to the peptide-MHC complex on the APC surface. The second signal is an unspecific signal transmitted by costimulatory molecules on the APC like CD45 or molecules of the B7 family i.e. CD80 to stimulatory receptors on the T cells. The activation of both signals leads to an induction of cytokine production like IL-2. Consequently, T cell proliferation and clonal expansion of antigen-specific T cells is induced, generating both effector and memory T cells. Recent studies have described that in contrast to memory T cells an optimal activation of naive T cells additionally requires a third signal provided by inflammatory cytokines. In the case of CD4 T cells IL-1 is supposed to support T cell responses while CD8 T cell activation is triggered by IL-12, IFN-α and IFN-β (Curtsinger and Mescher 2010). The development of the primary immune response takes 2-3 days. After a subsequent encounter with the same antigen, memory cells are able to provoke rapid secondary immune responses. In contrast, T-cell anergy and apoptosis are initiated if the antigeneic signal is too weak or costimulatory signals are missing. Additionally, T-cell activation can be inhibited by binding of the costimulatory molecules to inhibitory receptors (Delves and Roitt 2000b).

2.1.1.3 T-Lymphocyte Maturation

The adaptive immune system developed a unique system to confront and remember pathogens. After antigen encounter naive T cells mature into effector cells provoking a primary immune response, and memory cells, which rapidly protect the host after reinfection in a secondary immune response.

T-cell maturation begins with homing of naive T cells to secondary lymphoid organs, where they meet their specific antigens presented by APC. After activation, the naive T cells proliferate by clonal expansion generating effector cells and memory cells. Effector T cells (T_{EFF}) migrate to inflamed tissues where they secrete cytokines and kill target cells in a primary immune response. After the pathogen is cleared effector cells senescent into terminally effectors, which die by apoptosis. Memory cells can be devided in two subsets according to their homing capacity and effector function. Effector memory cells (T_{EM}) are no longer able to proliferate. They migrate to peripheral tissues, where they contribute to the primary immune response. Central memory cells (T_{CM}) take part in secondary immune responses and long term protection. They are still able to home into lymphoid tissues, where they proliferate and become effector cells upon secondary stimulation. It was long believed that all antigen experienced T cells become effector cells and therefore that all memory T cells are descended from effector T cells following a linear pathway. Only the strongest effector cells were thought to persist as T_{CM} (less differentiated) and T_{EM} (most differentiated) (Lanzavecchia and Sallusto 2005). Today additional models for T-cell differentiation are discussed. One model describes that the differentiation of T cells into distinct T-cell subsets might be specified directly when a naive T cell is activated. Another model postulates that naive T cells differentiate into T_{CM}, which give rise to T_{EM} that migrate to sites of infection and differentiate further into effector T cells. Additionally, the question if T_{EM} may revert to T_{CM} is still discussed (Ahmed*, et al* 2009, Klebanoff*, et al* 2006, von Andrian and Mackay 2000). Long-term antigenic stimulation of T cells due to chronic viral infection or malignancies with continuous strong antigen burden drives effector as well as memory cells into exhaustion. The T cells lose all effector functions (cytokine production, proliferation, cell lysis) and upregulate inhibitory molecules leading to cell death after interaction with APC (Lanzavecchia and Sallusto 2005, Williams and Bevan 2007).

During maturation T cells accomplish different functional properties, which require the expression of distinct cell surface molecules such as homing or cytokine receptors. These molecules serve as phenotypical markers for T-cell subset identification. Examples are given in Figure 1: Naive T cells and

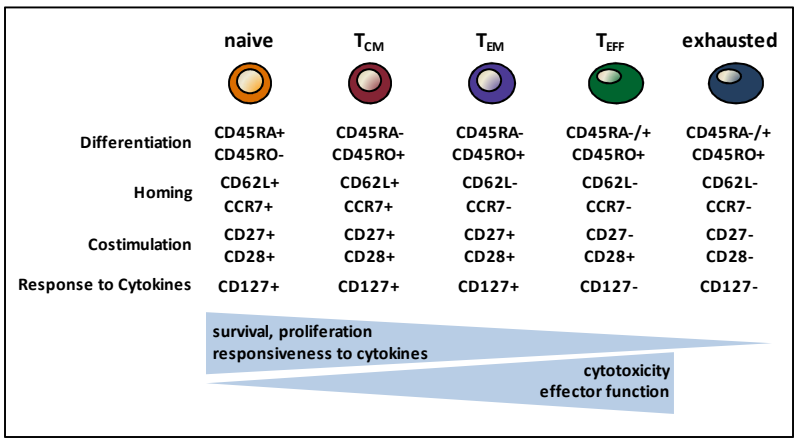


Figure 1. Phenotypic and functional properties of distinct CD4 T cell subsets. Shown is the expression of differentiation, homing and, co-stimulatory markers as well as cytokine receptors on naive and antigen experienced (T_{CM}, T_{EM}, T_{EFF}, exhausted T cells) CD4 T cells. Additionally given are functional differences between the respective T cell populations. Adapted and modified for CD4 T cells from Appay *et al*, 2008 & Klebanoff *et al*, 2006. T_{CM}, central memory T cell; T_{EM} effector memory T cell; T_{EFF}, effector T cell.

T_{CM} carry adhesion molecules like CD62L and chemokine receptors like CCR7 and CXCR4, which mediate T cell homing into lymphoid organs (CD62L, CCR7) and the bone marrow (CXCR4) (Appay*, et al* 2008, Sallusto*, et al* 1999). They additionally express CD45RA, which can also be found on terminally effector cells, a transmembrane protein tyrosine phosphatase taking part in T cell differentiation, while its isoform CD45RO can be found on effector and memory T cells (Henson*, et al* 2012, Michie*, et al* 1992). Specific for activated and memory T cells is the expression of the IL-2 receptor CD25, the IL-7 receptor CD127, and the IL-15 receptor showing their responsiveness to cytokines that are essential for T-cell proliferation and homeostatic survival also in the absence of antigen. Furthermore, receptors for costimulatory molecules like CD27 and CD28 are downregulated by T_{EFF} and T_{EM} (Appay*, et al* 2008, Lanzavecchia and Sallusto 2005, Sallusto*, et al* 2004, Williams and Bevan 2007).

2.1.1.5 Effector Mechanisms of T-Lymphocytes

CD4 and CD8 T cells are the two major types of effector lymphocytes, which can be categorized due to their interaction with MHC class I or II molecules, respectively. These two T-cell populations can be further devided into distinct lymphocyte subsets each fulfilling specific effector functions:

CD4 T Lymphocytes

Depending on the type of infection and the signals transmitted from APC, the responding T cells can make a range of responses. CD4 cells play an important role in the production of early effector cytokines giving them the name T helper cells (T_H). They enhance B as well as T cell responses and are able to directly kill infected cells. Nevertheless, CD4 T cell can also promote inhibitory effects. Today four CD4 T cell subsets are known including T_H1, T_H2, T_H17, and regulatory T cells (T_{reg}), which develop from naive T cells after antigen-specific activation (MacLeod*, et al* 2009).

T_H1. After activation T_H1 cells secrete predominantly inflammatory effector cytokines such as IFN-γ, TNF-α and IL-2. IL-2 induces CD8 T cells to proliferate and promotes their cytolytic function that is of special importance for memory CD8 T cells (Khanolkar*, et al* 2007). IFN-γ leads to the activation of macrophages facilitating the clearance of intracellular pathogens and induces NK-cell cytotoxicity. Additionally, T_H1 cells stimulate the production of antibodies of different immunoglobulin (Ig) G subclasses against extracellular pathogens by producing costimulatory signals to B lymphocytes (Parkin and Cohen 2001).

T_H2. T_H2 cells produce a number of cytokines like IL-4, IL-5, IL-6, and IL-10, which stimulate B lymphocytes to produce IgE or IgM immunoglobulins instead of IgG. In this way T_H2 cells contribute to the generation of humoral immune responses and the elimination of extracellular pathogens, but they take also part in the induction and persistence of asthma and other allergic reactions (Parkin and Cohen 2001, Zhu and Paul 2008).

T_H17. In addition to the classical T_H1 and T_H2 subsets T_H17 cells have recently been identified. They produce the effector cytokines IL-17, IL-21, and IL-22, which mediate host defensive mechanisms to extracellular bacterial and fungi infections by induction of inflammatory cytokine production and neutrophil activation. Furthermore, T_H17 cells are involved in the pathogenesis of many autoimmune diseases. Since the receptors for IL-17 and IL-22 are broadly expressed on various epithelial tissues T_H17 cells play an important role in the crosstalk between the immune system and tissues. IL-21 is important for T_H17 cell differentiation and also enhances proliferation of CD8 T cells, B cells, NK cells, and DC. The underlying mechanisms for T_H17 cell regulation and function are still not fully understood (Dong 2008, Ouyang*, et al* 2008, Zhu and Paul 2008).

T_{reg}. A crucial role in maintaining peripheral tolerance as well as in regulating immune responses is attributed to T_{reg}. For that reason, T_{reg} may be beneficial in preventing autoimmune diseases, limiting chronic inflammatory diseases, and preventing allograft rejection. Nevertheless, they also limit beneficial responses by suppressing complete removal of pathogens and limiting antitumor immunity implicating the interest in therapies with T_{reg} depletion and/or inhibiton strategies. The mechanisms of their suppressive functions are the expression of inhibitory cytokines like IL-10, IL-35 or transforming growth factor beta (TGF-β), effector cell cytolysis, inhibition of DC to stimulate T cells, and disruption of metabolites necessary for effector cell survival. T_{reg} can either be generated in the thymus (natural T_{reg}) or during immune responses (inducible T_{reg}) (Vignali*, et al* 2008, Zhu and Paul 2008).

Cytolytic CD4 T cells. The paradigm that cytolytic activity is a property only accomplished by CD8 T cells and not by CD4 helper T cells was overcome after the discovery of CD4 T cells with the ability to eradicate infected cells by direct killing. Cytotoxicity can either be mediated by granule-mediated cytolysis or by Fas - Fas ligand interactions. Cytotoxic T cells inducing cell lysis via the granule-mediated pathway contain cytolytic granule, which release perforin and granzymes (serine proteases) in a calcium-dependent manner. Perforin participates in the permeabilization of the plasma membrane allowing granzymes and other cytotoxic components to enter the target cells. Finally, granzymes cleave and activate intracellular caspases, resulting in DNA fragmentation and therefore in target-cell apoptosis. Caspases are also activated and lead to target cell apoptosis if cytolytic T cells express Fas ligand (CD178), which binds to the Fas receptor (CD95) on a variety of target cells (Barry and Bleackley 2002, MacLeod*, et al* 2009).

CD8 T Lymphocytes

CD8 T cells are directly cytotoxic to target cells presenting their specific antigen and are therefore called cytotoxic T lymphocytes (CTL). They kill target cells by distinct pathways. The granule-mediated and the Fas - Fas ligand pathway both involve direct cell-cell contact and are already described for CD4 cells. A third pathway is mediated by cytokines, such as IFN-γ and TNF-α, which are produced

and secreted as long as TCR stimulation continues. TNF-α triggers the caspase cascade on target cells, leading to target-cell apoptosis. IFN-γ, enhances the antigen presentation by MHC class I molecules and the expression of Fas on target cells, indirectly leading to target-cell lysis. The cytokine production can also activate macrophages and induce inflammation.
It is further postulated that specific CD8 T cell subsets have suppressor functions in downregulating lymphocyte immune responses (Andersen*, et al* 2006, Jiang and Chess 2006).

2.2 Leukemia – Malignancy of the Hematopoietic System

Leukemias, along with multiple myeloma and lymphoma, are hematological malignancies of the blood-forming organs. In 2008, leukemia was the tenth most common type of cancer in women and the ninth most common in men. The worldwide incidence of leukemia in 2008 was estimated to about 350,000 cases, with an incidence rate of 5.0/100,000 individuals/year. These numbers account for 2.8% of all cancer patients. Furthermore, leukemia caused the death of 3.4% of all cancer patients in 2008. However, leukemia is still the leading cause of death due to cancer in children and younger adults. (Kasteng*, et al* 2007)(http://GLOBOCAN.iarc.fr, 31-07-2012).
During leukemia the hematopoiesis of the patient is malfunctioning. Hematopoietic stem cells or their descendants transform into abnormal cells, which are immature, proliferate uncontrolled, and fail to fulfill their proper functions. The malignant cells spread out in the bone marrow (BM) but accumulate in the blood and within organs of the body. Leukemic cells can be categorized by the hematopoietic lineage, from which the cells derive, in myeloid and lymphoid leukemias. Each of these types includes both acute and chronic forms. Acute leukemias show a rapid onset. In acute myeloid leukemias (AML), the abnormal cells grow rapidly, and tend to die fast. In comparison, in acute lymphoid leukemias (ALL) cell growth is not as rapid, rather, the cells tend to accumulate. In both cases the cells lose their normal functions leading to death within weeks or months. In contrast, the onset of chronic leukemias tends to be slow. They may persist for months or years as it is the case for chronic lymphoid leukemias (CLL). In chronic myeloid leukemia (CML) an invariable conversion into the acute form occurs, leading to blast crisis and death (Kasteng*, et al* 2007)(www.cancer.org).
People of all ages are affected by leukemia. ALL accounts with 85% for the majority of leukemia cases in children. However, most leukemia patients are elderly. CML occurs in adults of all ages, while CLL primarily affects adults over 50 years of age. AML develops in early childhood and in later adulthood. It is the most common myeloid leukemia form in adults and shows the lowest survival rate of all leukemias. In 2002-2008, the overall 5-year relative survival rate of leukemia in the United States was

55%. Thereof, the four major types of leukemia were nearly 79% and 65% for CLL and ALL, respectively, 59% for CML, and only 23% for AML. While AML prognosis for younger patients has improved during the last years, the outcome in older patients is still very poor (Deschler and Lubbert 2006)(www.SEER.cancer/gov/statfacts, 31-07-2012).

2.2.1 Acute Myeloid Leukemia

The pathogenesis of AML begins during myelopoiesis, which is a highly controlled system of cell devision and differentiation. Myeloid blood cells constantly regenerate from hematopoietic stem cells. This process is driven by early and lineage-specific growth factors and their receptors. The differentiation of cells is regulated by early acting and lineage-specific transcription factors that control the expression of lineage-specific genes. The transformation of myeloid progenitor cells into leukemic blasts results from alterations in myeloid transcription factors, which regulate hematopoietic differentiation, and from activating mutations of regulators involved in signal transduction pathways. Thereby, normal myeloid differentiation is altered and immature cell clones with uncontrolled cell growth arise (Steffen*, et al* 2005).

AML is classified into subtypes using two systems: first, the French-American-British (FAB) classification and second, the newer World Health Organization (WHO) classification. In the FAB classification AML are divided into eight subtypes (M0 through M7) based on the lineage from which the leukemia developed and maturity of the cells. In contrast, the WHO proposed a system utilizing not only morphologic findings but also including genetic, immunophenotypic, biologic, and clinical features to define specific disease entities. The resulting classification divides AML into five broad groups: AML with certain genetic abnormalities, AML with multilineage dysplasia, AML related to previous chemotherapy or radiation, AML not otherwise specified and undifferentiated or biphenotypic acute leukemias. AML is diagnosed if more than 20% (WHO) / 30% (FAB) blasts are accumulated in the BM of the patient (Bennett*, et al* 1976, Estey and Dohner 2006, Vardiman*, et al* 2002)(www.cancer.org).

Development of AML is associated with distinct risk factors. These include previous hematological diseases, genetic disorders, exposure to ionizing radiation, chemicals or viruses, as well as chemotherapy (Deschler and Lubbert 2006). After AML has been diagnosed, prognostic factors can give evidence for the likelihood that a patient will show a response to treatment. Abnormalities in cytogenetics and molecular genetic features in AML blasts prior treatment can be found in 55% of adult AML patients. Cytogenetic abnormalities allow the subdivision of AML into three broad prognostic groups: favorable, intermediate, and adverse. Patients classified in the favorable group show specific mutations, which are associated with complete remission and low risk of relapse. The adverse prognostic group with poor outcome is defined by the presence of leukemic blasts with

complex karyotypes involving more than two chromosomes, monosomy or deletions. Patients with secondary AML belong to this group. In contrast, leukemic blasts of patients counting to the intermediate prognostic group have either a normal karyotype or cytogenetic abnormalities that are not included in the definition of the other subgroups. The presence of secondary chromosome abnormalities, gene mutations, and deregulated gene expression is also helpful for categorization and prognosis. One example of genetic abnormalities associated with poor outcome are internal tandem duplications and/or mutations in the gene of the growth factor receptor tyrosine kinase FLT3, which is important during lymphocyte development. Mutations in the gene encoding for the multifunctional nuclear acidic chaperone nucleophosmin (NPM1 mutations) are described to be found in patients with better overall survival (Estey and Dohner 2006, Estey 2012, Lowenberg*, et al* 1999).

Tumor Escape Machanisms. Today, the concept of tumor immune surveillance, which states the capability of the immune system to recognize and eliminate tumor cells, is well accepted. Since AML cells express MHC class I and II molecules and ligands for activating NK-cell receptors they can serve as targets for the adaptive and innate immune system. However, diverse abnormalities in AML lead to development of leukemia despite immune surveillance. Additionally, leukemia may acquire new characteristics to defeat immune control.

First, effector cells might be unable to reach leukemia blasts, which persist in immunological privileged sites, such as the central nervous system or the skin. Additionally, development of leukemic blasts might be favored by genetic characteristics. AML cells are found to strongly express inhibitory costimulatory molecules like cytotoxic lymphocyte antigen-4 (CTLA-4), which reduces T-cell proliferation. Furthermore, NK-cell activity can be blocked for example by upregulation of inhibitory killer cell immunoglobulin like receptors (KIR). It is also described that AML blasts mutate in order to circumvent immune responses. These mutations include the downregulation of costimulatory molecules, adhesion molecules or even of entire HLA molecules. Alterations in antigenic peptide sequences by deficient processing and presentation might also contribute to the prevention of AML clearance. Leukemic cells may also directly downregulate effector cells through secretion of inhibitory cytokines like IL-10 (Barrett and Le Blanc 2010, Kolb*, et al* 2004).

Therapeutic Strategies. Treatment of AML with chemotherapy facilitates remission but unfortunately the probability for disease relapse is very high. The development of strategies in order to prevent leukemia relapse like consolidation chemotherapy, stem cell transplantation and immunotherapy is studied intensely. The therapy of AML with autologous or allogeneic hematopoietic stem cell transplantation as well as the adoptive transfer of allogeneic or autologous T cells or NK cells is in detail discussed in chapter 2.3. Further immunotherapeutic approaches are vaccination, treatment with antibodies, cytokines, and immunomodulatory agents.

2.3 Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for several malignant and non-malignant diseases. It was originally developed for the treatment of sequelae due to radiation exposure (Lorenz*, et al* 1952) and was further ascertained for the cure of hematological malignancies. The initial focus was the application of intensive chemotherapy often combined with total body irradiation to eradicate tumor cells but this therapy strategy was limited by irreversible marrow aplasia and immune suppression of the patient. Transplantation of pluripotent hematopoietic stem cells (HSC) allowed the delivery of those myeloablative chemoradiotherapies by rescuing the hematopoietic system of the host in autologous HSCT (auto-HSCT) or by establishing donor hematopoiesis after allogeneic HSCT (allo-HSCT) (Boyiadzis and Pavletic 2004, Welniak*, et al* 2007). In auto-HSCT the stem cells of the patient are removed, stored and reinfused after conditioning treatment. Autologous transplants have the advantage of low risk for infections and graft rejection, but the disadvantage of higher relapse rates compared with allo-HSCT. In allo-HSCT the transferred stem cells are derived from a twin (syngeneic), sibling, relative, or unrelated donor (Boyiadzis and Pavletic 2004).

The immunological non-identity between donor and recipient after the application of allo-HSCT results in reciprocal immunological reactions of the graft against its new host and the host against the graft. After transplantation, the host may provoke an immunological attack against the graft, leading to graft rejection, which is mainly mediated by host T cells and NK cells that recognize allogeneic MHC molecules or minor histocompatibility antigens (mHAg) and mismatched KIR receptors, respectively, on transplanted cells of the donor. Besides the reactivity of recipient cells against the graft, immunocompetent cells in the graft can react against antigens of host tissues like skin, liver or gastrointestinal tract, which can result in life-threatening graft-*versus*-host disease (GvHD). GvHD results from T cells or NK cells transplanted with the graft or arising from it recognizing incompatible MHC molecules, mHAg or mismatched KIR receptors on host cells, respectively. In order to circumvent graft rejection and GvHD patients are treated with immunosuppressive agents given early after transplantation. Despite this prophylaxis, GvHD develops in 40-50% of HLA-matched recipients and in 40-85% of HLA-mismatched patients of allo-HSCT. The third effect after allo-HSCT is the graft-*versus*-tumor/leukemia (GvT/GvL) effect, which is closely intertwined with the development of GvHD. The GvL effect was discovered in murine models when eradication of leukemia occurred in mice receiving allogeneic transplants, but not in mice treated with syngeneic transplants. The existence of GvL effects in humans were confirmed in several studies demonstrating that relapse rates following allo-HSCT were less in patients with GvHD. The role of T cells in this process arose after the discovery of increased leukemia relapse when T cell depleted transplants were applied to prevent GvHD (Appelbaum 2001, Kolb*, et al* 2004).

With recognition of the strength of the GvL effect, clinical research began to focus on allo-HSCT more as an immunotherapeutic approach. Instead of using myeloablative conditioning regimens, which might lead to strong organ toxicity and the vulnerability of the patients to infections, the treatment has shifted to eradicating leukemia with alloimmune effector cells under non-myeloablative conditioning regimes. This platform of adoptive immune therapy for allo-HSCT is still under intense research (Kolb*, et al* 2004).

2.3.1 Separation of GvHD and GvL Reactions

One of the major challenges in allo-HSCT is the segregation of the deleterious GvHD and the beneficial GvL effects (Figure 2). This chapter outlines the underlying mechanisms of these immune reactions and highlights successful therapeutic approaches. Strategies for separation and therefore the improvement of allo-HSCT are discussed in detail giving examples of *in vitro* studies and of ongoing clinical trials.

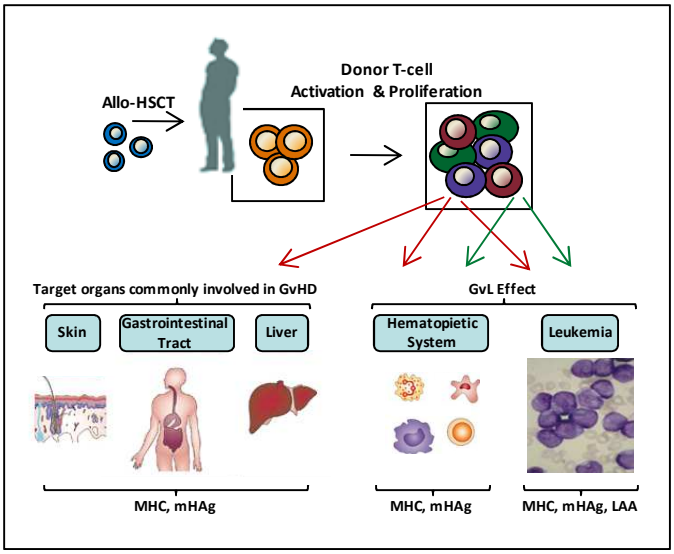


Figure 2. Separation of GvHD from GvL effects. After allo-HSCT donor T-cell activation and proliferation occur. Afterwards donor effector cells may attack both host tissues and leukemia cells. GvHD arises if donor T cells are specific to MHC molecules or mHAg broadly expressed on host tissues (red arrows) while GvL effects result from the recognition of MHC molecules, mHAg or LAA on leukemic as well as hematopoietic cells (green arrows). The separation of both effects might be possible if the targeted antigens are selectively expressed by leukemia cells. Adapted and modified from Jenq and van den Brink 2010 & Bleakley and Riddell 2004. allo-HSCT, allogeneic hematopoietic stem cell transplantation; GvHD, graft-*versus*-host disease; GvL, graft-*versus*-leukemia; MHC, major histocompatibility antigen; mHAg, minor histocompatibility antigen; LAA, leukemia-associated antigen.

2.3.1.1 The Pathophysiology of GvHD

GvHD represents intensified inflammatory responses against alloantigens. Donor T cells function adequately in the foreign and morbid environment of the host they encounter. The development of GvHD can be conceptualized in three sequential phases. In phase 1, the underlying disease, prior infections and the conditioning regimen with irradiation and chemotherapy damage recipient tissues. The harmed tissue (immune cells, endothelial and epithelial cells) responds with the secretion of proinflammatory cytokines like TNF-α, IL-1 and IL-6. This so-called cytokine storm increases the expression of adhesion molecules, costimulatory molecules, HLA-antigens and chemokine gradients on host tissues, which lead to the activation of host APC. In the second phase, donor T cells recognize

allogeneic HLA molecules and mHAg on primed host APC that leads to donor T cell activation. Once T cells are activated, an intracellular biochemical cascade ensues resulting in increased production of T_H1 cytokines (e.g. IFN-γ, IL-2) enhancing donor T and NK-cell activation, proliferation, differentiation and migration. In phase 3, the effector phase, cellular mediators (i.a. T and NK cells, monocytes, macrophages) and inflammatory mediators (cytokines like TNF-α, IFN-γ, IL-1 or LPS) synergize to amplify local tissue injury and further promote inflammation and target tissue destruction (Antin and Ferrara 1992, Levine 2011, Sun*, et al* 2007).

2.3.1.2 GvHD and GvL Target Antigens and their Use in Adoptive Immunotherapy

Major Histocompatibility Antigens. The development of GvHD and GvL is mainly based on the recognition of mismatched human leucocyte antigens (HLA) by alloreactive donor T cells. HLA genes, molecules and heredity as well as the phenomenon of alloreactivity are in detail described in chapter 2.1.1.2.

For allo-HSCT, the most influential genes are the HLA class I genes (HLA-A, HLA-B and HLA-C) and the HLA class II genes (HLA-DRB1, -DQB1 and -DPB1). These genes are highly polymorphic whereby finding a perfect match of all class I and II alleles between patient and donor in allo-HSCT is very difficult (Appelbaum 2001, Sun*, et al* 2007). Since there is a direct association between the number of HLA mismatches and the risk for mortality after allo-HSCT most transplantation have been between HLA-matched individuals. Before 1980, this exclusively meant HLA-matched siblings, but only one in three patients have such donors available (Appelbaum 2001). Nowadays, HLA-typing of unrelated donors for the five classical HLA loci, HLA-A, -B, -C, -DRB1 and -DQB1 and the coordinated work of international bone marrow donor registries allows the identification of HLA-matched unrelated donors for about 70% of patients lacking matched siblings (www.dkms.de, 31-07-2012). Retrospective studies report an improvement in survival and decrease in GvHD in patients matched for 10 of 10 alleles at 5 HLA loci compared to those patients with mismatches (Madrigal and Shaw 2008). Several groups showed that high-resolution matching for HLA-A/-B/-C and -DRB1 maximizes posttransplant survival (Morishima*, et al* 2002, Petersdorf*, et al* 2004). Of importance is the observation that mismatches at HLA-DQB1 do not show the same overall negative impact as those at the four other loci (Flomenberg*, et al* 2004). Mismatches at the sixth polymorphic HLA locus HLA-DPB1, which is to date not taken into account for the donor selection, do not seem to play a role in overall mortality. Lee *et al* discovered in a retrospective study that HLA-DPB1 mismatching was associated with an increased risk for GvHD. Nevertheless, there was no impact on overall survival, and low evidence for an increased risk for treatment-related mortality (Lee*, et al* 2007). In addition, Shaw *et al* showed a similar association between HLA-DPB1 mismatches and higher risk for GvHD. This negative impact may be outweight by a decreased risk for disease relapse (Bray*, et al* 2008,

Shaw, *et al* 2007). Although matching for HLA-DPB1 lowers overall risks after allo-HSCT, HLA-DPB1 matched donors are difficult to find due to HLA-DPB1 heredity. As described in chapter 2.1.1.2 already 5% of sibling donor/patient pairs and more than 80% of unrelated donor/patient pairs are mismatched for HLA-DPB1 (Buchler, *et al* 2002, Varney, *et al* 1999). Therefore, the identification of HLA-DPB1 mismatches that do not increase the risks (permissive mismatches) and avoid the use of donors with HLA-DPB1 mismatches that are associated with increased risk after transplantation (non-permissive mismatches) would be beneficial. It has already been described that HLA-DPB1 mismatches can mediate alloreactive T-cell responoses (Fleischhauer, *et al* 2001, Rutten, *et al* 2010b). Based on crossreactivity patterns of HLA-DPB1 alloreactive T-cell clones Fleischhauer *et al* developed a model for the identification of HLA-DPB1 mismatches as defined by the presence of T-cell epitope (TCE) mismatching. Therefore HLA-DPB1 alleles are classified initially into three TCE groups supposed to have high, intermediate, and low immunogeneic potential. In further studies the three TCE group model was expanded into a four-group model including two intermediate immunogenicity groups. If HLA-DPB1 alleles are mismatched and belong to the same TCE group they are permissive, if they belong to different TCE groups, they are defined as non-permissive. Permissive HLA-DPB1 mismatches might be tolerated, while non-permissive alleles would increase the risk after allo-HSCT (Crocchiolo, *et al* 2009, Fleischhauer, *et al* 2012, Zino, *et al* 2004). The validity of this algorithm is still under intense discussion (Rutten, *et al* 2010a, Sizzano, *et al* 2011, Touzeau, *et al* 2012, Vie, *et al* 2011).

An insight into the usefulness of HLA-DP specific T-cells in the adoptive immunotherapy of leukemia was gained in a study by Rutten *et al* about a patient with B-cell leukemia after unmodified donor lymphocyte infusion (DLI) administered late after T-cell depleted allo-HSCT. CD4 T-cells specific for HLA-DPB1 emerged in the patient, which were capable of recognizing and lysing HLA-DP expressing myeloid and lymphoid hematological malignant cells, while only minimal GvHD was observed (Rutten, *et al* 2008). The group further found that HLA-DP specific CD4 T cells frequently occur following HLA-DP mismatched T-cell depleted allo-HSCT and DLI and induce GvL reactivity in the absence or presence of GvHD (Rutten, *et al* 2012). These data were confirmed in a murine model, in which alloreactive CD4 T cells efficiently eradicated leukemic cells. HLA-DR, HLA-DQ as well as HLA-DP-specific T cells contributed to this GvL effect by acting as effector T cells and exerting T-helper functions by cytokine production (Stevanovic, *et al* 2011).

Minor Histocompatibility Antigens. Minor histocompatibility antigens (mHAg) do also play a significant role in GvHD and GvL development. These antigens are immunogenic peptides presented in conjunction with HLA class I or II molecules. They are derived from endogenous proteins encoded by polymorphic genes and are inherited separately from HLA. Disparities in mHAg nucleotide polymorphisms between donor and recipient induce allogeneic T-cell responses, which lead to GvHD

as well as GvL effects. For that reason about 40% of HLA matched allo-HSCT patients develop GvHD (Rezvani and Storb 2008, Sun*, et al* 2007).

Leukemia-Associated Antigens. Additionally to mHAg non-polymorphic proteins that are overexpressed or aberrantly expressed in tumor cells as a consequence of molecular dysregulation may serve as potential targets for GvL effects. In rare situations tumors may express unique antigens (leukemia-specific antigens) capable of generating T-cell responses. More frequently, malignant cells express leukemia-associated antigens (LAA), which are found on normal cells but are overexpressed in malignant tissues (Bleakley and Riddell 2004, Kantarjian*, et al* 2007, Rezvani and Storb 2008).

2.3.1.3 From T-Cell Depletion to T-Cell Therapy

T-cell depletion (TCD) of the donor stem-cell-graft offers the most effective strategy for prevention of GvHD. In clinical studies the incidence and severity of GvHD after TCD is diminished. However, TCD is associated with an increased rate of severe and often fatal infections due to delayed immunreconstitution, a higher incidence of graft rejection based on missing eradication of residual host immune cells by donor T cells, and an increased risk of leukemia recurrence because of the missing GvL effect. To lessen the negative impact of TCD, selective TCD, cytokine stimulations, and a combination with (delayed) donor lymphocyte infusions (DLI) are studied (Appelbaum 2001, Baron and Beguin 2002, Ho and Soiffer 2001).

DLI was first described by Kolb and colleagues in 1990 (Kolb*, et al* 1990) for the treatment of relapsed disease after allo-HSCT. Already in 1995 a retrospective survey of the European Group for Blood and Marrow Transplantation demonstrated that complete remission of relapsed disease was induced by DLI in 80% of patients with CML and 26% of patients with AML (Kolb*, et al* 2004). Unfortunately, disease regression due to the promoted GvL responses after DLI is often attended by the development of GvHD. Therefore, it is advantageous to use delayed DLI in combination with TCD grafts. The attraction of this approach is that it allows combining the benefits of both therapies; the decreased GvHD early after TCD allo-HSCT and the restoration of the GvL effect at a later time by DLI (Ho and Soiffer 2001, Slavin*, et al* 1998).

Further investigations concentrate on the selective depletion of distinct T lymphocyte populations involved in the development of GvL and GvHD effects. Animal models showed that both donor CD4 and CD8 T cells play a significant role in GvHD, but donor CD4 T cells in the absence of CD8 T cells can still mediate GvL activity. Until now several groups have demonstrated in clinical trials that depletion of CD8 T cells from either the allotransplant or from DLI efficiently reduces the incidence and severity of GvHD. However, disease remissions after CD8-depleted DLI in patients with myeloma or CML relapse are remarkable. Several clinical studies confirmed that CD8-depletion of DLI or BM maintains

considerable antileukemic activity while reducing GvHD (Alyea*, et al* 1998, Champlin*, et al* 1990, Ho and Soiffer 2001, Meyer*, et al* 2007, Meyer*, et al* 2010).

These studies are very encouraging to use CD4 T cells in adoptive immunotherapies. Nevertheless, the pathophysiologic differences between GvHD and GvL can not only be reduced to the various features of CD4 and CD8 T cells. Therefore, current research is ongoing to further break down alloreactivity to distinct T-cell subsets with naive or memory phenotype. The differences of T cells at distinct maturation stages may account to their respective ability to induce GvHD.

Naive as well as T_{CM} cells express homing markers like CCR7 and CD62L on their cell surface. These markers mediate T-cell homing into lymphoid organs, where the lymphocytes are encountered to allogeneic antigens leading to T cell activation. Primed T cells down-regulate the expression of homing markers and migrate from the lymph nodes into the periphery and sites of inflammation. While T_{CM} still express both CD62L and CCR7, T_{EM} already lack CCR7 expression and carry only low amounts CD62L on their cell surface, which is additionally down-regulated on effector T cells. Furthermore, memory T-cell populations have an increased specificity towards antigens and consequently a diminished TCR repertoire due to previous T-cell priming. In contrast naive T cells have a bride diversity of the TCR repertoire and should contain most alloreactive precursors. This may result in an increased or reduced ability of naive or memory T cells, respectively, to encounter and recognize allogeneic antigens. However, memory T cells show a lower activation threshold and mediate rapid effector responses when recalled by antigens (Chandok and Farber 2004, Foster*, et al* 2004, Nikolich-Zugich*, et al* 2004, Sallusto*, et al* 1999).

Anderson *et al* found in a murine model with HLA-identical and mHAg-incompatible donor/recipient pairs that CD4 memory T cells ($CD62L^{neg}CD44^{pos}$) did not induce GvHD, whereas naive T cells ($CD62L^{pos}CD44^{neg}$) produced lethal GvHD after the adoptive transfer into recipient animals. Additionally, they demonstrated that this effect was not due to T_{reg} ($CD4^{pos}CD25^{pos}$), which are known to promote self-tolerance and suppress GvHD (Anderson*, et al* 2003). Moreover, Chen *et al* demonstrated that $CD62L^{pos}$-depleted T cells failed to proliferate against allogeneic antigens and were unable to induce GvHD. In both studies, memory T cells were still able to recognize antigens to which the mice had previously been primed (Chen*, et al* 2004). These findings were confirmed by Beilhack *et al* who characterized trafficking and proliferation of naive ($CD62L^{pos}CD44^{neg}$) and memory ($CD62L^{neg}CD44^{pos}$) CD4 T-cell subsets *in vivo*. Only T cells with the naive phenotype proliferated in secondary lymphoid organs with subsequent homing to the intestines, liver, and skin and caused GvHD, while memory T cells did not proliferate and induced GvHD (Beilhack*, et al* 2005). In a human *in vitro* study by Foster *et al* T-cell populations were isolated by flow cytometric cell sorting according to the expression or absence of the maturation marker CD62L. Consequently, the T-cell subsets were expanded for up to three weeks by OKT3/IL-2 stimulation prior to MLR. As stimulator cells in MLR a

mixture of several HLA-mismatched B-LCL was applied. In proliferation and cytotoxicity assays stronger alloreactivity was found in $CD62L^{pos}$ compared to $CD62L^{neg}$ T-cell subsets (Foster*, et al* 2004). In contrast, several studies demonstrated that virus-specific T cells of memory phenotype can cross-react against mismatched HLA molecules – a phenomenon called heterologous immunity. These T cells can be reactivated by infectious agents after allo-HSCT. The rapid expansion and cytokine production of the virus-specific T cells may lead to the induction of severe GvHD and graft rejection and might explain the association between reactivation of viral infections during organ transplantation and increased graft rejection (Amir*, et al* 2010, Burrows*, et al* 1994, Cainelli and Vento 2002, Umetsu*, et al* 1985). Additionally, recent studies using a murine GvHD model identified a new T-cell subset of memory stem cells. These T cells persist in recipients with GvHD, are able to self-renew, proliferate and differentiate into effector and memory T cells, which cause GvHD. Hence, memory stem cells play an important role in the maintenance of GvHD responses over time (Zhang*, et al* 2005a, Zhang*, et al* 2005b).

Another approach for the selective depletion of alloreactive donor T-cells is based on the *ex vivo* elimination of alloreactive T cells by an immunotoxin that reacts with the cell surface antigen CD25 on activated T cells. The benefit of this immune reconstitution procedure with less GvHD has already been confirmed in clinical trials (Amrolia*, et al* 2006, Andre-Schmutz*, et al* 2002, Solomon*, et al* 2005). In a similar study T-cell anergy is successfully induced by blocking the interaction of costimulatory molecules on donor HSC *ex vivo* preventing key signaling events for lymphocyte activation (Guinan*, et al* 1999). Furthermore, studies are ongoing dealing with the genetic manipulation of donor lymphocytes by introduction of an inducible suicide gene, which allows the exploitation of the antitumor activity of donor T cells after allo-HSCT without GvHD (Berger*, et al* 2006, Bonini*, et al* 1997). Activated T cells could also be suppressed by the adoptive transfer of T_{reg} cells. T_{reg} cells exert a suppressive effect on the T-cell immune response, controlling autoreactivity and maintaining self-tolerance. The critical role of these cells in the outcome of allo-HSCT has been demonstrated in animal models. The infusion of T_{reg} cells exerts a potent suppressive effect on immune effector cells reactive to host antigens and prevent GvHD and graft rejection while maintaining GvL responses (Edinger*, et al* 2003). Additionally, first clinical trials confirmed the prevention of GvHD after adoptive transfer of T_{reg} cells. They present a basis for the knowledge on how to use T_{reg} cells to control and modulate immune responses in patients after allo-HSCT (Blazar*, et al* 2012, Brunstein*, et al* 2011, Zorn 2006).

3 Motivation & Aim of this Study

Today, the only curative treatment for patients with high-risk acute myeloid leukemia (AML) is allogeneic hematopoietic stem cell transplantation (allo-HSCT). Thereby, alloimmune effector lymphocytes of the stem cell graft recognize antigens on leukemia blasts of the patient mediating the beneficial graft-*versus*-leukemia (GvL) effect. However, donor lymphocytes also respond to allogeneic antigens on non-hematopoietic recipient tissues leading to the unwanted graft-*versus*-host disease (GvHD) – the major complication after allo-HSCT. The separation of these reciprocal effects is challenging and under intense research.

One of the first and most effective approaches to reduce GvHD is the elimination of alloreactive donor T cells by T-cell depletion of the graft. As a consequence patients show a high vulnerability to infections based on reduced immunreconstitution, graft rejection and furthermore an increased risk of leukemia relapse due to the additionally diminished GvL effects. Leukemia reactivity can be restored by (delayed) donor lymphocyte infusions (DLI), which for their part might again induce GvHD (Ho and Soiffer 2001, Kolb*, et al* 2004). Therefore, a more selective depletion of distinct T lymphocyte populations might be advantageous in allo-HSCT. It has already been demonstrated that CD8-depletion of the stem cell graft or DLI products provokes less severe GvHD while the remaining CD4 T cells sustain antileukemic activity (Alyea*, et al* 1998, Meyer*, et al* 2007). One advantage of CD4 T cells in allo-HSCT is that HLA class II molecules are predominantly expressed on hematopoietic cells under non-inflammatory conditions meaning that CD4 T cells mainly target hematopoietic and thereby leukemic cells while non-hematopoietic recipient tissues might be spared. Altogether, the application of CD4 T lymphocytes in adoptive immunotherapies is very promising. Nevertheless, remaining alloreactivity of CD4 T cells can be further broken down due to their differentiation stage into distinct T-cell subsets with naive or memory phenotype. Naive T cells should contain most alloreactive precursors because of their enormous T-cell receptor repertoire leading to an increased ability to recognize alloantigens compared to memory T cells (Chandok and Farber 2004, Nikolich-Zugich*, et al* 2004). These findings were promoted by data obtained in mouse models and *in vitro* studies, in which more severe GvHD was induced by naive T cells (Anderson*, et al* 2003, Beilhack*, et al* 2005, Foster*, et al* 2004). Hence, the elimination of alloreactive naive CD4 T-cell populations from the allograft could be valuable for retaining GvL effects while avoiding GvHD. Until now, respective ongoing studies are technical very complex and require intensive *in vitro* culture of donor T cells (Amrolia*, et al* 2006, Berger*, et al* 2006, Guinan*, et al* 1999).

For that reasons, the first part of this PhD thesis comprises the development of a novel screening protocol for the identification of CD4 T-cell reactivity of healthy donors to single alloantigens. Thereby, the alloreactivity of naive, memory and total CD4 T-cell populations isolated by flow cytometry from healthy donor PBMC was investigated. Furthermore, it was analyzed which T-cell

differentiation marker is most suitable for a clinical separation procedure to minimize alloreactive precusers in the allograft. Since HLA molecules are the major targets of alloreactive T cells the HLA class II molecules HLA-DRB1 and HLA-DQB1, which are currently considered for donor selection in allo-HSCT were chosen as alloantigens. The HLA-deficient cell line K562 transfected with the respective HLA allele was utilized as standard antigen-presenting cells in order to detect pure alloreactive T-cell responses of individual donors against the HLA allele of interest. Alloreactivity was analyzed in short-term mixed lymphocyte reaction (MLR) cultures followed by IFN-γ ELISpot assays and determination of T-cell expansion.

There is a direct association between the number of HLA mismatches and the risk for mortality after allo-HSCT. Therefore, patient and donor are usually matched for the HLA class I molecules (HLA-A, -B, -C) and the class II molecules HLA-DRB1 and HLA-DQB1, while the third HLA class II molecule HLA-DPB1 is still not taken into account for the donor selection. Interestingly, clinical studies demonstrated that disparities at HLA-DQB1 and permissive HLA-DPB1 alleles are better tolerated and do not influence overall mortality after allo-HSCT compared to mismatch constellations at the other four HLA loci (Bray*, et al* 2008, Flomenberg*, et al* 2004, Shaw*, et al* 2007). However, non-permissive HLA-DPB1 mismatches are described to increase the risk of GvHD while permissive HLA-DPB1 alleles may even decrease the risk for a disease relapse (Fleischhauer*, et al* 2012). Furthermore, HLA-DP specific CD4 T cells have been found to mediate GvL effects *in vitro* and *in vivo* (Ibisch*, et al* 1999, Rutten*, et al* 2012, Stevanovic*, et al* 2011). Additionally, HLA-DPB1 matched donors are rare based on the heredity of this locus. This leads to mismatches at the HLA-DP locus in 5% of sibling and more than 80% of unrelated donor/patient pairs matched for all other five HLA loci (Buchler*, et al* 2002, Varney*, et al* 1999). In clinical settings, in which patient and donor are matched in all HLA alleles except for one single HLA-DQB1 or permissive HLA-DPB1 allele, the application of CD4 T cells with specificity to this mismatch allele might be very advantageous. Under non-inflammatory conditions they might mediate anti-leukemic reactivity without the induction of severe GvHD.

Therefore in the second part of this thesis we developed a fast applicable protocol for the generation of allo-HLA-DQ/-DP specific CD4 T cells. It was based on MLR cultures performed with strong alloreactive naive T-cell subsets of healthy donor origin stimulated against autologous mature monocyte-derived dendritic cells transfected with the HLA-DQ/-DP mismatch allele of the patient. Resulting HLA-DQ/-DP specific CD4 T cells were analyzed for specificity, HLA-restriction and effector function against a broad panel of hematopoietic and non-hematopoietic cells. Additionally, the HLA class II expression pattern on these cell types was analyzed by flow cytometry under inflammatory (i.e. after pre-incubation with cytokines) and non-inflammatory conditions in order to determine if the HLA-DQ/-DP specific T cells would be able to recognize leukemic cells, while sparing non-hematopoietic recipient tissues.

4 Materials & Methods

4.1 Materials

4.1.1 Instruments & Equipment

Autoclave (VX-150)	Systec, Wettenberg, D
Beaker (250ml, 500ml, 1000ml)	Schott, Mainz, D
^{137}Caesium source (Gammacell-GC2000)	Molsgaard Medical, Gansloe, DK
Cell counting chamber (Fuchs-Rosenthal)	Marienfeld, Lauda-Königshofen, D
Cell culture tube-racks	VWR, Darmstadt, D
Centrifuges:	
Megafuge 1.0R	Hereaus, Hanau, D
Centrifuge 5415 R	Eppendorf, Hamburg, D
Sorvall RC 6 Plus	Thermo Scientific, Waltham, USA
Centrifuge tubes (COREX® No. 8445 + Adapter)	Gencompare, Brussels, B
CO_2-Incubators (9040-0007 & Heracell 240)	Binder, Tuttlingen, D; Heraeus, Hanau, D
Cover slip	Menzel, Braunschweig, D
Cryo freezing Containers (Nalgene)	Nunc, Wiesbaden, D
Cryo tube-racks	Greiner, Frickenhausen, D
Dispenser (Ceramus classic 0.4-2ml)	Hirschmann Laborgeräte, Herrenberg, D
Duran glass Flasks	Schott, Mainz, D
Electroporation Cuvettes 4 mm	peqlab, Erlangen, D
Electrophoresis Chamber & Equipment	NeoLab, Heidelberg, D
Electrophoresis Power Supply	NeoLab, Heidelberg, D
FACS tube-racks (Nalgene)	Nunc, Wiesbaden, D
Flow Cytometer (BD FACS Canto II, BD FACS Aria)	Becton Dickinson Biosciences, Heidelberg, D
Forceps	Carl Roth, Karlsruhe, D
Freezer (Herafreeze -80°C)	Heraeus, Hanau, D
Gene Pulser Xcell™ Electroporation System	Bio-Rad Hercules, CA, USA
GeneQuant II RNA DNA Calculator	Pharmacia Biotech, Cambridge, UK
Heater & Mixer (MR 3001)	Heidolph Instruments, Schwabach, D
Heating cabinet (BE 500)	Memmert, Schwabach, D
Incubator for Microbiology Research:	
Heraeus B15	Heraeus, Hanau, D
Infors HT Aerotron®	Infors AG, Bottmingen, CH
Inoculating loop	Carl Roth, Karlsruhe, D
Ice machine (UBE 50/35)	Ziegra, Isernhagen, D
MACS Multistand	Miltenyi Biotec, Bergisch Gladbach, D
Mastercycler Gradient	Eppendorf, Hamburg, D
MidiMACS Separator unit	Miltenyi Biotec, Bergisch Gladbach, D
Microscope (Axiovert 25 & Axio Imager M1)	Carl Zeiss AG, Jena, D
Nitrogen cryo bank (Espace 331 Gaz)	Air Liquide DMC, Marne-la-Vallée, F
Nitrogen reservoir tank (Taylor-Wharton XL-180)	Tec Lab, Husum, D
PH Meter (Five Easy)	Mettler Toledo, Schwarzenbach, CH
Pipettor (Pipetboy acu)	Integra Biosciences, Fernwald, D
Precision scale (EW150-3M)	Kern, Balingen-Frommern, D
Refrigerator Freezer Combo (+4°C / -20°C)	Liebherr, Ochsenhausen, D & Bosch, München, D
Scissors	Carl Roth, Karlsruhe, D

Single-channel & multi-channel pipettes:	
0.5-10 µl (Research)	Eppendorf, Hamburg, D
2-20µl, 20-200µl, 100-1000µl (Pipetman)	Gilson, Limburg, D
10-100µl, 30-300µl (ErgoOne 10 & 30)	Starlab, Ahrensburg, D
Sterile work bench (NU-440-601E & Herasafe HS18)	Nuaire, Plymouth, USA & Haereus, Hanau, D
Thermomixer 5436	Eppendorf, Hamburg, D
UV Transilluminator UST-30M-8PC, 312nm	Biostep, Jahnsdorf, D
Vortex mixer (MS2 Minishaker)	IKA, Staufen, D
Water bath heater (Typ 1013)	GFL, Burgwedel, D
Water deionization machine (Purelab Classic)	Elga LabWater, Celle, D
Wizard^2 gamma counter	Perkin Elmer, Rodgau, D

4.1.2 Consumables

Cell culture flasks:	
Cellstar 25 cm^2, 75 cm^2 with or without filter	Greiner, Frickenhausen, D
Cell culture plates:	
6-/24-/48-well flat bottom	Greiner, Frickenhausen, D
96-well V-/U-/flat bottom	Greiner, Frickenhausen, D
Cell culture tubes (15ml, 50ml)	Greiner, Frickenhausen, D
Cell Scraper (25cm)	Sarstedt, Nümbrecht, D
Cell Strainer (100µm)	Becton Dickinson Biosciences, Heidelberg, D
Cryo tubes (Cryo.S)	Greiner, Frickenhausen, D
Cytotox tubes (0.6ml)	Greiner, Frickenhausen, D
ELISPot Plate (MultiScreen HTS Filter Plates)	Millipore, Eschborn, D
FACS tubes (Falcon 5ml)	Becton Dickinson Biosciences, Heidelberg, D
Ficoll separation tubes (Leucosep™ 50ml)	Greiner, Frickenhausen, D
LS columns	Miltenyi Biotec, Bergisch Gladbach, D
Microtiter plates (96-well flat-, round-, V-bottom)	Greiner, Frickenhausen, D
Petri dishes (35/60/95/145mm)	Greiner, Frickenhausen, D
Pipette tips (TipOne 10µl, 200µl, 1000µl)	Starlab GmbH, Ahrensburg, D
Pipette Filter tips (TipOne 20µl, 100µl, 200µl, 1000µl)	Starlab GmbH, Ahrensburg, D
Preseparation filter (30µm)	Miltenyi Biotec, Bergisch Gladbach, D
Reaction tubes (0.2ml, 0.5mL, 1.5mL, 2mL)	Eppendorf, Hamburg, D
Safe-Lock Tubes (0.5ml, 1.5ml) Eppendorf Biopur®	Eppendorf, Hamburg, D
Scalpel (Disposable scalpel No.23 pfm)	Feather Safety Razor CO., Osaka, J
Serological pipettes (Cellstar 1/2/5/10/25/50mL)	Greiner, Frickenhausen, D
Sterile filter (Stericup 0.22µm, 0.45µm)	Millipore, Eschborn, D

4.1.3 Chemicals and Reagents

Cell Culture

Acetic Acid (99%)	Carl Roth, Karlsruhe, D
Bovine Serum Albumin (BSA)	Carl Roth, Karlsruhe, D
Dimethyle Sulfoxide (DMSO)	Carl Roth, Karlsruhe, D
Dispase®	Invitrogen, Karlsruhe, D
Dulbecco´s Phosphate Buffered Saline 1x (PBS)	Invitrogen, Karlsruhe, D
Ethanol (>99,8%)	Carl Roth, Karlsruhe, D

Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich, Steinheim, D
Formaldehyde (37%)	Sigma-Aldrich, Steinheim, D
Trypan Blue	Merck, Darmstadt, D
^{51}Sodium Chromate ($Na_2{}^{51}CrO_4$)	Amersham Biosciences, Freiburg, D
Triton X-100	Sigma-Aldrich, Steinheim, D
3-Amino-9-Ethylcarbazole (AEC) Tablets	Sigma-Aldrich, Steinheim, D
PBS Instamned® Powder (9.55 g/l)	Biochrom, Berlin, D
Sodium Acetate	Sigma-Aldrich, Steinheim, D
Sodium Chloride	Sigma-Aldrich, Steinheim, D
N,N-Dimethylformamide (DMF)	Sigma-Aldrich, Steinheim, D
H_2O_2 30% Solution	Sigma-Aldrich, Steinheim, D
Tween 20	AppliChem, Darmstadt, D

Molecular Biology

Agar-Agar	Carl Roth, Karlsruhe, D
Agarose (for routine use)	Sigma-Aldrich, Steinheim, D
Boric Acid	Carl Roth, Karlsruhe, D
Diethyl Pyrocarbonate	Sigma-Aldrich, Steinheim, D
2-Propanol Rotipuran®	Carl Roth, Karlsruhe, D
RNaseZap®	Sigma-Aldrich, Steinheim, D
Sodium Chloride	Carl Roth, Karlsruhe, D
StarPure Agarose Low EEO Standard > 1kb	Starlab GmbH, Ahrensburg, D
Tris Base	Carl Roth, Karlsruhe, D
Trypton Pepton	Carl Roth, Karlsruhe, D
Yeast Extract	Carl Roth, Karlsruhe, D

4.1.4 Buffers & Solutions

Cell Culture

Dispase Solution:
PBS
+ 2.2IU/ml Dispase®
(sterile filtrated, 0.22µm)

ELISpot Acetate Buffer:
615.8mL H_2O
+ 1.8g Sodium Acetate
+ 9.2mL Acetic Acid 99%

ELISpot AEC Solution:
20mg AEC (1 Tablet)
+ 2.5ml DMF
+ 47.5ml ELISpot Acetate Buffer
(sterile filtered, 0.45µm)

ELISpot Secondary Antibody Buffer:
PBS
+ 0.5% BSA
(sterile filtrated, 0.22µm)

ELISpot Washing Buffer:
PBS (ELISpot PBS)
+ 0.05% Tween 20

ELISpot PBS:
95.5g PBS Instamned® Powder
ad 10.0L H_2O

FACS Buffer:
PBS
+ 0.1% BSA
(sterile filtrated)

FACS Fixation Solution:
PBS
+ 1% Formaldehyd

Fibroblast Wash Buffer:
PBS
+ 2% Fluconazol
+ 1% P/S
(sterile filtrated, 0.22µm)

MACS buffer:
PBS
+ 0.5% BSA
+ 2mM EDTA
(sterile filtrated, 0.22µm)

Trypan Blue Staining Solution:
750ml Trypan Blue Stock Solution (A)
+ 250ml Trypan Blue Stock Solution (B)

Trypan Blue Stock Solutions:
(A) H_2O + 2‰ Trypan Blue
(B) H_2O + 4.5% Sodium Chloride

SORT Buffer:
PBS
+ 1% HS
+ 2mM EDTA
(sterile filtrated, 0.22µm)

Molecular Biology

Nuclease-Free Water:
Quiagen, Hilden, D

MOPS-EDTA-Sodium Acetate Buffer:
Sigma-Aldrich, Steinheim, D

TAE-Buffer (50x):
121g Tris Base
+ 22ml Acetic Acid (99%)
+ 50ml 0.5M EDTA (pH 8.3)
ad 500ml Aqua Destillata

TBE-Buffer (5x):
54g Tris Base
+ 27.5g Boric Acid
+ 20ml 0.5M EDTA (pH 8.0)
ad 1000ml Aqua Destillata

4.1.5 Additives

Cell Culture

BD Cytofix/Cytoperm™	BD Biosciences, Heidelberg, D
Fetal Calf Serum (FCS)	PAA Laboratories, Pasching, A
Fluconazol 2mg/ml solution for infusion	HIKMA Pharma GmbH, Klein Winternheim, D
Heparin-Natrium-25000	Ratiopharm, Ulm, D
Human Albumin (Alburex 20)	CSL Behring, Marburg, D
Human Serum (HS): (heat-inactivated, sterile filtrated)	Isolated from healthy donor blood
Phytohaemagglutinin (PHA)	Murex Biotech, Kent, UK
Penicillin/Streptomycin (P/S)	Invitrogen, Karlsruhe, D
Trypsin-EDTA (0.05%)	Invitrogen, Karlsruhe, D
AL-Lysis Buffer	Institut für Immunologie und Genetik Kaiserslautern, D

Molecular Biology

Ampicillin	Sigma-Aldrich, Steinheim, D
Kanamycin	Sigma-Aldrich, Steinheim, D
Deoxynucleotide Solution Mix	NEB GmbH, Frankfurt am Main, D
1kb DNA Ladder	NEB GmbH, Frankfurt am Main, D
100bp DNA Ladder	NEB GmbH, Frankfurt am Main, D
Gel Loading Dye Blue (6x)	NEB GmbH, Frankfurt am Main, D
GelRed Nucleic Acid Stain	Biotium, Hayward, USA
ssRNA Ladder (500-9000bp)	NEB GmbH, Frankfurt am Main, D

4.1.6 Media

Cell Culture

AIM-V® Medium:
Invitrogen, Karlsruhe, D

DMEM
(+4,5g/L Glucose +1% L-Glutamine + Phenol Red)
Invitrogen, Karlsruhe, D

Opti-MEM® I Medium:
Invitrogen, Karlsruhe, D

RPMI Medium 1640
(+ 4,5g/L Glucose + 1% L-Glutamine + Phenol Red)
Invitrogen, Karlsruhe, D

Freezing Medium:
AIM-V® Medium
+ 8% Human Albumin
+ 10 IU/mL Heparin-Natrium
+ 10% DMSO (added prior to use)

Immortalized Cell Line Culture Medium:
RPMI Medium 1640
+ 10% FCS (+ 20% FCS for NKC)
+ 1% P/S

T-Cell Culture Medium:
AIM-V® Medium
+ 10% HS (+ 5% HS for PHA blasts)

Defined Keratinocyte Serum Free Medium (K-SFM):
Invitrogen, Karlsruhe, D

Lymphocyte Separation Medium (LSM 1077):
PAA Laboratories, Pasching, A

RPMI Medium 1640 (without Phenol Red):
(+ 4,5g/L Glucose + 1% L-Glutamine)
Invitrogen, Karlsruhe, D

DC Culture Medium:
RPMI Medium 1640
+ 2% HS
+ 1% P/S

Fibroblast Culture Medium:
DMEM
+ 10% FCS
+ 2 % Fluconazol
+ 1% P/S

Keratinocyte Culture Medium:
Defined K-SFM
+ 2% Fluconalzol
+ 1% P/S

Molecular Biology

LB-Agar:
15g Agar-Agar
ad 1L LB-Medium

LB-Medium
10g Trypton Pepton
5g Yeast Extract
0.5g Sodium Chloride
ad 1L Aqua Destillata

4.1.7 Cytokines

hrIFN-γ	R&D Systems, Wiesbaden, D
hrIL-1β	Miltenyi Biotec, Bergisch Gladbach, D
hrIL-2 (Proleukin™)	Novartis, Nürnberg, D
hrIL-4	Miltenyi Biotec, Bergisch Gladbach, D
hrIL-6	Miltenyi Biotec, Bergisch Gladbach, D
hrIL-7	R&D Systems, Wiesbaden, D
hrIL-12	R&D Systems, Wiesbaden, D
hrIL-15	R&D Systems, Wiesbaden, D
GM-CSF (Leukine™)	Bayer, Leverkusen, D
PGE_2 (Prostaglandin E_2)	Sigma-Aldrich, Steinheim, D
TNF-α (Tumor necrosis factor alpha)	PromoCell, Heidelberg, D

4.1.8 Enzymes

Alkaline Phosphatase Calf Intestinal (CIP) (10000U/ml) + 10x NEBuffer 3	NEB GmbH, Frankfurt am Main, D
EciI (2000U/ml) + 10x NEBuffer 4	NEB GmbH, Frankfurt am Main, D
SpeI (2000U/ml) + 10x NEBuffer 4 + 100x BSA	NEB GmbH, Frankfurt am Main, D
T4 DNA Ligase (20000U/ml) + 10x Reaction Buffer	NEB GmbH, Frankfurt am Main, D
XbaI (20000U/ml) + 10x NEBuffer 4 + 100x BSA	NEB GmbH, Frankfurt am Main, D
XhoI (20000U/ml) + 10x NEBuffer 4 + 100x BSA	NEB GmbH, Frankfurt am Main, D
Platinum® Pfx DNA Polymerase	Invitrogen, Karlsruhe, D

4.1.9 Antibodies & Fluorescent Dyes

Flow Cytometry

For immunophenotyping of cell populations the following mouse anti-human (if not otherwise stated) monoclonal antibodies (mAb) and fluorescent dyes were used.

Antibody Specificity	Fluorochrome Conjugation	Provider
7-amino-actinomycin D (7AAD)	-	Sigma-Aldrich, Steinheim, D
IgG1	APC, FITC, PE, PerCp	Beckman Coulter, Krefeld, D
	Alexa Fluor 647, Horizon V450, PE-Cy7	BD Biosciences, Heidelberg, D
IgG2a	FITC, PE	BD Biosciences, Heidelberg, D
IgG3	PE	Abcam, Cambridge, UK
CD3	APC, FITC, PE	Beckman Coulter, Krefeld, D
	Horizon V450	BD Biosciences, Heidelberg, D
CD4	APC, FITC, PE	Beckman Coulter, Krefeld, D
	PerCp	BD Biosciences, Heidelberg, D
CD8	APC, FITC, PE	Beckman Coulter, Krefeld, D
	PerCP	BD Biosciences, Heidelberg, D
CD11c	APC	BD Biosciences, Heidelberg, D
CD14	PE	BD Biosciences, Heidelberg, D
CD16	FITC, PE	Beckman Coulter, Krefeld, D
CD19	FITC, PE	Beckman Coulter, Krefeld, D
CD25	FITC, PE-Cy7	Beckman Coulter, Krefeld, D
CD27	FITC	Beckman Coulter, Krefeld, D
CD28	PE	Beckman Coulter, Krefeld, D
CD33	PE	Beckman Coulter, Krefeld, D
CD45	APC	Beckman Coulter, Krefeld, D
CD45RA	APC	Miltenyi, Bergisch Gladbach, D
CD45RO	PE	BD Biosciences, Heidelberg, D
CD56	FITC, PE	Beckman Coulter, Krefeld, D
CD62L	PE	Beckman Coulter, Krefeld, D
CD90	PE	Beckman Coulter, Krefeld, D
CD127	PE	Beckman Coulter, Krefeld, D
CD197 (CCR7)	APC, FITC	R&D Systems, Wiesbaden, D
CXCR4	PE	BD Biosciences, Heidelberg, D
D7-Fib	-	BIOZOL, Eching, D
FOXP3	FITC	eBioscience, Frankfurt, D
Goat-anti-Mouse (GAM) IgG1	FITC, PE	Beckman Coulter, Krefeld, D
Granzyme A	PE	BD Biosciences, Heidelberg, D

Granzyme B	Alexa Fluor 647	BD Biosciences, Heidelberg, D
HLA-A/B/C	APC	BD Biosciences, Heidelberg, D
HLA-DP	PE	Abcam, Cambridge, UK
HLA-DQ	FITC	BD Biosciences, Heidelberg, D
HLA-DR	PE	Beckman Coulter, Krefeld, D
Perforin	FITC	BD Biosciences, Heidelberg, D

ELISpot

The following mouse anti-human antibodies were used in ELISPot Assays. Hybridoma supernatants of murine monoclonal antibodies were used for HLA-blocking experiments, antibodies were purified from hybridoma supernatants, aliquoted and stored until use in PBS at -20°C.

Antibody Specificity	**Conjugation**	**Provider**	**Reference**
IFN-γ 1-DIK	-	tech AB, Nacka, S	-
IFN-γ 7-B6-1	Biotin	tech AB, Nacka, S	-
HB55	-	Hybridoma	Lampson and Levy 1980
B7/21	-	Hybridoma	Watson*, et al* 1983
SPV-L3	-	Hybridoma	Spits*, et al* 1983
W6/32	-	Hybridoma	Parham*, et al* 1979

4.1.10 Manufactured Articles

Cell Culture

CD14 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, D
Vectastain ABC-Kit (reagents A + B)	Vector Laboratories, Burlingame, USA
Anti-Human FOXP3 Staining Set FITC	eBioscience, Frankfurt, D

Molecular Biology

EndoFree® Plasmid Midi / Maxi Kit	Quiagen, Hilden, D
mMASSAGE mMACHINE® T7 Ultra	Ambion, Darmstadt, D
QIAshredder™	Quiagen, Hilden, D
QIAprep® Spin Miniprep Kit	Quiagen, Hilden, D
QIAquick® PCR Purification Kit	Quiagen, Hilden, D
RNeasy Mini Kit	Quiagen, Hilden, D
Superscript III CellsDirect cDNA Synthesis System	Invitrogen, Karlsruhe, D

4.2 Methods

4.2.1 Cell Culture

This study was approved by the local Ethics Committee of the Ärztekammer Rheinland-Pfalz. Healthy donors and patients participated after informed consent according to the Declaration of Helsinki.

4.2.1.1 Primary Cell Culture

4.2.1.1.1 Isolation of PBMC by Density Gradient Centrifugation

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coat products of healthy donors by standard Ficoll density centrifugation. The isolation was performed in Leucosep™ 50ml tubes containing a porous barrier. 15ml of Ficoll Lymphocyte Separation Medium were brought underneath the frit by centrifugation before 30ml of a 1:2 dilution of blood with PBS (RT) was given onto the barrier. During centrifugation for 20min at 836g without brake PBMC were collected in the interphase above the separation medium and thereby isolated from erythrocytes, polymorpho-nuclear leukocytes, and granulocytes.

PBMC were harvested, washed twice with PBS (4°C), and counted. For high-resolution HLA class II typing $1x10^7$ cells were lysed in a 1:2 dilution of AL-Lysisbuffer with PBS. HLA typing was performed from genomic DNA by Dr. med. W. E. Hitzler, University Medical Center, Mainz, D or Dr. med. B. Thiele, Institute for Immunology and Genetics, Kaiserslautern, D.

Until use $50\text{-}100x10^6$ PBMC were frozen in 1ml Freezing Medium and kept in liquid nitrogen. In this work PBMC were utilized for the isolation of distinct CD4 T-cell populations, the generation of PHA blasts and mDC or they were transfected with HLA class II molecules and served as APC in MLR stimulations.

4.2.1.1.2 Generation of PHA-Activated T Lymphocytic Blasts

For the generation of phytohemagglutinin-activated T lymphocytic blasts (PHA blasts) $1x10^6$ healthy donor PBMC were seeded in 2ml T-Cell Culture Medium containing 1µg/ml PHA and 250IU/ml IL-2 in a 24-well plate. They were incubated at 37°C, 5% CO_2 in humidified atmosphere. Confluent grown wells were splitted with IL-2 (250IU/ml) containing T-Cell Culture Medium. Between day 10 and day 14 cells were grown to blasts. If necessary, $0.5\text{-}5x10^6$ cells were frozen in 1ml Freezing Medium and kept in liquid nitrogen until use. During this study PHA blasts were transfected with single HLA class II molecules and function as APC in MLR stimulations followed by ELISpot assays.

4.2.1.1.3 Generation of Monocyte-derived Mature Dendritic Cells

Mature dendritic cells (mDC) were generated from monocytes in 48h based on a "FAST-DC" protocol developed by Dauer and colleagues (Dauer*, et al* 2003). Monocytes were either gained from PBMC via plastic adherence or by isolation of $CD14^{pos}$ cells by MACS technology (chapter 4.2.2.3). For isolation by plastic adherence $15x10^6$ PBMC/well were incubated in 3ml DC Culture Medium in 6-well plates at 37°C, 5% CO_2 in humidified atmosphere. After 90min the plates were washed carefully with PBS (RT) for the removal of suspended lymphocytes. Adherent monocytes or $CD14^{pos}$ cells isolated by MACS separation ($5x10^6$/well) were further cultured in 3ml DC Medium containing 1000IU/ml GM-CSF and 500IU/ml IL-4. 24h later 1ml culture medium was replaced by 1ml fresh DC Medium supplemented with IL-1ß [30ng/ml], TNF-α [30ng/ml], IL-6 [3000IU/ml] and PGE_2 [3µg/ml]. mDC maturation is continued for additional 24h before the cells were harvested by washing the culture plates on ice with PBS (4°C). Purity of mDC populations without T-, B- or NK-cell contaminations was confirmed in flow cytometry by the cell surface marker phenotyp $CD11c^{pos}$, $CD19^{neg}$, $CD3^{neg}$, $CD16^{neg}$, and $CD56^{neg}$. If necessary, $0.2\text{-}0.5x10^6$ cells were frozen in 1ml Freezing Medium and kept at -196°C in liquid nitrogen until use. In further experiments mDC were transfected with HLA class II molecules and applied as APC in MLR stimulations followed by ELISpot assays.

4.2.1.1.4 Recovery and Cultivation of Primary AML Blasts

Recovery. Leukemia blasts were obtained from peripheral blood or leukapheresis products of AML patients who were initially diagnosed for leukemia. The isolation was performed by Ficoll density centrifugation as described in chapter 4.2.1.1.1 with the following modifications. First, the concentration of white blood cells was determined and adjusted to a maximum of $150x10^6$ cells/ml with PBS (RT). 35ml diluted blood were stacked carefully directly onto the separation medium in 50ml tubes without porous barrier since the resulting broad ring of white blood cells might be set in the height of the barrier. After harvesting and washing $50\text{-}200x10^6$ leukemic cells were frozen in 1ml Freezing Medium and stored at -196°C in liquid nitrogen until use.

Cultivation. AML blasts were required as target cells (APC) in IFNγ ELISpot and ^{51}chromium-release assays, as well as for phenotypical analysis in flow cytometry assays. Therefore, leukemic cells were thawed and pre-cultured for four days under three different culture conditions in a concentration of $2x10^6$ cells/ml in 6-well plates at 37°C, 5% CO_2 in humidified atmosphere. First, AML blasts were pre-incubated in T-Cell Culture Medium without additives. Leukemia cells treated like that simulated non-inflammatory conditions. Second, 500IU/ml IFN-γ were added to the culture to reconstitute an inflammatory environment (Schreiber and Farrar 1993). Third, the culture medium was supplemented with IL-1β [10ng/ml], IL-6 [1000IU/ml], and TNF-α [10ng/ml] to mediate the cytokine milieu of GvHD patients (Deeg 2001, Hill*, et al* 1999, Levine 2011, Mihara*, et al* 2012). For cross-

reactivity IFN-γ ELISpot analysis AML blasts were thawed and pre-incubated overnight in T-Cell Culture Medium without additives in a concentration of 3-5x10^6 cells/ml in petri dishes at 37°C, 5% CO_2 in humidified atmosphere.

4.2.1.1.5 Generation and Cultivation of Primary Fibroblasts

Generation. Human primary fibroblasts (FB) were generated using human foreskin from 0.5 to 10 year old children which underwent surgery due to medical indications. The foreskin was washed thoroughly in Fibroblast Wash Buffer. The tissue was cutted into small pieces with 5mm in diameter, which were digested overnight at 4°C in Dispase Solution. Upon the proteases cleaved the basement membrane zone region between epidermis and dermis, they were separated. The epidermis was used for the generation of keratinocytes (KC) as described in the next section. For growth of FB four pieces of dermis were transferred into one 25cm^2 culture flask and air-dried until they adhered to the bottom of the culture flask. The dermis was covered with 4ml Fibroblast Culture Medium and incubated at 37°C, 5% CO_2 in humidified atmosphere. Approximately after one week adherent FB were grown out of the dermis. The skin was removed from the culture flask. FB were rinsed with PBS (RT) and provided with fresh Fibroblast Culture Medium.

Cultivation. When cells were grown to confluent monolayers they were passaged. Therefore the culture medium was removed. The cells were rinsed with PBS (RT) and incubated with 0.5-1ml Trypsin-EDTA solution for 5min at 37°C, 5% CO_2 in humidified atmosphere. Detached FB were washed with DMEM before 2x10^5 cells/75cm^2 culture flask were seeded in 8ml Fibroblast Culture Medium and cultured at 37°C, 5% CO_2 in humidified atmosphere. FB were expanded and HLA typed (chapter 4.2.1.1.1). Their identity was verified in flow cytometry by the characteristic fibroblast phenotype D7-Fib^{pos}, CD90^{pos}, HLA-A/B/C^{pos} while contaminations with CD45^{pos}, CD33^{pos}, and CD14^{pos} cells were rare (Kisselbach*, et al* 2009, Min*, et al* 2002, Nonn*, et al* 2008, van Osch*, et al* 2001). FB were utilized as stimulator cells in functional assays and for the analysis of HLA expression on mesenchymal cells between passage four to twelve. For the experiments FB were pre-cultured in three different cytokine environments (chapter 4.2.1.1.4): First, 5x10^5 cells were cultivated for four days in a 25cm^2 culture flask in 4ml Fibroblast Culture Medium. Second, 500IU/ml IFN-γ were added to the culture medium. Third, the culture medium was supplemented with the cytokines IL-1β [10ng/ml], IL-6 [1000IU/ml], and TNF-α [10ng/ml].

4.2.1.1.6 Generation and Cultivation of Primary Keratinocytes

Generation. Human primary KC were generated from the same human foreskin that was used for the production of FB cultures. The epidermis was gained as described above (chapter 3.2.1.1.5). It was intensely mashed with a scalpel. The resulting suspension of small cell clusters was digested in 6ml

Trypsin-EDTA for 15min in a water bath at 37°C. Every 5min the cells were severely vortexed. The reaction was stopped by addition of 3ml FCS. Single cells were separated from cell clusters by passing the suspension through a 100µm filter. The filter was rinsed with 20ml Fibroblast Wash Buffer. Cells were centrifuged and the pellet was resuspended thoroughly in Keratinocyte Medium. KC were seeded in a density of $1x10^6$ cells/$75cm^2$ culture flask in 8ml Keratinocyte Medium and incubated at 37°C, 5% CO_2 in humidified atmosphere. For removal of dead cells that did not adhere to the culture flask the medium was changed four hours after seeding and at day one of culture. Afterwards the cells were provided with fresh media twice a week whereby KC were additionally rinsed with Fibroblast Wash Buffer. HLA typing was performed with the respective FB cultures of the same patients.

Cultivation. The potential of primary KC as stimulator cells to provoke allorecognition in IFN-γ ELISpot assays and the analysis of their HLA expression pattern by flow cytometry was investigated under non-inflammatory and inflammatory culture conditions. Therefore cells were examined after cultivation in Keratinocyte Medium alone and after pre-culture with 500IU/ml IFN-γ. Since KC tended to degenerate with proceeding *in vitro* culture passages (Nonn*, et al* 2008, Rheinwald and Green 1975) the experiments were performed with first passage KC at day 11-15 of culture, which were only available in limited amounts. IFN-γ was given into the culture medium during medium exchange 4 days previous to analysis at day 8-11 of culture. KC were detached from the culture flask using a cell scraper after 15min incubation with 1.5ml Trypsin-EDTA at 37°C, 5% CO_2 in humidified atmosphere and stopping the digest by addition of 2ml FCS. The cells were washed with Keratinocyte Medium prior to use in the experiments.

4.2.1.1.7 Generation and Cultivation of Primary Non-Malignant Kidney Cells

Generation: Human primary non-malignant kidney cells (NKC) were gained from non-malignant kidney tissues adjacent to the tumor of renal cell carcinoma patients. The processing from tissue into single-cell suspensions was performed in our research group according to standard methods as previously described by Kausche *et al* (Kausche*, et al* 2006).

Cultivation: NKC were seeded in a density of $2x10^5$ cells/$75cm^2$ culture flask in 10ml Immortalized Cell Culture Medium and incubated at 37°C, 5% CO_2 in humidified atmosphere. When cells were grown to confluent monolayers they were passaged by trypsinization followed by washing in RPMI Medium 1640 as it is described for FB in chapter 4.2.1.1.5.

In this thesis NKC served as representatives for mesenchymal cells and were screened for HLA expression by flow cytometry. For the experiments fourth to seventh passage NKC were pre-incubated under three different cytokine conditions (chapters 4.2.1.1.4 and 4.2.1.1.5): First, $5x10^5$ NKC were cultivated for four days in a $25cm^2$ culture flask in 4ml Immortalized Cell Culture Medium.

Second, 500IU/ml IFN-γ were added to the culture medium. Third, the culture medium was supplemented with the cytokines IL-1β [10ng/ml], IL-6 [1000IU/ml], and TNF-α [10ng/ml].

4.2.1.2 Cell Lines

4.2.1.2.1 K562

The human cell line K562 derived from a female patient with chronic myelogeneous leukemia in terminal blast crisis (Lozzio and Lozzio 1975). Based on the fact that the cells were described to lack HLA-class I and HLA-class II expression under non-inflammatory conditions they were established as *in vitro* targets for natural killer cells (Kaplan and Callewaert 1978, Saksela*, et al* 1979, Ziegler*, et al* 1981). Over the years K562 cells gained more and more prominence as artificial APC (Suhoski*, et al* 2007, Turtle and Riddell 2010). For example our group demonstrated that K562 cells transfected with HLA class I molecules could serve as stimulators for CD8 T cells (Britten*, et al* 2002, Wehler*, et al* 2007). Additionally, genetically engineered K562 cells in good manufacturing practice (GMP) quality were already used in clinical trials (Butler*, et al* 2007, Smith*, et al* 2010).

K562 cells were cultivated in Immortalized Cell Culture Medium in a density of $0.4x10^6$ cells/ml in a total volume of 30ml/75cm^2 cell culture flasks at 37°C, 5% CO_2 in humidified atmosphere. Sub-cultivation was conducted twice a week if cell concentration exceeded $1x10^6$ cells/ml. In this work K562 cells were applied as standard antigen-presenting cells expressing single HLA class II alleles on their surface for studies with distinct CD4 T-cells subsets.

4.2.1.2.2 B-Lymphoblastoid Cell Lines

Epstein-Barr Virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCL) were derived from PBMC of leukemia or renal cell carcinoma patients according to standard protocols (Tatsumi 1992). The immortalized suspension cell lines were cultivated in Immortalized Cell Culture Medium in a density of $0.4x10^6$ cells/ml at 37°C, 5% CO_2 in humidified atmosphere in a total volume of 30ml/75cm^2 cell culture flasks. Sub-cultivation was conducted if the cell concentration exceeded $1x10^6$ cells/ml.

In order to analyze the HLA expression pattern on lymphatic hematopoietic cells B-LCL were investigated by flow cytometry. For application in flow cytometric screening assays B-LCL were pre-cultured for four days under three different cytokine conditions (chapter 4.1.1.1.4). $2x10^6$ cells/ml were incubated in 5ml Immortalized Cell Culture Medium in 6-well plates at 37°C, 5% CO_2 in humidified atmosphere. In one experimental arm the incubation was performed without the addition of cytokines, in the second experimental arm 500IU/ml IFN-γ were added to the culture medium, and in the third arm the medium was supplemented with IL-1β [10ng/ml], IL-6 [1000IU/ml], and TNF-α [10ng/ml].

4.2.2 Immunological Methods

4.2.2.1 Mixed Lymphocyte Reaction

In a classical mixed lymphocyte reaction (MLR) T cells from one donor are mixed with irradiated lymphocytes from a second individual. If the T cells recognize the HLA molecules on the lymphocytes of the second donor they proliferate and mediate effector functions, which can be explained by alloreactivity (chapter 2.1.1.2).

During this study we investigated the alloreactive potential of distinct CD4 T-cell populations by working with a variation of the classical allogeneic MLR. As responder cells $CD3^{pos}$ $CD4^{pos}$ T cell-subsets isolated from healthy donor PBMC by flow cytometric cell sorting according to their expression or absence of the respective T cell maturation markers CD45RA, CD45RO, CD62L or CCR7 were applied (chapter 4.2.2.2). T cells were either stimulated with K562 cells expressing single mismatch HLA-DR or HLA-DQ molecules (K562-HLA) or autologous mDC, PHA blasts or PBMC carrying single allogeneic HLA-DQ or HLA-DP proteins on their cell surface (mDC-HLA, PHA blasts-HLA, PBMC-HLA) (chapter 4.2.3.3). The stimulation was performed as follows: After sorting, $1x10^6$ T cells were seeded in 2ml T-Cell Culture Medium in 24-well plates and incubated at 37°C, 5% CO_2 in humidified atmosphere. The stimulator cells were freshly thawed and lethally irradiated (K562-HLA 100Gy, mDC-HLA 70Gy, PHA blasts-HLA and PBMC-HLA 35Gy). The effector-to-target (E:T) ratio differed between the stimulator cells: K562-HLA and PBMC-HLA stimulators were added in an E:T ratio of 10:1, while mDC-HLA and PHA blasts-HLA were added in a ratio of 20:1. In the first week irradiated autologous PBMC ($1x10^6$/well) were given to the culture as feeder cells. MLR responder populations were weekly restimulated with irradiated APC. The culture medium was supplemented with 10ng/mL IL-7, 1ng/mL IL-12, and 5ng/mL IL-15 during the first week. From d7 on, IL-12 was omitted, and IL-2 was added at 100IU/ml. If necessary, confluent grown cells were splitted with T-Cell Culture Medium without cytokines. T-cell expansion was measured by counting viable cells every week using trypane blue staining. Effector functions were analyzed in IFN-γ ELISpot and ^{51}chromium-release assays. Statistical analysis between different experimental arms was performed with SPSS 15.0 software. Wilcoxon signed-rank test was used to calculate *P*-values, which were regarded as significant if $P < 0.05$.

4.2.2.2 Flow Cytometry

Flow cytometry (FACS, fluorescence-activated cell sorting) is a technique used for the analysis of cellular characteristics of individual cells within heterogeneous populations. It can be used for a variety of applications like phenotypic characterization, expression analysis, determination of cell viability and proliferation, cell counting, cell sorting, or cell cycle analysis.

By hydrodynamic focusing suspended cells are forced to pass one by one through a laser beam of different wavelengths. Meanwhile each single cell emerges light which is directed through a series of filters and mirrors, so that particular wavelength ranges are delivered to appropriate detectors. While forward scattered light is proportional to the size of the cell, light that scatters to the side reflects the granularity and structural complexity inside the cell. Additionally, if the cells were stained with fluorochrome labeled antibodies or fluorescently active substances, which bind to specific surface markers or inside the cell, the fluorochrome can be excitated by a designated wavelength followed by emission of a fluorescent signal. Analysis of the emerging fluorescence intensity allows the identification of the respective markers, their expression level and the amount of cells, which carry these markers.

Flow cytometry cell sorters are used to separate a specific cell subset from a heterogeneous population. For this purpose cells of interest were analyzed and identified as described above. Subsequently, after the cell stream passes the laser beam the stream breaks off into drops containing single cells. Thereby drops including selected cells were electrostatically charged. When the drops pass through two high-voltage deflection plates charged droplets are deflected into collection vessels whereas uncharged drops are aspirated to waste.

Immunofluorescent Labeling. Immunofluorescent stainings of cells can either be directly or indirectly. For direct immunofluorescent staining $0.1x10^6$ cells were washed with FACS Buffer. Afterwards, fluorochrome-conjugated monoclonal antibodies (mAb) with specificity to distinct cell surface antigens were given onto the cell pellet and incubated for 15min at 4°C in the dark. In advance the optimal concentration of each antibody was determined by titration. Unbound antibodies were removed by washing the cells with FACS Buffer before the cells were resuspended in 0.2ml FACS Fixation Solution. In indirect staining protocols unlabed primary bind to the particular cell surface antigens. A secondary , which is fluorescently labeled, binds specifically to the primary antibody. The stainings were performed in analogy to direct cell labeling with the following modifications: Primary and secondary were incubated for 30min at 4°C in the dark one after another. In between cells were washed twice with FACS Buffer. Unconjugated primary antibodies applied herein were either hybridoma supernatants of HLA-specific antibodies or the fibroblast marker D7-Fib. As secondary antibody fluorochrome-labeled goat-anti-mouse IgG was used. The vitality of cells was investigated using 7-amino-actinomycin D (7-AAD), a fluorescent dye, which intercalates into the DNA after passing through the cell membrane of dead and apoptotic cells (Philpott*, et al* 1996). Cell labeling with 7-AAD was performed like direct antibody stainings. Since 7-AAD stained cells cannot be fixed the cells were resuspended in 0.2ml FACS Buffer and immediately analyzed. Cell staining was analyzed on a BD FACSCanto flow cytometer (BD Biosciences, D). After gating on viable cells, 10^4 events were evaluated using BD FACSDiva™, FlowJo™ or EXPO32™

software for analysis. For analysis either percentage positive cells or the median fluorescence intensity (MFI) were quoted. In some cases the relative median fluorescence intensity (rMFI) was calculated by division of the MFI of the antigen staining by the MFI of the isotypic control staining. Statistical analysis between different experimental arms was performed with SPSS 15.0 software. Wilcoxon´s signed-rank test was used to calculate *P*-values.

Intracellular Cell Staining. If intracellular staining was applied the cells were first stained for cell surface markers as described above. After washing the cells in FACS Buffer the intracellular staining was performed: The cells were incubated for 20min in 250µl Cytofix/Cytoperm Solution at 4°C in the dark. Afterwards the cells were washed twice with Saponin Buffer before the intracellular antibody was incubated for 30min at 4°C in the dark. Unbound antibodies were removed by washing the cells with Saponin Buffer before the cells were resuspended in 0.2ml FACS Buffer and immediately analyzed. Analysis and evaluation were performed as previously described. Intracellular staining was utilized for the analysis of enzymes contained in intracellular granule (granzyme A and B, perforin). Furthermore the transcriptional regulator FOXP3 as marker on regulatory T cells was stained. For this purpose the Anti-Human FOXP3 Staining Set FITC (eBioscience, D) was used according to manufacturer´s instructions.

Flow Cytometric Cell Sorting. For flow cytometric cell sorting 60-80Mio PBMC were washed with Sort Buffer (4°C), pelletized, and stained with 50µl of fluorochrome-conjugated for CD3 and CD4, in combination with those for CD45RA, CD45RO, CCR7, or CD62L, respectively. After 15min incubation on ice in the dark PBMC were washed with Sort Buffer. Afterwards the cells were resuspended in 3ml Sort Buffer and the cell suspension was passed through a 30µm nylon mesh for separation of cell agglomerates. Additional buffer was added to adjust the cell concentration to 10-15Mio/ml. Cell sorting was performed using the BD FACSAria cell sorter. $CD3^{pos}$ $CD4^{pos}$ T-cell subsets were selected based on the expression or absence of the particular T-cell maturation marker and collected in T-Cell Culture Medium.

4.2.2.3 Magnetic Activated Cell Sorting (MACS)

Magnetic activated cell sorting (MACS) is a method for separation of various cell populations depending on their surface antigens (Miltenyi*, et al* 1990). Magnetic MicroBeads are linked to monoclonal antibodies, which bind to specific cell surface antigens on target cells in a heterogeneous population. The cell suspension is loaded onto a column which is placed in a magnetic field. Magnetically labeled target cells are retained within the column while unlabeled cells pass the column. After removal of the column from the magnetic field, labeled cells can be eluted as positively selected cell fraction.

In this thesis the isolation of $CD14^{pos}$ monocytes from healthy donor PBMC was performed via MACS technology following the manufacturer´s instructions. Briefly, healthy donor PBMC were labeled with CD14 MicroBeads. The positive immunomagnetically selection of $CD14^{pos}$ cells was performed with LS columns. Purity of freshly isolated $CD14^{pos}$ cells without T-, B- or NK-cell contaminations was verified in flow cytometry by the cell surface marker phenotyp $CD11c^{pos}$, $CD19^{neg}$, $CD3^{neg}$, $CD16^{neg}$, and $CD56^{neg}$ before the cells were directly employed for the generation of mDC as described in chapter 4.2.1.1.3.

4.2.2.4 IFN-γ ELISpot Assay

Cytokine secretion after recognition of APC is an effector function of immune cells that gives valuable information about cell reactivity. The IFN-γ Enzyme-Linked Immunosorbent Spot (ELISpot) assay provides a potent tool of measuring IFN-γ cytokine production of T cells on the single cell level. The principle of an IFN-γ ELISpot assay operates as follows: First, a primary antibody with specificity to IFN-γ is bound to an ethanol-activated hydrophobic high protein binding polyvinylidene fluorid membrane of a 96-well ELISpot plate. Unspecific binding is blocked by serum incubation before stimulated T cells and target cells are co-cultured in the 96-well plate for 16-18h. T cells, which respond to the stimulator cells, produce IFN-γ. The secreted IFN-γ is captured by the primary antibodies on the membrane. After cell removal a secondary antibody is added. This antibody is directed against another epitope of IFN-γ than the primary antibody and is biotinylated. The resulting cytokine-antibody-biotin complex is bound by an avidin-enzym complex that binds specifically to biotin. At last the enzyme of the avidin-enzym complex converts an uncolored substrate into a colored molecule that binds to the membrane and forms a spot. With the spot number the reactivity and frequency of responding cells can be calculated.

The IFN-γ ELISpot assays were performed as follows: 96-well multiscreen HTS™ IP plate membranes were activated by prewetting with 35% by volume ethanol. After washing with PBS 60µl/well primary antibody (anti-hIFN-γ 1-DIK) in PBS [10µg/ml] were used for coating overnight at 4°C. Plates were washed with PBS and blocked with T-Cell Culture Medium for 2h at 37°C, 5% CO_2 in humidified atmosphere to avoid unspecific binding. 0.5-2x10^4 T cells/well were seeded together with 1-5x10^4 target cells/well in T-Cell Culture Medium in a total volume of 100µl. As control for spontaneous cytokine release effector cells and APC were also cultivated separately in the culture plate. If necessary, the stimulator cells were employed after pre-incubation with 500IU/ml IFN-γ for four days as it is described in chapter 4.2.1.1. Information on HLA-restriction of target cell recognition could be obtained by incubation of APC with saturating concentrations of HLA blocking for 30min at 37°C, 5% CO_2 in humidified atmosphere before addition of responder cells. If monoclonal antibodies, which block CD4-interactions, were applied the T cells were pre-incubated with the antibodies instead of the target cells. The cultures were incubated for 16-18h at 37°C, 5% CO_2 in humidified atmosphere.

Subsequently, plates were washed with ELISpot Washing Buffer before incubation with 60µl/well biotinylated secondary antidody (anti-hIFN-γ 7-B6-1) in ELISpot Secondary Antibody Buffer [2µg/ml] for 2h at 37°C, 5% CO_2 in humidified atmosphere. For detection the Vectastain® Elite ABC Kit (Vector Laboratories, USA) was applied. The ABC working solution was prepared as described in the manufacturer´s protocol. After removal of the secondary antibody by washing with ELISpot Washing Buffer 100µl ABC working solution were given into each well and incubated for 1h at RT in the dark. Again the plates were washed with ELISpot Washing Buffer followed by washing with PBS only. Addition of 100µl/well ELISpot AEC Solution provoked the final substrate conversion into the colored dye. The reaction was stopped after 8-10min by rinsing with water. Spots were counted and evaluated with the Axio Imager M1 microscope and the corresponding KS ELISpot 4.9 software (Herr*, et al* 1997) or the CTL ImmunoSpot® S5 Versa UV Analyzer and the pertinent ImmunoCapture™ 6.4. and ImmunoSpot® 5.0 software. Reactivity of T-cell cultures was determined by subtraction of spot numbers observed in wells with parental cells from that with HLA transfectant cells. Shown results are means ± standard deviation (SD) of duplicates. Statistical analysis was performed with SPSS15.0 software. Wilcoxon signed-rank test was used to calculate *P*-values, which were regarded as significant if $P < 0.05$.

4.2.2.5 ^{51}Chromium-release Assay

One effector function of T cells - mainly of CD8 T cells - is their ability to kill target cells. This cytolytic activity of T cells can be investigated in a ^{51}Chromium-release assay. For this purpose target cells are incubated with radioactive sodium chromate ($Na_2{}^{51}CrO_4$), which emits gamma radiation. Living cells take up the sodium chromate and store it inside the cell. If a T cell recognizes and kills these target cells, the radioactive content is released into the medium. The resulting radioactive radiation of the cell supernatant can be quantified in a gamma counter and is consulted for calculation of the specific lysis mediated by the T cells.

The ^{51}chromium-release assay was performed with HLA-DQ and HLA-DP specific T cells which were stimulated as described in chapter 4.2.2.1. The assay was conducted at day 5 after the last T-cell restimulation (d19 or d26). Primary AML blasts and primary fibroblasts were applied as target cells and were either incubated in medium with 500IU/ml IFN-γ for 4 days or cultured without IFN-γ as described in chapter 4.2.1.1. Respectively, 0.5Mio targets were incubated for 2h at 37°C, 5% CO_2 in humidified atmosphere with 75µCi $Na_2{}^{51}CrO_4$ and 25µl FCS to provide the cells with serum proteins and to ensure optimal osmotic conditions for the cells both enhancing cell survival. Subsequently, cells were washed and plated in 96-well plates (V-bottom) in a density of 1-1.5x10^3 cells/well. T cells were added in duplicates in designated (E:T) ratios in a total volume of 160µl T-Cell Culture Medium per well. If the reaction was blocked by monoclonal antibodies the cells (T cells for CD4-blockade

including isotypic control, targets for HLA-blockade including isotypic control) were incubated with the respective antibodies for 30 minutes at 37°C before they were given into the wells. To evaluate the maximum release of target cells a detergent solution (1% Triton X 100 in PBS) instead of T cells was incubated with the targets. The spontaneous release of target cells was measured by adding PBS instead of T cells to the wells. The plates were incubated for 5h at 37°C, 5% CO_2 in humidified atmosphere before centrifugation and collection of 80µl supernatant. The radioactive radiation in the supernatant was counted in a Wizard^2 Gamma Counter. Percent specific lysis was calculated as follows: specific lysis [%] = 100 x (experimental ^{51}Cr-release – spontaneous ^{51}Cr-release) / (maximum ^{51}Cr-release – spontaneous ^{51}Cr-release). Data are mean ± SD of duplicates.

4.2.3 Biomolecular Methods

For this study APC that express specific HLA-DR (DRA1*01:01, DRB1*07:01), HLA-DQ (DQA1*01:02, DQB1*06:02) and HLA-DP (DPA1*01:03 and DPB1*02:01) molecules were generated. This was accomplished by different biomolecular methods. cDNA vectors containing full-length HLA-DP alpha and beta chain genes were newly synthesized by molecular cloning, while cDNA plasmids for HLA-DR and HLA-DQ alleles were kindly provided by Dr. U. Sahin, University Medical Center, Mainz, D. The vectors were used for the generation of *in vitro* transcriped (IVT) mRNA, which was electroporated into the cells of interest. Consequently, mRNA transfection resulted in transient HLA class II expression on APC.

4.2.3.1 Cloning of HLA-DP Alpha and Beta Chain Alleles

PBMC of healthy donor 225 were used for cloning the HLA-DP alpha and beta chain alleles. Donor 225 is positive for the HLA-DPB1*02:01 allele. The HLA-DP alpha chain has not been typed and was picked randomly during the cloning procedure. Based on the fact that 87% of the kaukasian population are positive for the HLA-DPA1*01:03 allele the primers were designed for this allele. HLA nucleotide coding sequences were obtained from *www.hla.alleles.org*.

4.2.3.1.1 Total RNA Isolation and cDNA Synthesis

Total RNA isolation was performed with 3x10^6 PBMC using the RNeasy Mini Kit (Quiagen, D) according to manufacturer´s instructions. 1µg of freshly isolated RNA was used for cDNA synthesis. Therefore the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen, D) was employed following the instruction manual. RNA and DNA concentrations were measured on a GeneQuant II UV spectrophotometer (Pharmacia Biotech, UK).

4.2.3.1.2 PCR

Primers were designed for the amplification of the HLA-DP alleles and ordered from Eurofines MWG Operon, D. Besides the coding nucleotide sequence of the alleles a recognition site for the restriction endonuclease XbaI for the sense primer and XhoI for the antisense primer was integrated into the primer sequence. The sequences are shown in the following:

HLA-DPA1*01:03for: ATGCGCCCTGAAGACAGAATGTTC(5'→3'); HLA-DPA1*01:03rev: TCACAGGGTCCCCTGGGC(5'→3')
HLA-DPB1*02:01for: ATGATGGTTCTGCAGGTTTCTGC(5'→3'); HLA-DPB1*02:01rev: TTATGCAGATCCTCGTTGAACTTTC(5'→3')

As template for the polymerase chain reaction (PCR) the newly synthesized cDNA (chapter 4.2.3.1.1) was used. The PCR was performed with the Platinum Pfx DNA Polymerase in a gradient mastercycler (Eppendorf, D). The PCR mix was composed of 1.0µl forward primer [10pmol] + 1.0µl reverse primer [10pmol] + 1.5µl dNTP [10mM] + 1.0µl $MgSO_4$ [50mM] + 1.0µl cDNA [1:10 dilution = 65ng] + 5.0µl polymerase buffer (10x) + 1.0µl Pfx polymerase [2.5U/µl] in a total volume of 50µl. The PCR was run with a temperature gradient to find the most stringent annealing temperature for each primer set (95°C/2min; 35 cycles: 95°C/30s, 55.5°C|58°C|60.5°C/30s, 68°C/50s; 4°C/hold). The success of the PCR was verified in an agarose gel (1.0%) electrophoresis showing that 60.5°C annealing temperature gave a clear band with the expected size of 810bp (783bp coding sequence + 27bp primer sequence) for HLA-DPA1*01:03. For the amplification of HLA-DPB1*02:01 an annealing temperature of 55.5°C leaded to a specific 804bp (777bp coding sequence + 27bp primer sequence) band.

4.2.3.1.3 Linearization & Purification

The entire PCR products were purified with the QIAquick PCR Purification Kit (Quiagen, D) as described in the manufacturer´s manual. Afterwards the DNA was digested overnight with restriction endonucleases. For the digest the total PCR product (50µl) was mixed with 3µl XbaI and 3µl XhoI in NEB Buffer 4 (7µl 10x Buffer) including BSA (7µl 10x BSA) in a total volume of 70µl in an overnight reaction at 37°C. The enzymes were inactivated by incubation at 65°C for 20 minutes. As vector the modified pGEM4Z-64A cDNA plasmid, which was kindly provided by Dr. S. Thomas, University Medical Center, Mainz, Germany, was utillized (Boczkowski*, et al* 2000). The plasmid was linearized under the same conditions as the PCR products: 5µg vector (10.0µl) + 3.0µl XbaI + 3.0µl XhoI + 7.0µl NEB Buffer 4 (10x) including 7.0µl BSA (10x) in a total volume of 70.0µl, over night at 37°C. The linearized plasmid was purified with the QIAquick® PCR Purification Kit (Quiagen, D) and resuspended in 50.0µl endotoxin free water. Afterwards a dephosphorylation was performed to prevent recircularisation during the ligation step. Therefore 50µl vector were incubated with 3µl calf intestinal phosphatase (CIP) and 6.0µl CIP buffer (10x) in a total volume of 60µl for 2h at 37°C. Vector and insert DNA were purified using the QIAquick® PCR Purification Kit (Quiagen, D) and resuspended in 30.0µl endotoxin free water.

4.2.3.1.4 Ligation, Transformation, DNA Preparation & Sequencing

A ligation of the linearized vector with the HLA-DPA1 or the HLA-DPB1 PCR product was performed using the following protocol: 1.0µl vector + 15.0µl Insert + 1.0µl T4-Ligase + 2.0µl T4-Ligase Buffer (10x) in a total volume of 20.0µl, overnight at 16°C. Following standard molecular methods 10.0µl ligation product were used for a transformation with 100µl E. coli K12 JM 109 competent cells (kindly provided by the research group of M. Theobald, University Medical Center, Mainz, D). The transformation product was plated on selective LB-Agar plates containing 100mg/l ampicillin, which were incubated overnight at 37°C. To confirm that grown clones contain the DNA fragment of interest the colonies were screened. Therefore an overnight culture in selective LB-Medium containing 100mg/l ampicillin and a DNA minipreparation with the QIAprep® Spin Miniprep Kit (Quiagen, D) were performed. The resulting DNA was used in a control digest with XbaI and XhoI as performed for the PCR products before (1.0µg DNA (5.0µl) + 1.0µl XbaI + 1µl XhoI + 2.0µl NEB Buffer 4 (10x) + 2.0µl BSA (10x) in a total volume of 20.0µl). Afterwards an agarose gel (1.0%) electrophoresis was performed. Clones showing the correct pattern of bands (backbone 2833bp, insert 810bp alpha chain, 804bp beta chain) were sent in for sequencing to Genterprise Genomics, D to confirm the correct DNA sequence. Primer used for the DNA sequencing were:

for_pGEM4Z_S: CGC CCA GCT CTA ATA CGA CTC(5'→3'); rev_pGEM4Z_S: GGA GCA GAT ACG AAT GGC TAC(5'→3')

DNA of the clones with the right DNA sequence was chosen for a DNA midipreparation with the EndoFree® Plasmid Midi Kit (Quiagen, D) and was used for the following *in vitro* transcription.

4.2.3.2 *In Vitro* Transcription of HLA Allele cDNA

As described in the previous section the HLA-DP alleles (DPA1*01:03 and DPB1*02:01) were cloned into the pGEM4Z cDNA vector. Full length cDNA gene sequences of the HLA-DR (DRA1*01:01 = 765bp, DRB1*07:01 = 801bp) and HLA-DQ (DQA1*01:02 = 768bp, DQB1*06:02 = 786bp) alpha and beta chain alleles were integrated into pST1-2β-globin UTR-A(120) cDNA plasmids (Holtkamp*, et al* 2006). The plasmids were linearized for *in vitro* transcription: 20µg pGEM4Z vectors were linearized with 4.0µl SpeI in NEB Buffer 4 (5µl 10x Buffer) containing 1x BSA (5µl 10x BSA) in a total volume of 50.0µl in an overnight digest. pST1 vectors were linearized with EciI. Therefore 20µg pST1 plasmids were digested for 4.5h at 37°C with 10.0µl EciI in NEB Buffer 2 (10µl 10x Buffer) including BSA (10µl 10x BSA) in a total volume of 100.0µl. Restriction endonucleases were inactivated by incubation at 65°C for 20-30 minutes. For DNA purification the QIAquick® PCR Purification Kit (Ambion, D) was used and the purified DNA was resuspended in nuclease-free water. Subsequently, RNA was synthesized by *in vitro* transcription with the mMESSAGE mMACHINE T7 Ultra Kit according to manufacturer´s instructions including the enzymatic DNaseI digestion for removal of template DNA. IVT RNA was purified with the RNeasy Mini Kit (Quiagen, D) according to the instruction manual. RNA

concentration was measured spectrophotometrically and the RNA was frozen at -80°C in units of 10µg. The purity of the *in vitro* transcribed RNA was verified by a denaturing agarose gel (1.0%) electrophoresis. The RNA of each HLA molecule gave a single defined band corresponding to the expected size of the RNA molecule (HLA-DRA1*01:01 = 1246b, HLA-DRB1*07:01 = 1282b, HLA-DQA1*01:02 = 1249b, HLA-DQB1*06:02 = 1267b, HLA-DPA1*01:03 = 1147b, HLA-DPB1*02:01 = 1146b).

4.2.3.3 mRNA Electroporation of HLA Class II Alleles

HLA class II transfectant cells were generated by mRNA electroporation into different cell types. During an electroporation the cells are brought into an electric field that induces the formation of small membrane pores, which allow polar molecules to pass before the membrane reseals (Rols 2006, Rols and Teissie 1998).

Two different approaches were applied: On the one hand, the K562 cell line was emploid to develop standard antigen-presenting cells expressing single HLA-DR (DRA1*01:01, DRB1*07:01) or HLA-DQ (DQA1*01:02, DQB1*06:02) molecules, respectively. Therefore 1-2x10^7 K562 cells were washed twice in RPMI Medium 1640 without phenol red and once in Opti-MEM® I Medium. The cells were resuspended in 200µl Opti-MEM® I Medium and were given into a 4mm cuvette containing 10µg RNA encoding the alpha and beta chains of HLA-DR or HLA-DQ, respectively. Since HLA class II molecules consist of an alpha and a beta chain, which need to be expressed simultaneously for the reassembling of functional molecules on the cell surface the alpha and the beta chains were electroporated simultaneously. The electroporation was performed with the Gene Pulser Xcell system (Bio-Rad Hercules, USA) applying a square wave pulse of 400V for 5ms as it has been similarly shown for T lymphocytes by Schaft and colleagues (Schaft*, et al* 2006). As controls MOCK electroporations were included in the experiments. Transfectant cells were transferred into a 6-well plate and incubated in 3ml T-Cell Culture Medium at 37°C, 5% CO_2 in humidified atmosphere. HLA expression kinetics on K562 cells after electroporation was monitored for one week. Transfectant cells were counted after 4h, 8h, 12h, 24h, 48h, 96h or 120h, and 168h and stained with HLA class II for flow cytometry analysis. Aliquots of HLA class II transfectant cells (2-5x10^5 cells) were frozen 12h after electroporation and were used directly after thawing and if necessary, lethal irradiation. Hence, HLA expression was also monitored after freezing, thawing and irradiation cycles (4h, 8h, 12h, 24h after electroporation). On the other hand, mDCs, PHA blasts or PBMCs carrying HLA-DQ (DQA1*01:02, DQB1*06:02) or HLA-DP (DPA1*01:03 and DPB1*02:01) on their cell surface were utilized. The electroporation was conducted under the same conditions as described for K562 cells except for PBMC. PBMC were exposed to a single square wave pulse of 350V for 12ms as it has been previously described by Kleemann *et al* (Kleemann*, et al* 2012).

5 Results

5.1 Cell sorting of PBMC for naive & memory CD4 T-cell subsets

In the first part of this study we investigated the allorecognition and alloproliferation of distinct CD4 T-cell subsets that were enriched for naive or memory T cells, respectively. The T-cell differentiation markers CD45RA, CD45RO, CD62L and CCR7 were chosen to define the particular T-cell subsets. CD45RA is a protein tyrosine phosphatase, which is mainly expressed by naive T cells and by late effector cells. In contrast, the isoform CD45RO is a cell surface marker on memory T cells. The adhesion molecule CD62L and the chemokine receptor CCR7 can be found on naive and antigen experienced central memory T cells (Appay*, et al* 2008, Sallusto*, et al* 2004). Figure 3 displays the reciprocal expression of CD45RA and CD45RO (Michie*, et al* 1992) as well as the unequal distribution of CD62L and CCR7 co-expression on CD4 T cells demonstrating that each marker represents a unique T-cell population. Each subset is defined by just one single maturation marker to facilitate future establishment of a clinical grade depletion procedure under GMP conditions.

In order to isolate the CD4 T-cell subsets from total PBMC we used flow cytometric cell sorting. PBMC from six healthy donors were stained with monoclonal antibodies for CD3 and CD4 together with those for the T-cell surface markers CD45RA, CD45RO, CD62L and CCR7, respectively. Note that the PBMC of donor 053 were only stained and sorted for $CD4^{pos}$ and the respective differentiation $marker^{pos/neg}$ T-cell populations, but not additionally for $CD3^{pos}$ T-cell subsets (data not shown). Since the resulting T-cell populations showed a contamination with NK cells (chapter 5.3.5), CD3 was added to the staining protocol. $CD3^{pos}CD4^{pos}$ T cells of the viable lymphocyte population were gated and sorted due to the strong or absent expression of the differentiation markers. The difference in fluorescence intensity between the gate for the maturation marker positive population and the gate for the respective negative counterpart fraction was at least 0.5 log units (Figure 4). Using this approach, we obtained four different naive-enriched $CD4^{pos}$ T cell subsets ($CD45RA^{pos}$, $CD45RO^{neg}$,

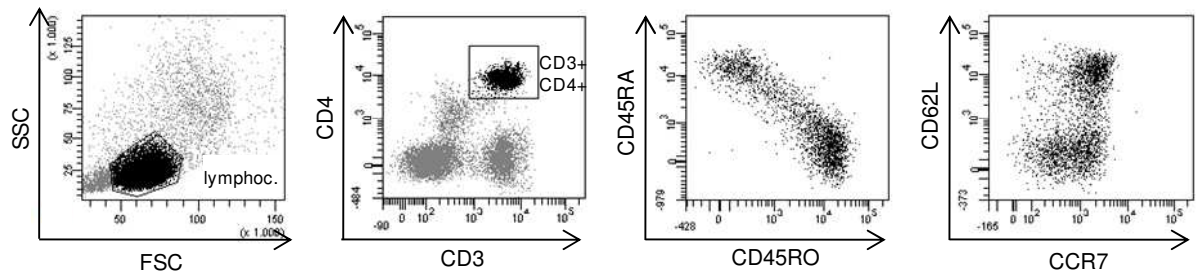


Figure 3. Flow cytometric staining of T-cell subsets. PBMC of healthy donors were stained with mAb for CD3 and CD4 together with mAb for T-cell differentiation markers (i.e. CD45RA, CD45RO, CD62L, CCR7). CD3 CD4 T cells of the lymphocyte population were gated with subsequent analysis of CD45RA and CD45RO as well as CD62L and CCR7 co-expression. Note the reciprocal expression of CD45RA and CD45RO, and the unequal distribution of CD62L and CCR7 expression, indicating that CD62L and CCR7 describe different T-cell subsets. Shown are representative results with PBMC of donor 927. Data partly published in Distler, Bloetz, *et al* 2011.

CD62L^{pos}, CCR7^{pos}) and additionally four distinct memory-enriched CD4 counterpart fractions (CD45RA^{neg}, CD45RO^{pos}, CD62L^{neg}, CCR7^{neg}). The eight different T-cell subsets of six donors (i.e. n=48 fractions in total) had a median purity of 99.8% (range, 82.8%-100%). Sorted total CD4^{pos} T cells were also included in the experiments for the comparison with the sorted subsets. Median purity of total CD4 populations was 99.6% (range, 74.4%-99.8%, n=6). In further experiments sorted T cell subsets were analyzed for allorecognition and alloproliferation to single HLA-DR and HLA-DQ mismatch alleles by allogeneic MLR *in vitro*.

In the second part of this work CD4 T cells specific for mismatched HLA-DQ or HLA-DP alleles were generated in order to investigate their capacity to recognize and kill leukemia cells without the destruction of non-hematopoietic tissues. For this purpose T-cell subpopulations were applied that showed naturally a strong alloreactive potential in order to gain the strongest possible reactivity against patient cells. We knew from the first part of this study that naive T cells derived from the CD4^{pos}CD45RA^{pos} compartment provoked strong alloreactivity against mismatch HLA alleles. Therefore, PBMC of seven healthy donors were sorted for the CD4^{pos}CD45RA^{pos} T-cell subsets with the strategy described above and had a median purity of 99.5% (range, 95.0%-100%). During this study they were stimulated *in vitro* against single HLA-DQ/-DP alleles in MLR.

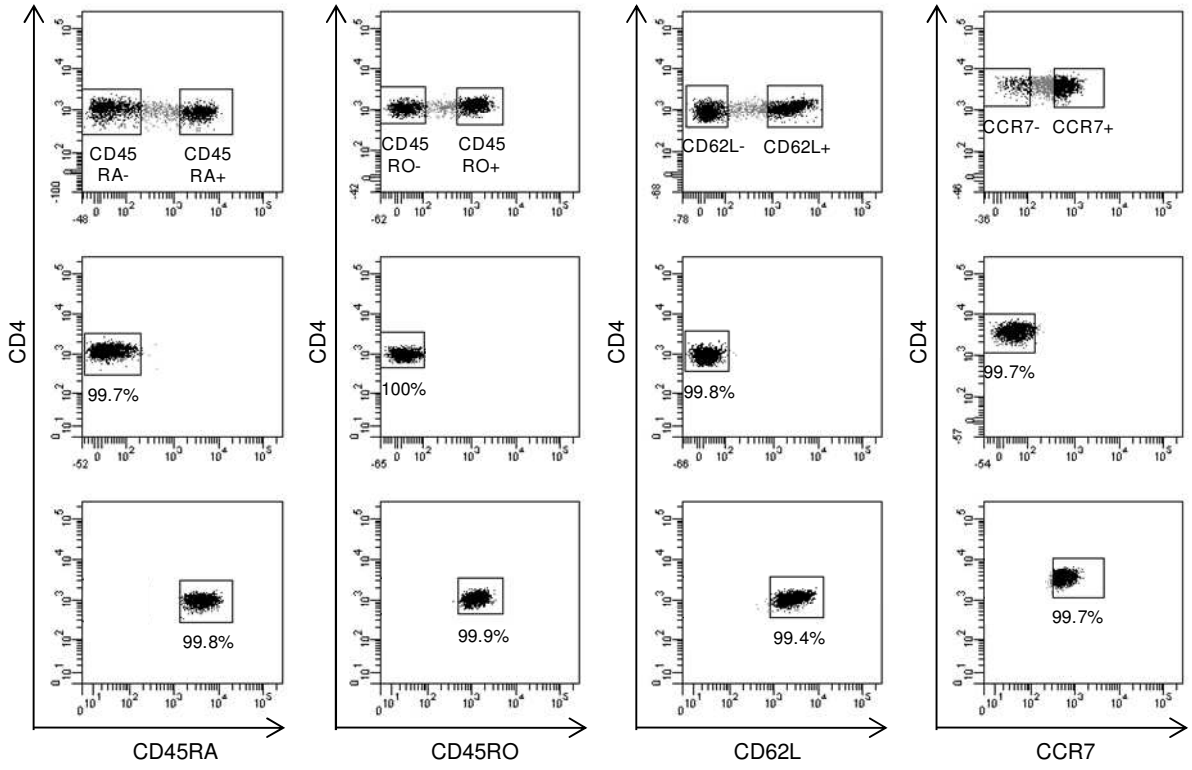


Figure 4. Flow cytometric staining and sorting of T-cell subsets. PBMC of healthy donors were stained with mAb for CD3 and CD4 together with mAb for T-cell differentiation markers (i.e. CD45RA, CD45RO, CD62L, CCR7). Gating strategy for cell sorting: After gating of CD3 CD4 T cells of the lymphocyte population (see Figure 3), the gates for a single T-cell differentiation marker were set according to strong or absent expression of this marker (>0.5 log difference in fluorescence intensity). Shown are the sorting gates for CD3 CD4 T cells of donor 372. Purity of sorted T-cell subsets was >99% in re-analysis. Data partly published in Distler, Bloetz, *et al* 2011.

5.2 APC for distinct HLA class II alleles

5.2.1 K562 cells as standard APC for HLA-DR or HLA-DQ molecules

In this study standard APC that express single HLA-DR and HLA-DQ molecules were generated. For this purpose the CML cell line K562, which naturally lacks HLA expression under non-inflammatory conditions (Kaplan and Callewaert 1978), was transfected by electroporation with mRNA encoding for the alpha and beta chains of distinct HLA-DR (DRA1*01:01, DRB1*07:01) and HLA-DQ (DQA1*01:02, DQB1*06:02) molecules, respectively. Consequently, RNA transfection resulted in transient HLA class II expression on K562 cells. Kinetics of the HLA expression was followed for seven days by flow cytometry. HLA molecules could be detected on the cell surface already 4h after electroporation (e.g. for Batch A: HLA-DR positive cells = 48%, HLA-DQ positive cells = 21%) with a maximum expression level after 12h to 24h. HLA-DR and HLA-DQ surface expression could even be measured seven days after transfection (e.g. for Batch A: HLA-DR positive cells = 12%, HLA-DQ positive cells = 5%). In order to perform all experiments, which were conducted with T cells from one donor with exactly the same K562 transfectant cells, one batch of K562-HLA transfectant cells was generated for each donor. The batches were frozen in aliquots 12h after electroporation, when the cells showed strong HLA molecule expression. The median value of HLA positive cells for the used batches of K562-HLA-DR and -DQ transfectant cells was 70% (range, 22-85%) (Figure 5). Prior to use the cells were thawed and if necessary lethally irradiated. The K562-HLA class II mRNA transfectants did not lose HLA class II expression upon freezing, thawing and irradiation cycles (data not shown).

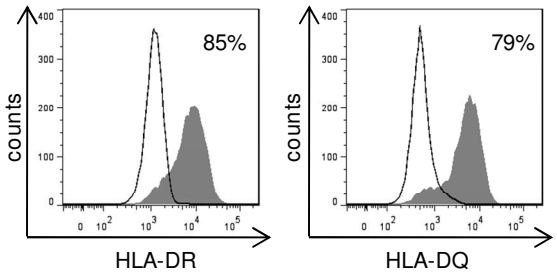


Figure 5. Flow cytometric analysis of HLA class II allele expression on K562 cells. K562 cells were co-transfected with alpha and beta chain mRNA of single HLA-DR/-DQ molecules by electroporation. Shown are stainings of transfectant cells (filled histograms) and mock controls (black lines) with anti-HLA-DR/-DQ mAb 12h after electroporation. Given numbers are the percentage of HLA-positive cells. Data published in Distler, Bloetz, *et al* 2011.

5.2.2 mDC as APC stocks expressing allo-HLA-DQ or allo-HLA-DP alleles

In a second approach mature monocyte-derived dendritic cells (mDC) expressing single mismatch HLA class II molecules, which were not endogenously expressed by the cells, were used as APC. As described in the previous chapter for K562 cells mDC were transfected by electroporation with mRNA, here encoding for the alpha and beta chains of the class II molecules HLA-DQ (DQA1*01:02, DQB1*06:02) and HLA-DP (DPA1*01:03, DPB1*02:01), respectively.

The mDC were generated from monocytes in 48h based on a "FAST-DC" protocol (Dauer*, et al* 2003). First the monocytes were gained from PBMC of healthy donors after applying MACS technology with $CD14^{pos}$ MicroBeads (n=4). Since the yield of mDC after the monocyte isolation and consequent DC maturation was very low (median percentage of mDC derived from PBMC = 1.0% (range, 0.7-2.3%)) we changed the protocol and used plastic adherence for the monocyte isolation. In this way 3.4 times more mDC were obtained (median percentage of mDC derived from PBMC = 3.4% (range, 2.1-5.6%), n=5) with comparable mDC purity for both protocols (data not shown). Additionally, the generation of monocytes via plastic adherence is time- and cost-saving compared to MACS technology.

The experiences made during the transfection of K562 cells as APC and the class II molecules HLA-DR and HLA-DQ as integrated proteins were transferred to the electroporation of mDC as target cells and the HLA-DP proteins. Hence, APC stocks were generated for each donor by freezing the HLA-DQ/-DP mismatch allele carrying mDC in aliquots 12-14h after electroporation, when the cells showed strong HLA molecule expression. Figure 6 indicates the expression of HLA-DQ and HLA-DP molecules on mDC before and after the electroporation. Since HLA class II molecules were already expressed on mDC prior transfection the exact electroporation efficiency could not be determined. Transfectant cells were applied for further experiments if the MFI values of the HLA expression on mDC did at least increase 2.2-fold after electroporation (median increase in MFI = 9.1-fold, range, 2.2-25.1-fold; n=6).

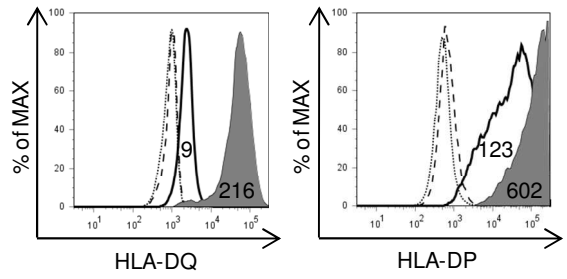


Figure 6. Flow cytometric analysis of HLA class II allele expression on mature dendritic cells. DCs were co-transfected with alpha and beta chain mRNA of single HLA-DQ/-DP molecules by electroporation. Shown are stainings of transfectant cells (filled histograms) and untransfected cells (black lines) with anti-HLA-DQ/-DP mAb. Isotype-matched IgG mAb were used as control (dotted lines for DC, dashed lines for DC-HLA). Given numbers are MFI values.

5.3 Alloreactive potential of naive- & memory-enriched allo-HLA-DR/-DQ specific CD4 T-cell subsets

5.3.1 Separation and allostimulation of naive- & memory-enriched CD4 T-cell subsets

In the first part of this thesis the alloreactivity of naive- and memory-enriched CD4 T-cell populations against single HLA-DR and HLA-DQ mismatch alleles was analyzed in MLR. In this way the CD4 T-cell compartment with the strongest alloreactive potential was determined. Stem cell transplants or DLI

products containing this subset would presumably trigger severe GvHD and reduce survival of patients in HLA-DR/-DQ incompatible HSCT. Once identified the T-cell subset could be considered for depletion in prospective clinical studies.

The novel approach developed in the PhD thesis for the generation of allo-HLA-DR/-DQ specific CD4 T cells is shown in Figure 7. PBMC of healthy donor origin were separated by flow cytometric cell sorting into subsets enriched for naive and memory CD4 T cells due to the expression or absence of the differentiation markers CD45RA, CD45RO, CD62L and CCR7 (chapter 5.1, Figure 4). After sorting the T cells were weekly stimulated *in vitro* against allo-HLA molecules. For this purpose the HLA-deficient cell line K562 was transfected by electroporation with single HLA-DR (DRB1*07:01) and HLA-DQ (DQB1*06:02) molecules, respectively, using off-the-shelf IVT mRNA (chapter 5.2.1). The K562-HLA transfectant cells carrying single mismatch HLA molecules were utilized as standard APC in MLR with the sorted T-cell fractions. Naive- and memory-enriched allo-HLA-DR/-DQ specific CD4 T-cell populations were weekly restimulated. The cytokines IL-7, IL-12 and IL-15 were added to the culture, from day 7 on IL-12 was replaced by IL-2. The alloreactivity of the distinct populations was analyzed by IFN-γ ELISpot assays and determination of T-cell proliferation by trypan blue counting.

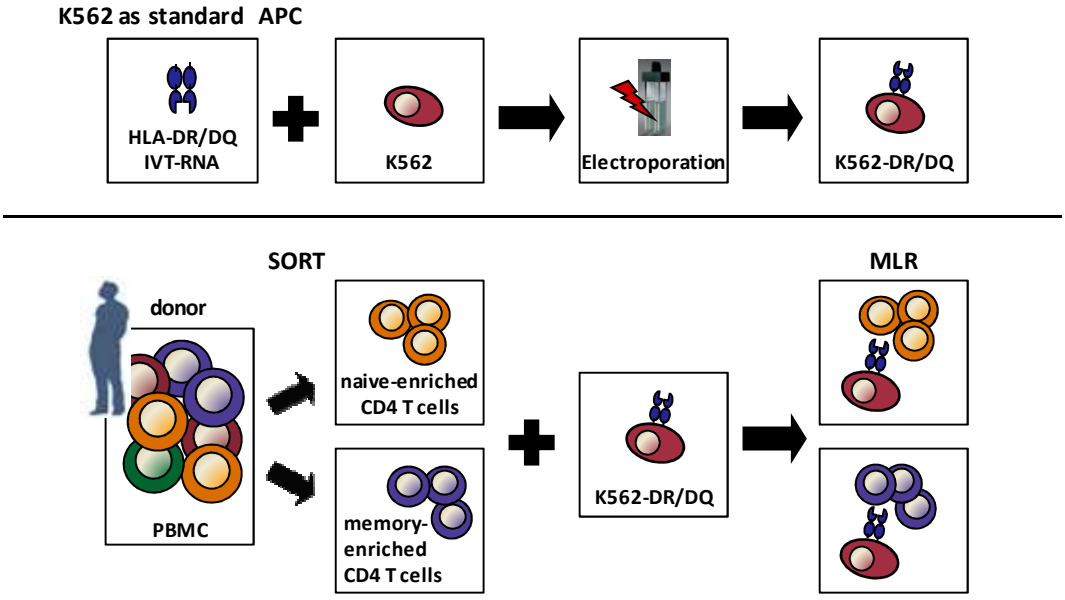


Figure 7. Schematic representation of the separation and allostimulation of naive- and memory-enriched CD4 T-cell subsets. Upper panel, production of standard APC: IVT mRNA of distinct HLA-DR/-DQ alleles was synthesized. HLA-DR/-DQ deficient K562 cells were transfected with the IVT mRNA of HLA-DR/-DQ alleles by electroporation. Resulting standard APC express a single HLA class II allele. Lower panel, sorting & MLR: Healthy donor PBMC were sorted by flow cytometry for naive- and memory-enriched CD4 T-cell populations due to the expression or absence of distinct maturation markers. CD4 T-cell subsets were stimulated *in vitro* in MLR against K562-DR/-DQ cells. Allo-HLA-DR/-DQ specific naive- and memory-enriched CD4 T-cell populations were obtained and analyzed for alloreactivity.

5.3.2 HLA-DR/-DQ specific allorecognition of CD4 T-cell subsets

Nine donor/allo-HLA combinations (HLA-DR: n=5, HLA-DQ: n=4; Table 1) derived from PBMC of six healthy donors were investigated for allorecognition by IFN-γ ELISpot assays five days after the first restimulation at day 12 of culture. This early culture time point was chosen in order to match the *in vivo* situation as close as possible and avoid *in vitro* bias. Furthermore, sufficient numbers of CD4 T cells were obtained for the analysis and following restimulations.

Table 1. HLA types of healthy donors and transfectant K562 cells used for *in vitro* stimulation.

			HLA class II-mismatched K562 transfectant cells	
	HLA-DRB1*	**HLA-DQB1***	**DRB1*07:01**	**DQB1*06:02**
Don 053	10:01 / 11:04	03:01 / 05:01	×	n.d.
Don 073	03:01 / 11:01	02:01 / 03:01	×	×
Don 372	11:01 / 13:01	03:01 / 06:03	×	×
Don 079	04:01 / 15:01	03:02 / 06:02	×	n.d.
Don 454	04:01 / 14:54	03:02 / 05:03	×	×
Don 225	01:01 / 11:01	03:01 / 05:01	n.d.	×

Don, donor; n.d., not determined

Allo-HLA-DR/-DQ reactive T cells could be detected basically in those CD4 T-cell subsets, which were enriched for naive T cells compared to their memory counterparts (Figure 8). Therefore the HLA-restriction and strong specificity of the MLR/K562-HLA screening approach is exemplarily shown for the naive-enriched CD4 T-cell populations of donor 372 in Figure 8. It could be demonstrated that

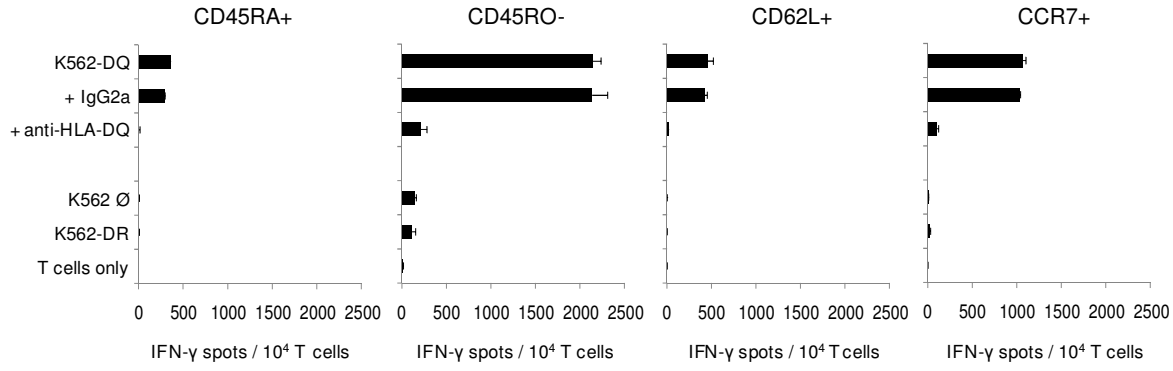


Figure 8. Specificity of allo-HLA-DR/-DQ mismatch recognition of CD4 T-cell subsets. Shown are representative results with naive-enriched CD4 T-cell subsets of donor 372 specific for allo-HLA-DQB1*06:02. MLR populations were screened in IFN-γ ELISpot assays on d12 (i.e. 5 days after first allo-HLA restimulation on d7) for reactivity to original K562-DQ mismatch stimulator cells, as well as to parental K562 cells and K562 transfectant cells carrying an irrelevant HLA-DR allele. To demonstrate HLA-restriction of detected reactivity, mAb blocking the TCR-HLA-DQ interaction (and IgG2a isotypic control) were used. Similar data from memory counterpart fractions are not presented. Data published in Distler, Bloetz, *et al* 2011.

significant alloresponses were only observed if K562-HLA transfectant cells, which were utilized for the T-cell priming, were applied as target cells. If parental K562 cells as well as transfectant cells carrying third-party HLA alleles served as stimulators no significant spot production of CD4 T cells was detected. In all experiments, allo-HLA class II reactivity could be blocked by mAb binding to the class II molecule used for primary *in vitro* stimulation, whereby IgG isotypic controls did not lead to any spot reduction (Figure 8).

5.3.3 Stronger allorecognition in naive compared to memory CD4 T cells

Alloresponses of MLR responders derived from naive- and memory-enriched CD4 T-cell subsets as well as from entire CD4 T cells stimulated against mismatched K562-HLA alleles were explored five days after the first restimulation at day 12 of culture for five donor/allo-HLA-DR pairs as well as for four donor/allo-HLA-DQ combinations in IFN-γ ELISpot assays. T-cell populations for donors 073, 372 and 454 were used in stimulations against mismatched K562-DR and K562-DQ, respectively. Due to the limited number of PBMC from donors 053, 079 and 225 T-cell subsets derived from those donors were either stimulated against mismatched HLA-DR or HLA-DQ alleles, only.

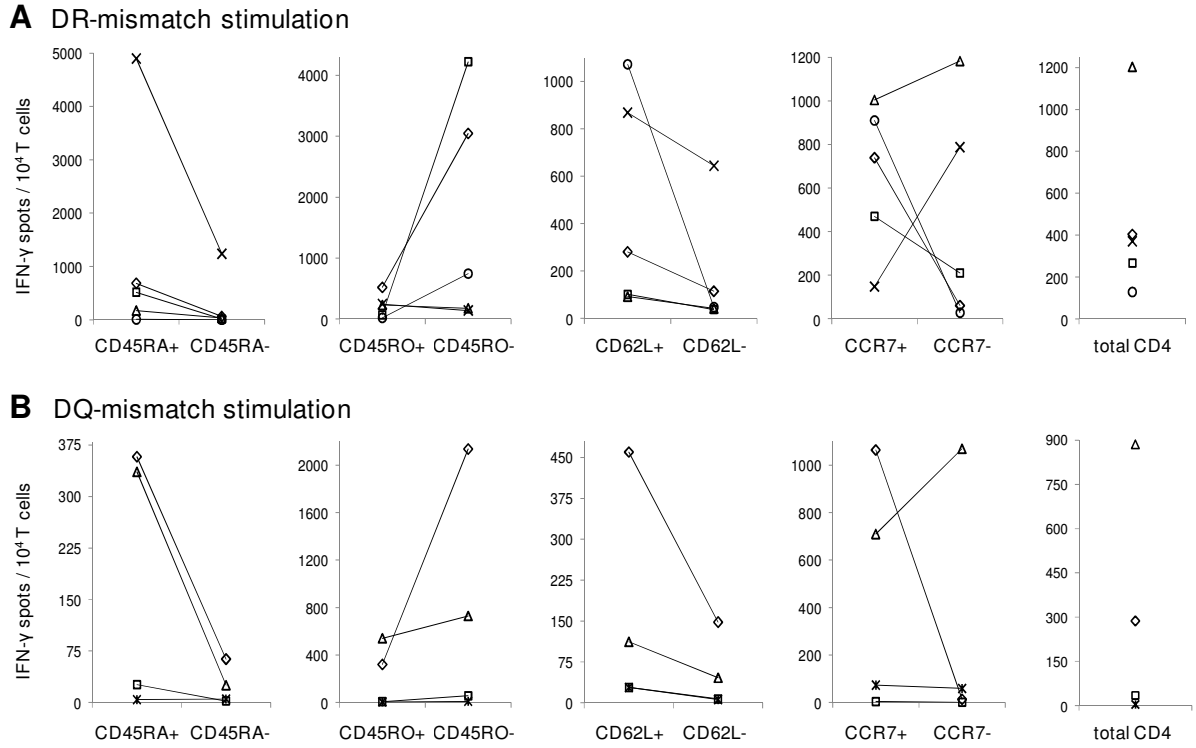


Figure 9. HLA-DR/-DQ specific allorecognition of CD4 T-cell subsets. MLR cultures were tested for allo-HLA reactivity in IFN-γ ELISpot assays against the original K562 mismatch stimulator cells. Shown are numbers of spot-forming cells from d12 cultures (i.e. 5 days after first allo-HLA restimulation on d7) initiated with naive- and memory-enriched sorted subsets (CD45RA, CD45RO, CD62L, CCR7) or entire CD4 T cells of 6 healthy donors (Don 053 (✕), Don 073 (△), Don 372 (◇), Don 079 (○), Don 454 (□), and Don 225 (✳)). Allo-HLA class II mismatch alleles used for *in vitro* stimulation were HLA-DRB1*07:01 **(A)** and HLA-DQB1*06:02 **(B)**, respectively. If PBMC numbers were limited, MLR stimulations were restricted only to one allo-HLA allele. Data published in Distler, Bloetz, *et al* 2011.

We observed that the T-cell fractions enriched for naive CD4 T cells (CD45RA^{pos}, CD45RO^{neg}, CD62L^{pos}, CCR7^{pos}) were more responsive to the allo-HLA stimulation than their memory counterpart subsets (Figure 9). In all nine donor/allo-HLA pairs for CD45RA populations the CD4 T cells derived from the naive compartments showed stronger alloresponses than the memory fractions with median IFN-γ spot-forming cell (SFC) numbers of 336 (range, 4-4900) to 25 (range, 2-1237) per 10^4 MLR responder cells. This was also the case for CD62L subsets (median SFC numbers of CD62L^{pos} populations 112 (range, 28-1072) *versus* 46 (range, 6-644) for CD62L^{neg} subsets). A similar reactivity pattern could be found for CD45RO with one exception. CD45RO^{pos} memory cells of donors 053 and 073 showed slightly stronger reactivity to K562-HLA-DR than the CD45RO^{neg} naive fraction. However, the median SFC numbers for the naive CD45RO^{neg} populations was 730 (range, 9-4225) *versus* 226 (range, 4-540) for CD45RO^{pos} memory counterparts. The difference of donors 053 and 073 in allo-HLA-DR recognition reoccurred in the experiments with CCR7 populations. Additionally for donor 073 alloreactivity of T-cell subsets enriched for CCR7^{neg} memory cells exceeded that for the CCR7^{pos} naive counterparts. The median SFC numbers for CCR7^{pos} naive subsets was 710 (range, 4-1065) *versus* 61 (range, 1-1183) for CCR7^{neg} counterpart fractions.

Figure 10 shows the statistical analysis of the combined data for HLA-DR and HLA-DQ mismatch stimulations. The Wilcoxon signed-rank test was used to calculate *P*-values. Statistical significance was found for CD62L (*P*=0.008) and CD45RA (*P*=0.011) subsets. Data for CD45RO (*P*=0.066) and CCR7 (*P*=0.314) MLR were not significant because of the different reactivity pattern for the individual donors 053 and 073 as described above. Alloreactivity of MLR cultures derived from memory CD4 T-cell subsets was altogether substantially lower compared to that of entire CD4 T cells. Median SFC

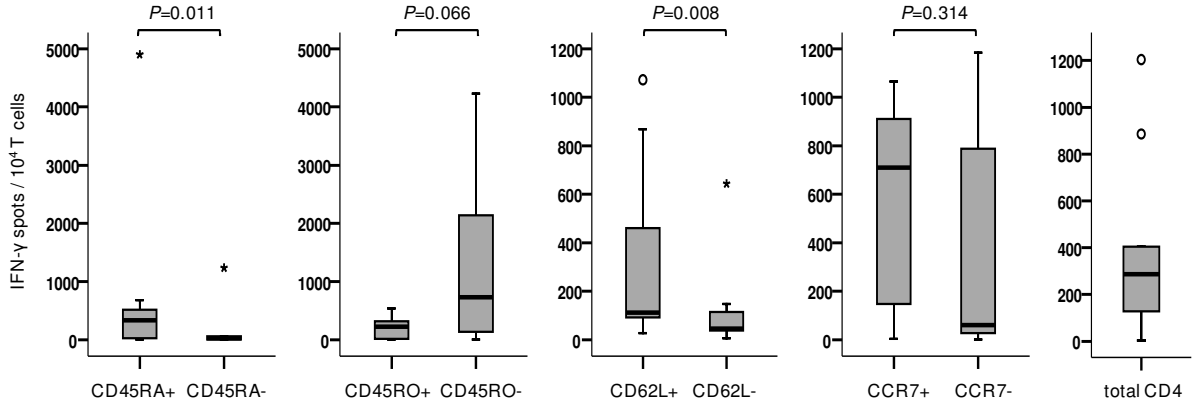


Figure 10. Statistical evaluation of HLA-DR/-DQ specific allorecognition of CD4 T-cell subsets. MLR cultures were tested for allo-HLA reactivity in IFN-γ ELISpot assays against the original K562-DR/-DQ mismatch stimulator cells. Numbers of spot-forming cells from d12 cultures (i.e. 5 days after first allo-HLA restimulation on d7) initiated with naive- and memory-enriched sorted subsets (CD45RA, CD45RO, CD62L, CCR7) or entire CD4 T cells of 6 healthy donors as presented in Figure 9 were statistically reviewed. Box plots with median (line), 25th to 75th percentile (box), and minimum and maximum values (error bars) are indicated. The Wilcoxon signed-rank test was used to calculate *P*-values. Data published in Distler, Bloetz, *et al* 2011.

numbers per 10^4 MLR/allo-HLA responder cells were 25 (range, 2-1237) for memory CD45RA^{neg} CD4 T cells, 46 (range, 6-644) for memory CD62L^{neg} CD4 T cells, 61 (range, 1-1183) for memory CCR7^{neg} CD4 T cells, 226 (range, 4-540) for memory CD45RO^{pos} CD4 T cells and 287 (range, 4-1204) for entire CD4 T cells, respectively. This means that the allorecognition of CD45RA^{neg} CD4 T-cell populations was16.6-fold lower than the allorecognition mediated by entire CD4 T cells (CD62L^{neg}: 3.5-fold, CD45RO^{pos}: 1.6-fold, CCR7^{neg}: 1.3-fold). These factors were determined by calculating the median of the ratios of SFC numbers for memory fractions to SFC numbers of total CD4 T cells of each donor. However, statistical significance in reduction of allorecognition for memory-enriched CD4 T-cell fractions compared to entire CD4 T cells was not observed for all four differentiation markers (data not shown). Interestingly, the median level of mismatch reactivity was clearly higher for the used HLA-DR than for the HLA-DQ allele (601 *versus* 93 SFC per 10^4 MLR responders for all naive CD4 subsets).

As additional read-out-assays for analysis of the allorecognition of CD4 T-cell subsets to HLA class II mismatch alleles we determined the secretion of the T_H1 cytokine TNF-α and the T_H2 cytokine IL-4 by TNF-α ELISpot assays and IL-4 ELISA, respectively. CD4 MLR responder populations of individual donors secreted low levels of IL-4 and TNF-α, with a tendency of stronger production in MLR derived from naive-enriched subsets (data not shown).

5.3.4 HLA-DR/-DQ specific alloproliferation in naive and memory CD4 T cells

Additionally to allorecognition we determined CD4 T-cell proliferation as a further parameter of T-cell activation. Alloproliferation was investigated for naive- and memory-enriched CD4 T-cell subsets as well as entire CD4 T cells derived from MLR against mismatched K562-HLA-DR/-DQ alleles for the same nine donor/allo-HLA pairs, which were used for the determination of allorecognition by IFN-γ ELISpot assays Figure 8, 9, 10. CD4 T-cell expansion of MLR cultures was followed over three weeks because potential differences in alloproliferation between the different counterpart fractions tended to broaden with increasing time. T-cell numbers were counted weekly by trypan blue staining for dead cell exclusion.

Figure 11 shows the total numbers of MLR responder cells at d21 of CD4 T-cell cultures initiated with $1x10^6$ CD4 T cells at day zero. An overall strong proliferation could be observed for all subsets analysed: for naive- and memory-enriched CD4 T-cell populations as well as for total CD4 T cells. The median cell numbers for the different counterpart fractions were very close to each other: CD45RA (median d21: $48.1x10^7$ CD45RA^{pos} and $37.8x10^7$ CD45RA^{neg} cells), CD45RO (median d21: $14.2x10^7$ CD45RO^{neg} and $24.0x10^7$ CD45RO^{pos} cells), CD62L (median d21: $19.8x10^7$ CD62L^{pos} and $19.0x10^7$

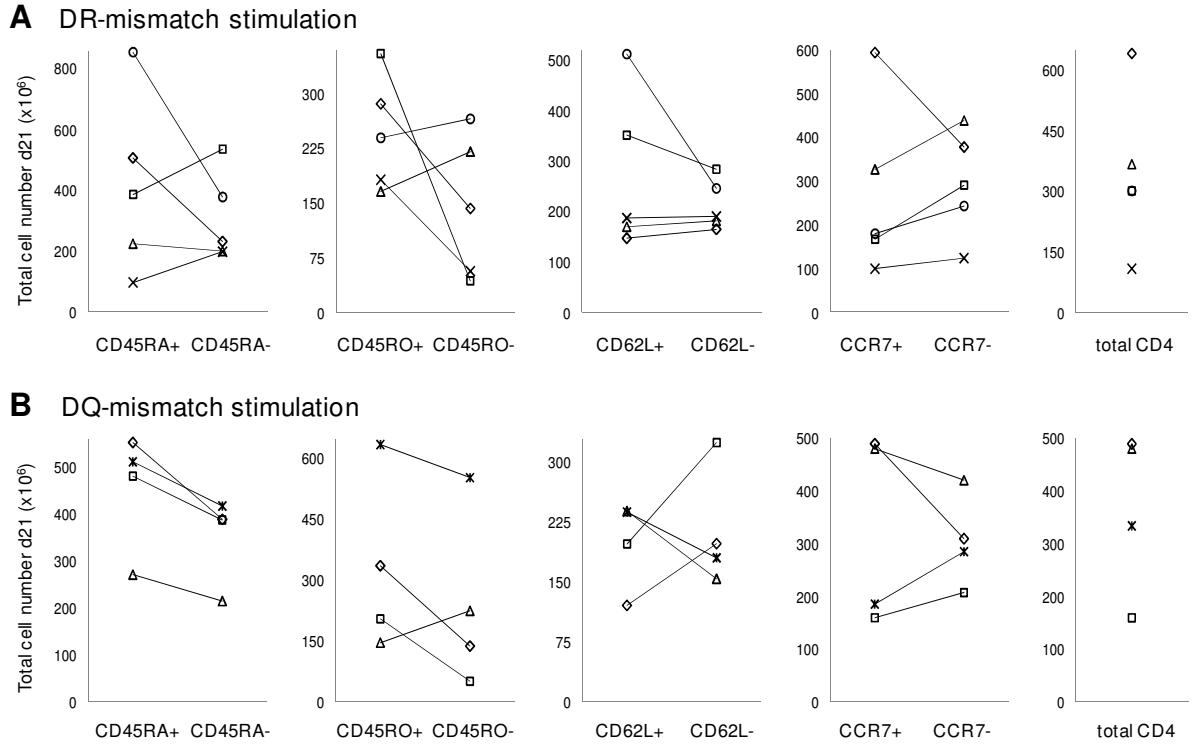


Figure 11. Alloproliferation to HLA-DR/-DQ alleles in CD4 T-cell subsets. Cell counts of MLR cultures were weekly determined. Total numbers of MLR responder cells at d21 if cultures were initiated at d0 with 1x10^6 cells of sorted naive-enriched, memory-enriched or entire CD4 T cells, respectively. Data from 6 healthy donors (Don 053 (✕), Don 073 (△), Don 372 (◇), Don 079 (○), Don 454 (□), and Don 225 (✳)) screened against mismatched HLA-DRB1*07:01 **(A)** or HLA-DQB1*06:02 **(B)** are shown. If PBMC numbers were limited, MLR stimulations were restricted only to one allo-HLA allele. Data shown in Figure 9 and Figure 10 are derived from the same MLR experiments. Data published in Distler, Bloetz, *et al* 2011.

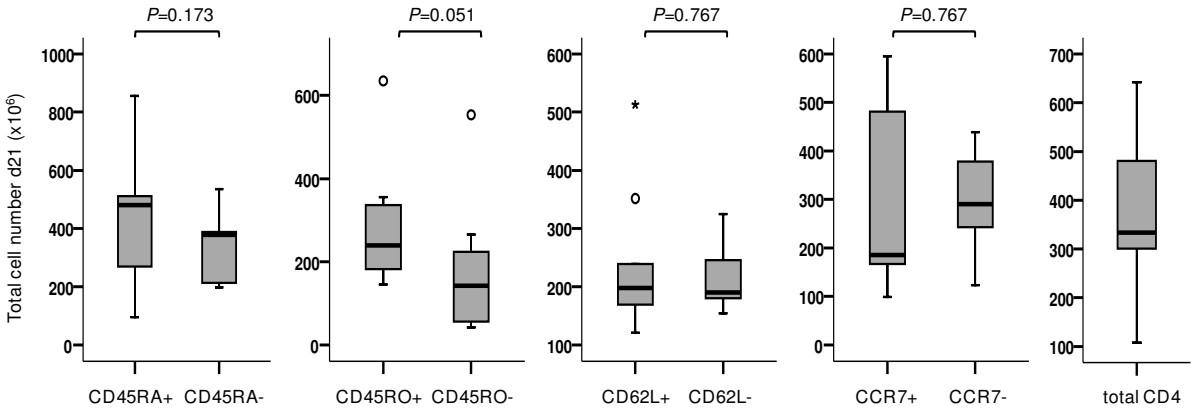


Figure 12. Statistical evaluation of alloproliferation of HLA-DR/-DQ specific CD4 T-cell subsets. Cell counts of MLR cultures were weekly determined. Total numbers of MLR responder cells at d21 if cultures were initiated at d0 with 1x10^6 cells of sorted naive-enriched, memory-enriched or entire CD4 T cells, respectively. Data from 6 healthy donors screened against mismatched HLA-DR and HLA-DQ alleles as presented in Figure 11 were statistically reviewed. Box plots with median (line), 25th to 75th percentile (box), and minimum and maximum values (error bars) are indicated. The Wilcoxon signed-rank test was used to calculate *P*-values. Data shown in Figure 9 and Figure 10 are derived from the same MLR experiments. Data published in Distler, Bloetz, *et al* 2011.

CD62L^{neg} cells) and CCR7 (median d21: 18.5x10^7 CCR7^{pos} and 29.0x10^7 CCR7^{neg} cells). In median 33.4x10^7 of total CD4 T-cell were obtained after 21 days.

The statistical evaluation of the combined data for HLA-DR and HLA-DQ mismatch proliferation is shown in Figure 12. The Wilcoxon signed-rank test was used to calculate *P*-values. Statistical significance in alloproliferation between MLR cultures derived from subsets enriched for naive or memory CD4 T cells was not observed for any of the four differentiation markers.

5.3.5 Phenotypic characterization of allo-HLA-DR/-DQ specific CD4 T-cell subsets

The phenotypic characteristics of the naive- and memory-enriched allo-HLA-DR/-DQ specific CD4 T cell-subsets would be important for the adoptive transfer into the patient, because the cells need to survive, expand, and accomplish effector functions *in vivo* for a successful therapy. Therefore the expression of distinct cell surface markers on the T-cell populations was investigated by flow cytometry analysis on d14 of culture. CD45RA, CD45RO, CD27, and CD28 were chosen as differentiation markers, CD62L, CCR7, and CXCR4 as markers for T-cell homing, and CD25 (IL-2Rα) and CD127 (IL-7Rα) as markers for the responsiveness to cytokines.

All MLR responder populations of donor 053 stimulated with K562-DR and of donor 073 stimulated with K562-DR and K562-DQ, respectively, were exemplarily tested. The results demonstrated that subsets enriched for naive CD4 T cells retained the expression of early differentiation markers in a higher proportion of MLR responders compared to their memory counterparts. In addition and as expected, if the T-cell subsets were sorted for high expression of one of the differentiation markers this molecule was stronger expressed on the resulting T-cell subsets compared to populations sorted for other differentiation markers. Figure 13 shows results for CD4 T-cell populations of donor 073, which were sorted for the expression and absence of CD45RA. While the naive-enriched T-cell fraction showed a central memory phenotype with the expression of CD62L as well as CCR7, the memory-enriched T cells carried less CD62L and very low amounts of CCR7 on their cell surface and had already an effector memory phenotype.

Furthermore the naive- and memory-enriched CD4 T-cell populations of all six donors were screened for contaminations with NK cells. The different T-cell subsets were co-stained with CD3, CD16 and CD56 and viable lymphocytes were examined for CD3^{neg}CD16^{pos}CD56^{pos} NK cells (data not shown). Flow cytometric analysis was not performed, if PBMC numbers were limited. Only minor amounts of NK cells could be detected in the different CD4 T-cell fractions. The median percentage of NK-cells was 0.8% (range, 0.0-46.0%) for 75 tested subsets.

The outliers were mostly due to the different sorting strategy for PBMC of donor 053, which were only stained and sorted for CD4^{pos} and the respective differentiation marker$^{pos/neg}$ T-cell populations,

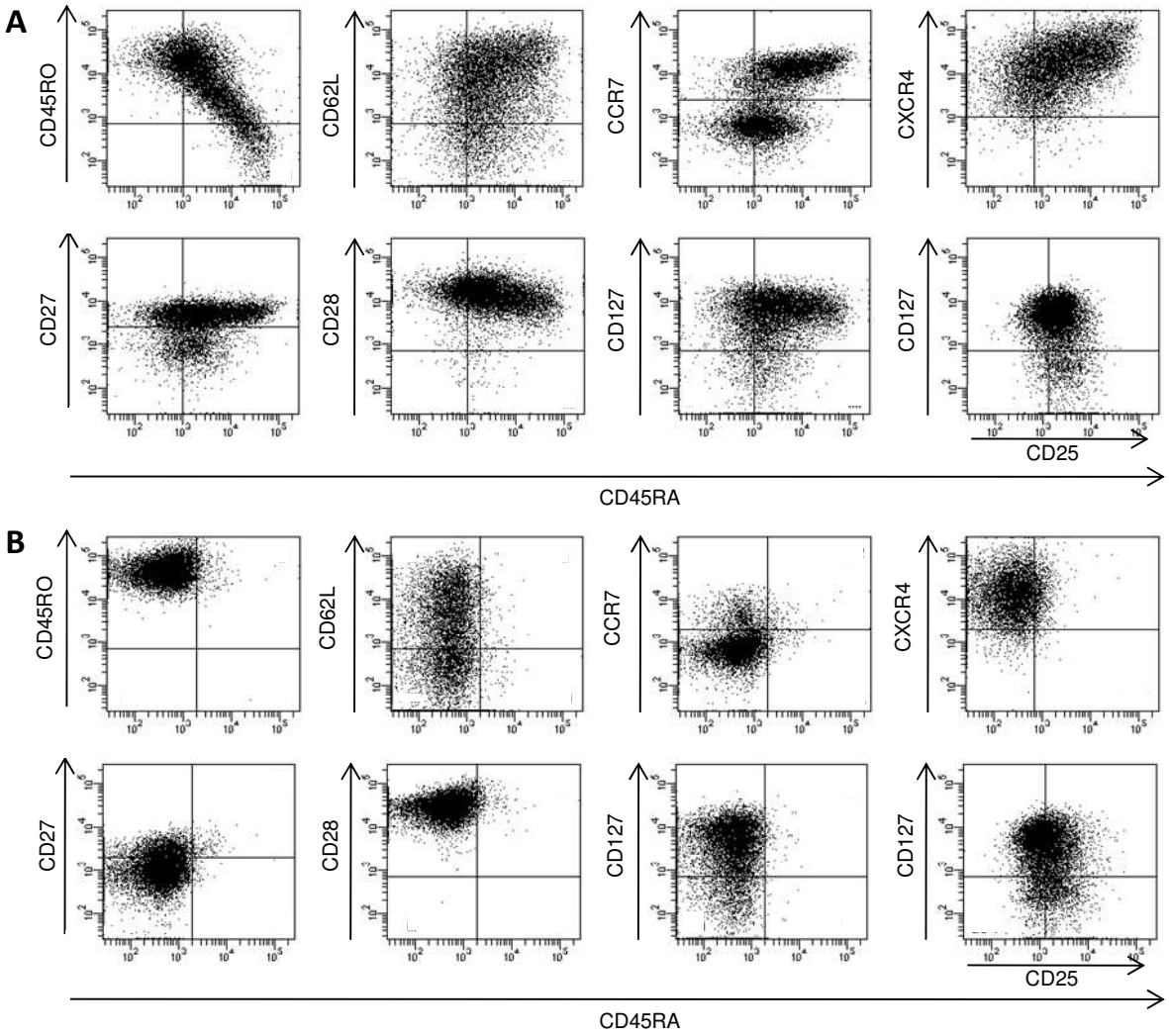


Figure 13. Comparison of the maturation state of naive- and memory-enriched CD4 T-cell subsets by flow cytometry. CD4 T-cell populations sorted for the expression **(A)** or absence **(B)** of CD45RA were stained at d14 of culture with mAb for CD4 and CD45RA along with mAb for T-cell differentiation (CD45RO, CD27, CD28), homing (CD62L, CCR7, CXCR4) as well as for markers indicating the responsiveness to cytokines (CD25, CD127). CD4 T cells of the lymphocyte population were analyzed for co-expression of CD45RA and a third T-cell marker. If CD25 is stained CD4 T cells were analyzed for co-expression of CD127 and CD25. Shown are representative results with CD4 T-cell subsets of Don 073 stimulated against K562-DQ.

but not additionally for $CD3^{pos}$ T-cell subsets. Since the observed results for allorecognition and alloproliferation of outlier populations (chapter 5.3.3) were in accordance with the results for populations without NK-cells, the NK-cell contaminations were neglected.

Additionally, we explored the distribution of naturally occurring T_{reg} to memory *versus* naive CD4 T-cell subsets (Figure 14). The cell surface marker expression $CD4^{pos}CD25^{highpos}CD127^{low}$ (Liu*, et al* 2006, Sakaguchi 2002) and the intracellular expression of the transcription factor $FOXP3^{pos}$ (Fontenot*, et al* 2003) was choosen for T_{reg} identification. Exemplarily, the $CD45RA^{pos}$ and $CD45RA^{neg}$ fractions of $CD3^{pos}CD4^{pos}$ lymphocytes of five different donors were analyzed for their expression of CD25, FOXP3, and the absence of CD127. The median percentage of T_{reg} was 3.4% (range, 2.8-3.9%) in total CD4 T cells. Of entire T_{reg}, a median of 33.5% (range, 19-41%) was found in $CD45RA^{pos}$ T cells and a median of 66.5% (range, 59-81%) in $CD45RA^{neg}$ T cells.

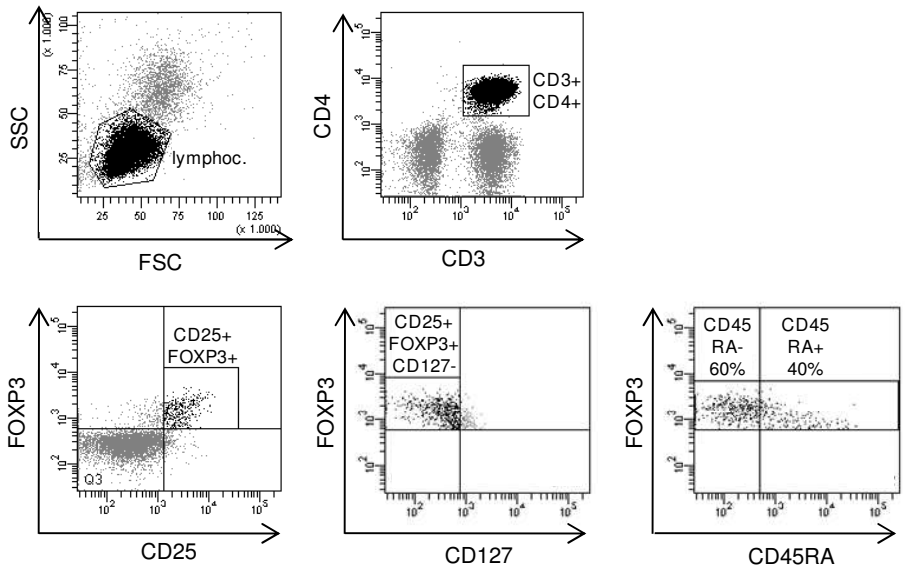


Figure 14. Flow cytometric staining for T_{reg} in memory versus naive CD4 T-cell subsets. PBMC of healthy donors were stained with mAbs for CD3 and CD4 along with mAbs for T_{reg} identification markers (i.e. CD25, CD127, FOXP3) and the T-cell differentiation marker CD45RA. CD3 CD4 T cells of the lymphocyte population were gated for co-expression of CD25 and FOXP3 as well as the absence of CD127 co-expression in order to identify T_{reg}. Given number is percentage T_{reg} in total CD4 T cells. In the next step entire T_{reg} were gated for the expression of CD45RA. Percentages T_{reg} with memory $CD45RA^{neg}$ and naive $CD45RA^{pos}$ phenotype are indicated. Shown are representative results with PBMC of donor 767 out of five donors tested (additionally analyzed were PBMC of donors 517, 850, 851, and 854).

5.4 *In vitro* generation & characterization of allo-HLA-DQ/-DP specific CD4 T cells

5.4.1 Generation of allo-HLA-DQ/-DP specific CD4 T cells

In the second part of this work a protocol for the generation of allo-HLA-DQ/-DP specific CD4 T cells was developed as it is displayed in Figure 15. It is supposed to be applied in a clinical setting in which patient and donor are matched for all HLA alleles except for one single HLA-DQ or permissive HLA-DP allele. T cells with specificity to this mismatch allele would recognize the hematopoietic and therefore leukemia cells of the patient while sparing non-hematopoietic recipient tissues, at least under non-inflammatory conditions.

First IVT mRNA of the chosen HLA-DQ/-DP mismatch allele of the patient had to be synthesized. During this project we worked with IVT mRNA of the HLA-DQB1*06:02 and HLA-DPB1*02:01 alleles in order to proof the principle. In the next step PBMC of healthy donor origin were used for the generation of mDC via a “FAST-DC” protocol. mDC were transfected with the IVT mRNA of the respective allogeneic HLA-DQ or HLA-DP allele by electroporation. Resulting transfectant allo-DQ/-DP mDC could be used as a stock of APC expressing a single HLA mismatch allele. Moreover, mDC could be replaced by PBMC, PHA blasts, or B-cells and were used as allo-DQ/-DP APC stocks.

In a MLR the allo-HLA-DQ/-DP mDC served as stimulator cells for T-cell populations of the same donor. In this way it was possible to generate allo-HLA-DQ/-DP specific CD4 T cells, which were

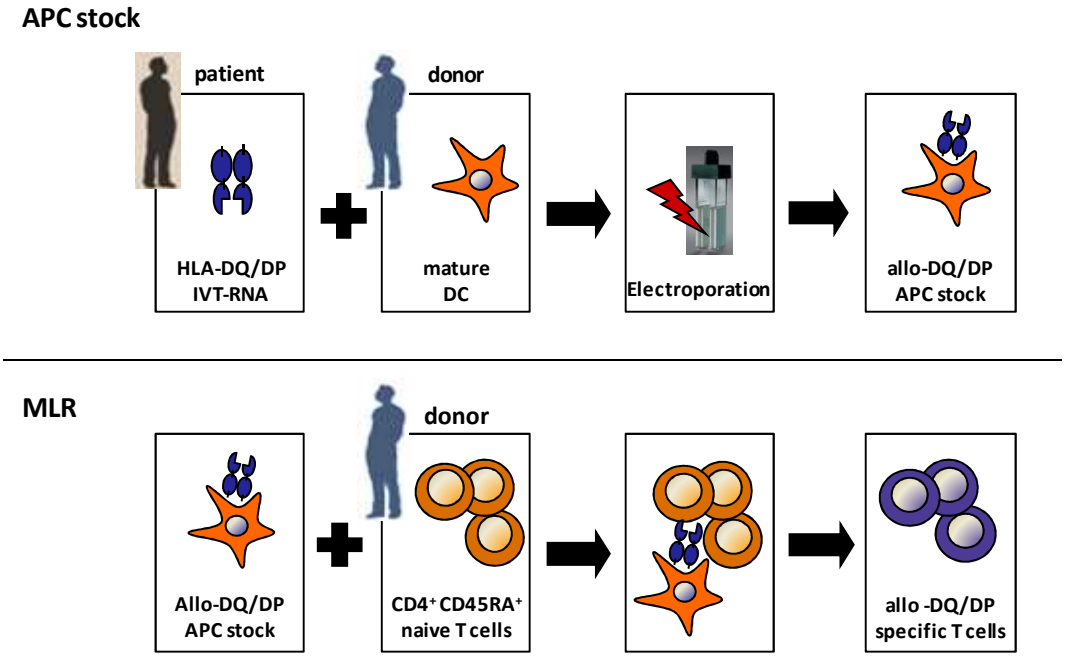


Figure 15. Schematic summary of the generation of allo-HLA-DQ/-DP specific CD4 T cells. Upper panel, production of APC stocks: IVT mRNA of distinct HLA-DQ/-DP alleles mismatched between donor and patient was synthesized. mDC of healthy donor origin were transfected with the IVT mRNA of the respective allogeneic HLA-DQ/-DP allele by electroporation. Resulting APC stocks expressed a single HLA mismatch allele. Lower panel, MLR: Donor derived $CD4^{pos}CD45RA^{pos}$ T-cell populations were stimulated *in vitro* in MLR against the single allo-HLA-DQ/-DP molecules on autologous mDC. Rapidly expanding allo-HLA-DQ/-DP specific CD4 T cells were obtained and further analyzed.

reactive against the mismatch allele of the patient, without the necessity of patient cells. In order to work with a T-cell population with a strong alloreactive potential the naive $CD4^{pos}CD45RA^{pos}$ T-cell subset was chosen because in the first part of this study the superior alloreactive potential of this T-cell compartment was demonstrated (chapter 5.3.3).

The $CD4^{pos}CD45RA^{pos}$ T cells were isolated by flow cytometric cell sorting and were weekly stimulated *in vitro* against the single HLA-DQ/-DP mismatch molecules expressed by transfected autologous mDC. The cytokines IL-7, IL-12 and IL-15 were added to the culture, from day 7 on IL-12 was replaced by IL-2. Afterwards the HLA-DQ/-DP specific CD4 T cells were analyzed for reactivity, specificity, HLA-restriction and effector function in IFN-γ ELISpot and ^{51}chromium-release cytotoxicity assays.

5.4.2 Specificity and HLA-restriction of allo-HLA-DQ/-DP specific CD4 T cells

Seven donor / allo-DQ/-DP mDC combinations (HLA-DQ = 4, HLA-DP = 3, Table 2) were stimulated with the protocol described above. Rapidly expanding MLR responders (median cell number of cultures initiated on day zero with $1x10^6$ cells after 14 days: $46.1x10^7$ cells; range, 6.1-190.7 $x10^7$ cells; data not shown) were investigated for allorecognition by IFN-γ ELISpot assays five days after the second restimulation at day 19 of culture. Figure 16 shows the HLA-restriction and specificity of the MLR / mDC-HLA approach exemplarily for the HLA-DQ specific T cells of donor 302 and the

Table 2. HLA types of healthy donors and transfectant mDC used for *in vitro* stimulation.

			HLA class II-mismatched transfectant dendritic cells	
	HLA-DQB1*	**HLA-DPB1***	**DQB1*06:02**	**DPB1*02:01**
Don 905	03:03 / 05:01	03:01 / 04:01	n.d.	×
Don 914	02:02 / 03:01	04:01	n.d.	×
Don 927	06:03 / 06:04 or 06:21 / 06:32 or 06:34	03:01 / 04:01	n.d.	×
Don 302	02:01 / 03:01	n. d.	×	n.d.
Don 409	03:01 / 05:01	04:01	×	n.d.
Don 492	02:02	02:01 / 17:01	×	n.d.
Don 569	03:01 or 03:19 / 04:02	n.d.	×	n.d.

Don, donor; n.d., not determined

HLA-DP specific T cells of donor 914. Significant alloresponses were only observed if mDC-HLA transfectant cells, which were utilized for the T-cell priming, were applied as target cells. If untransfected autologous mDC served as stimulators no significant IFN-γ spot production of the HLA-DQ/-DP specific CD4 T cells was detected. In all experiments, allo-HLA class II reactivity could be blocked by mAb binding to the class II molecule used for primary *in vitro* stimulation, whereby monoclonal antibodies binding to third party HLA-alleles did not lead to spot reduction.

At the beginning of the study two donor / allo-DQ/-DP combinations (HLA-DQ for donor 409, HLA-DP for donor 905) were additionally stimulated in MLR with PBMC-HLA and PHA blasts-HLA, respectively. The resulting HLA-DQ/-DP specific CD4 T cells showed the same specificity and HLA-restriction as it was observed for MLR performed with mDC-HLA. However, the overall reactivity of the T cells was much lower than for MLR stimulated with allo-DQ/-DP mDC (data not shown). For that reason the following MLR were only performed with allo-DQ/-DP mature monocyte-derived dendritic cells.

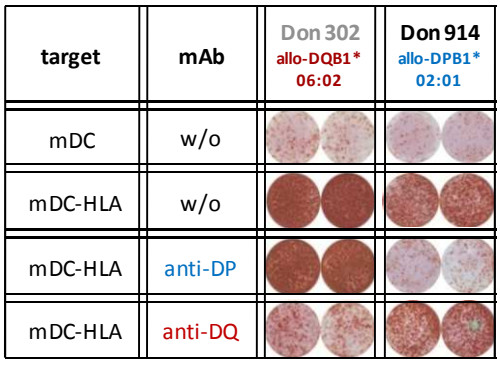


Figure 16. Specificity and HLA-restriction of HLA-DQ/-DP specific CD4 T cells. Shown are representative results for CD4 T cells of donor 302 specific to allo-HLA-DQB1*06:02 and donor 914 specific to allo-HLA-DPB1*02:01. MLR populations ($2x10^4$/well) were analyzed in IFN-γ ELISpot assays on d19 (i.e. 5 days after the second allo-HLA restimulation on d14) for reactivity to original allogeneic mDC-HLA stimulator cells, as well as to untransfected autologous mDC ($5x10^4$/well). HLA-restriction was investigated by use of mAb blocking the TCR-HLA-DQ or -DP interaction, respectively. w/o, without; anti-DP, B7/21 HLA-DP blocking antibody; anti-DQ, SPV-L3 HLA-DQ blocking antibody.

5.4.3 Reactivity of allo-HLA-DQ/-DP specific CD4 T cells against AML blasts

5.4.3.1 Allo-HLA-DQ/-DP specific CD4 T cells recognize AML blasts

In the following part of the project the capability of the HLA-DQ/-DP specific T-cells to recognize primary leukemic cells was examined. Supplementary Table 1 shows the results of all six donor / allo-DQ/-DP mDC combinations (HLA-DQ = 3, HLA-DP = 3), which were analyzed for recognition of primary acute myeloid leukemia (AML) blasts at day 19 or 33 of culture (five days after the second or fourth restimulation). In total a panel of 24 leukemia samples was screened, which were pre-incubated overnight in cytokine-free medium (chapter 4.2.1.1.4) prior to use. Figure 17 shows exemplarily the original IFN-γ ELISpot data for the HLA-DQB1*06:02 specific T cells of donor 302 and the HLA-DPB1*02:01 specific T cells of donor 927 analyzed against AML blasts of nine patients.

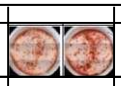
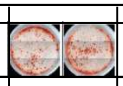
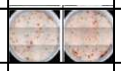
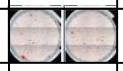
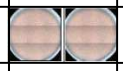
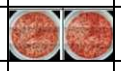
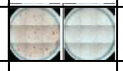
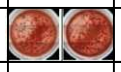
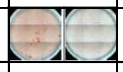
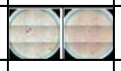
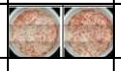
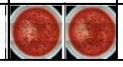
AML (n=24)	DQB1*	Don 302 allo-DQB1* 06:02	Don 927 allo-DPB1* 02:01	DPB1*
237	03:01 / 06:02			02:01 / 04:01
308	05:03 / 06:03			02:01 / 05:01
458	03:02 / 04:02			03:01 / 04:01
653	02:02 / 06:02			04:01 / 04:02
683	03:01 / 03:02			04:01 / 13:01
728	06:02			04:01
849	03:01			02:01 / 04:01
874	03:02 / 06:02			03:01 / 04:01
888	02:02 / 06:02			02:01 / 14:01

Figure 17. Recognition of primary AML blasts by allo-HLA-DQ/-DP specific CD4 T cells. Shown are results for CD4 T cells of donor 302 specific to allo-HLA-DQB1*06:02 and donor 927 specific to allo-HLA-DPB1*02:01. MLR populations ($2x10^4$/well) were analyzed in a 18h IFN-γ ELISpot assay on d19 (i.e. 5 days after the second allo-HLA restimulation on d14) for reactivity against AML blasts ($5x10^4$/well). Original IFN-γ ELISpot data are displayed for nine representative AML samples. HLA-DQ and HLA-DP typings of the respective AML patients are indicated. AML blasts were pre-incubated overnight in cytokine-free medium. For further data see Supplementary Table 1.

Significant alloresponses were only observed if the AML blasts carried the HLA-DQ/-DP allele that was used for the initial T-cell stimulation. For example MZ888-AML was HLA-DQB1*06:02 and HLA-DPB1*02:01 positive and was strongly recognized by the HLA-DQ and HLA-DP specific T cells. In contrast MZ458-AML and MZ683-AML were negative for the HLA molecules used for the T-cell priming and did not mediate significant IFN-γ spot production. The only exception was found for

MZ308-AML, which carried the distinct HLA-DPB1*02:01 allele but was not recognized by all three HLA-DP reactive T-cell populations. In a few cases T-cell populations showed donor-specific minor background reactivity to leukemic cells that did not express the HLA allele used for the T-cell priming (e.g. HLA-DP specific T cells of donor 927 to MZ114-AML; Supplementary Table 1).

The reactivity of the HLA-DQ specific T cells was also examined against leukemia cells expressing mismatch suballeles (Supplementary Table 1). Four AML blasts carried the HLA-DQB1*06:03 allele, which did not lead to significant spot production for MZ131-AML and MZ911-AML. In one case the suballele was recognized slightly (MZ308-AML) while for MZ903-AML strong IFN-γ spot production was detected. The DQB1*06:04 mismatch suballele of MZ632-AML resulted in low but significant recognition by the HLA-DQ specific T cells.

5.4.3.2 Allo-HLA-DQ/-DP specific CD4 T cells mediate cytotoxicity towards AML blasts

In order to determine if the allo-HLA specific CD4 T cells do not only respond to AML blasts by cytokine production (chapter 5.4.3.1) but would also be able to kill the leukemic cells their cytolytic activity was investigated in ^{51}chromium-release assays. The cytotoxicity assays were performed at d19 or d26 of culture five days after the second or third T-cell restimulation. We applied the AML blasts either after pre-incubation with medium containing IFN-γ for four days or after incubation in medium without addition of an inflammatory cytokine. Using this approach, it was possible to study if allo-HLA-DQ/-DP reactive T cells mediate cell lysis of AML blasts under inflammatory and non-inflammatory conditions.

Figure 18 A shows the results for MZ653-AML, which was positive for the HLA-DQB1*06:02 allele. HLA-DQ reactive CD4 T cells of three donors which had been stimulated with the respective HLA-DQB1*06:02 mismatch allele served as effector cells in a 5h ^{51}chromium-release assay. The leukemia

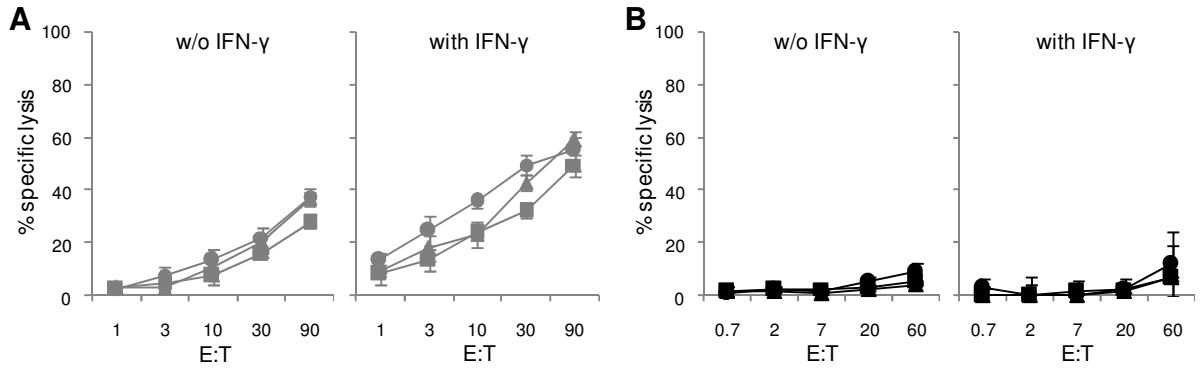


Figure 18. Cytolytic activity of HLA-DQ/-DP mismatch specific CD4 T cells against MZ653-AML blasts. HLA-DQB1*06:02 specific CD4^{pos} T-cell populations of Don 302 (▲), Don 492 (●) and Don 569 (■) **(A)** as well as HLA-DPB1*02:01 specific CD4^{pos} T cells of Don 905 (▲), Don 914 (●), and Don 927 (■) **(B)** were analyzed for cytolytic activity in ^{51}chromium release assays on d19 of culture (5 days after the second restimulation) against MZ653-AML blasts, which were positive for the HLA-DQB1*06:02 but negative for the HLA-DPB1*02:01 alleles. Cytotoxicity was analyzed with or without pre-incubation of the AML blasts for four days with IFN-γ. E:T, effector-to-target ratio; w/o without.

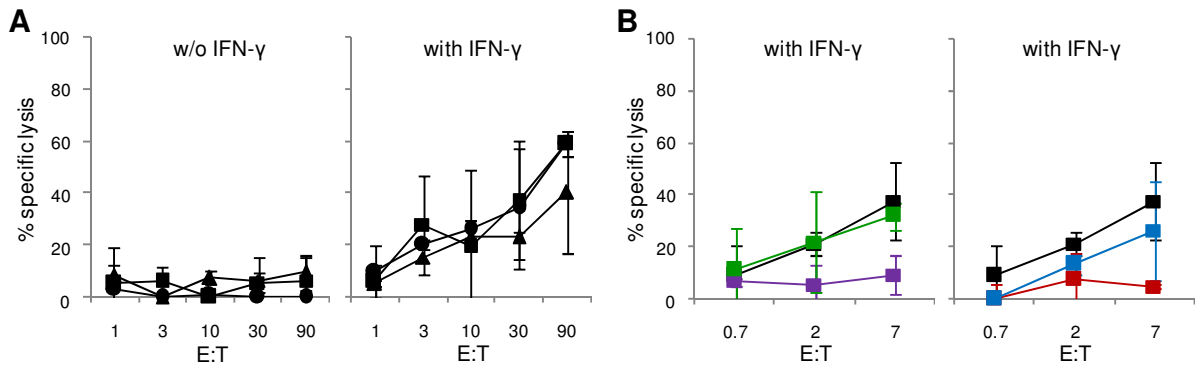


Figure 19. Specificity of the cytolytic activity of HLA-DP mismatch reactive CD4 T cells against MZ237-AML blasts. (A) HLA-DPB1*02:01 specific $CD4^{pos}$ T cells of Don 905 (▲), Don 914 (●), and Don 927 (■) were analyzed for cytolytic activity in ^{51}chromium release assays on d19 of culture (5 days after the second restimulation) against MZ237-AML blasts, which expressed the HLA-DPB1*02:01 allele. Cytotoxicity was observed with or without pre-incubation of the AML blasts for four days with IFN-γ. **(B)** Blocking of cytolysis mediated by HLA-DP specific CD4 T cells of Don 927 (■) on d26 of culture (5 days after the third restimulation) against IFN-γ pre-incubated MZ237-AML blasts using mAb binding to CD4 (■), HLA-DP (■), and HLA-DQ (■). Isotype-matched IgG mAb (■) were used as control. E:T, effector-to-target ratio; w/o, without.

cells were lysed by the T cells at low levels if the AML was not pre-incubated with IFN-γ. Cell lysis increased to intermediate levels if the target cells were pre-incubated with IFN-γ. MZ237-AML blasts, which naturally expressed the HLA-DPB1*02:01 allele, were killed at intermediate levels by HLA-DP specific CD4 T cells primed for HLA-DPB1*02:01 only after pre-incubation with IFN-γ (Figure 19 A). Without cytokine pre-treatment the leukemic cells were not lysed, although the HLA-DP expression on the AML blasts was verified under inflammatory and non-inflammatory conditions by flow cytometry (data not shown). Overall, two out of five AML blasts analyzed, that carried the HLA-alleles used for T-cell priming, were cytolysed without previous cytokine incubation. Cytolytic activity of allo-HLA class II T cells could be blocked by mAb binding to CD4 or to the HLA class II molecule used for primary *in vitro* stimulation, whereby mAb specific to third-party HLA class II alleles or IgG isotypic controls did not lead to efficient reduction in cell lysis (Figure 19 B). Figure 18 B shows the results for MZ653-AML as stimulator cells which were negative for the HLA-DPB1*02:01 allele. The AML blasts could not be lysed by the HLA-DP reactive T cells of the three donors independent of pre-treatment with IFN-γ. This was observed for all tested AML blasts that did not carry the respective HLA-allele (n=3). Altogether the results demonstrated the strong specificity and HLA-restriction of the cytotoxicity of HLA-DQ/-DP reactive CD4 T cells.

5.4.3.2.1 Mechanism of cell lysis

The mechanism of cytotoxicity of the alloantigen-specific CD4 T cells was evaluated by intracellular flow cytometry analysis. The expression of the pore-forming protein perforin and the serine proteases granzyme A and B, which belong to the main contents in granules that induce granule-mediated cell lysis (Fan and Zhang 2005), was determined.

Figure 20 displays the results of four donor / allo-DQ/-DP combinations (HLA-DQ = 2, HLA-DP = 2) analyzed at day 26 of culture five days after the third restimulation. The HLA-DQ/-DP specific CD4 T-cell populations were double positive for granzyme A (rMFI = 23.2; range, 14.8-25.3) and granzyme B (rMFI = 74.8; range, 25.4-197.4) and expressed low amounts of perforin (rMFI = 1.3; range, 1.1-1.5). In comparison the CD8 cytotoxic T-lymphocyte cell line 2E8 (MZ529AML/Don.730) expressed similar amounts of granzyme A (rMFI = 24.8) and granzyme B (rMFI = 113.7), while the perforin expression (rMFI = 9.2) was much stronger.

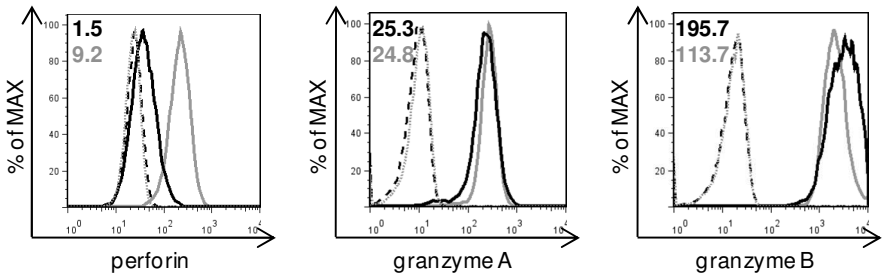


Figure 20. Intracellular flow cytometric staining of allo-HLA-DQ/-DP specific CD4 T-cells for cell lysis mediating granule contents. Allo-HLA-DQ/-DP specific CD4 T-cell populations of four donors (HLA-DPB1 specific: Don 914, Don 927; HLA-DQB1 specific: Don 302, Don 569) as well as the CD8 cytotoxic T-lymphocyte (CTL) line 2E8 (MZ529-AML/Don.730) as positive control were stained on d26 of culture (5 days after the third restimulation) with mAb for CD3 and CD4 or CD8, respectively. Additionally, the cells were stained intracellularly for granule contents using mAb for granzyme A and B as well as perforin. CD3 CD4 T cells of the lymphocyte population were analyzed for granule protein co-expression. Depicted are representative results for Don 927. Shown are stainings of CD4 T cells (black lines) and control CD8 CTL (grey lines). Isotype-matched IgG mAb were included (grey dotted lines for CD4 T cells, black dashed lines for control CD8 CTL). Given numbers are relative medians of fluorescence intensities (rMFI) calculated by division of the median fluorescence intensity (MFI) of the antigen staining by the MFI of the isotypic control staining.

5.4.3.3 Characterization of the HLA class II expression pattern on hematopoietic cells

We analyzed the expression of the three respective HLA class II molecules and as a control of total HLA class I molecules on hematopoietic cells under inflammatory and non-inflammatory conditions by flow cytometry. The expression pattern of the different HLA class II molecules on diverse cell types (see also chapter 5.4.4.3) was of importance for this study because we were interested, which cell types would be recognized by the newly generated HLA-DQ/-DP specific CD4 T cells under different inflammatory conditions.

As hematopoietic cells primary AML blasts and B-LCL were investigated. The cells were cultured for four days either with or without the addition of inflammatory cytokines. As cytokines in one experimental arm IFN-γ was used for pre-incubation, in the other experimental arm a cytokine cocktail containing the GvHD-relevant proinflammatory cytokines TNF-α, IL-1β, and IL-6 was utilized to simulate inflammatory conditions.

AML blasts. The HLA class I and II expression pattern was investigated for primary AML blasts of ten different patients exemplarily shown for MZ667-AML in Figure 21 A. Untreated AML blasts expressed all three HLA class II molecules. The strongest expression was detected for HLA-DP with a rMFI of

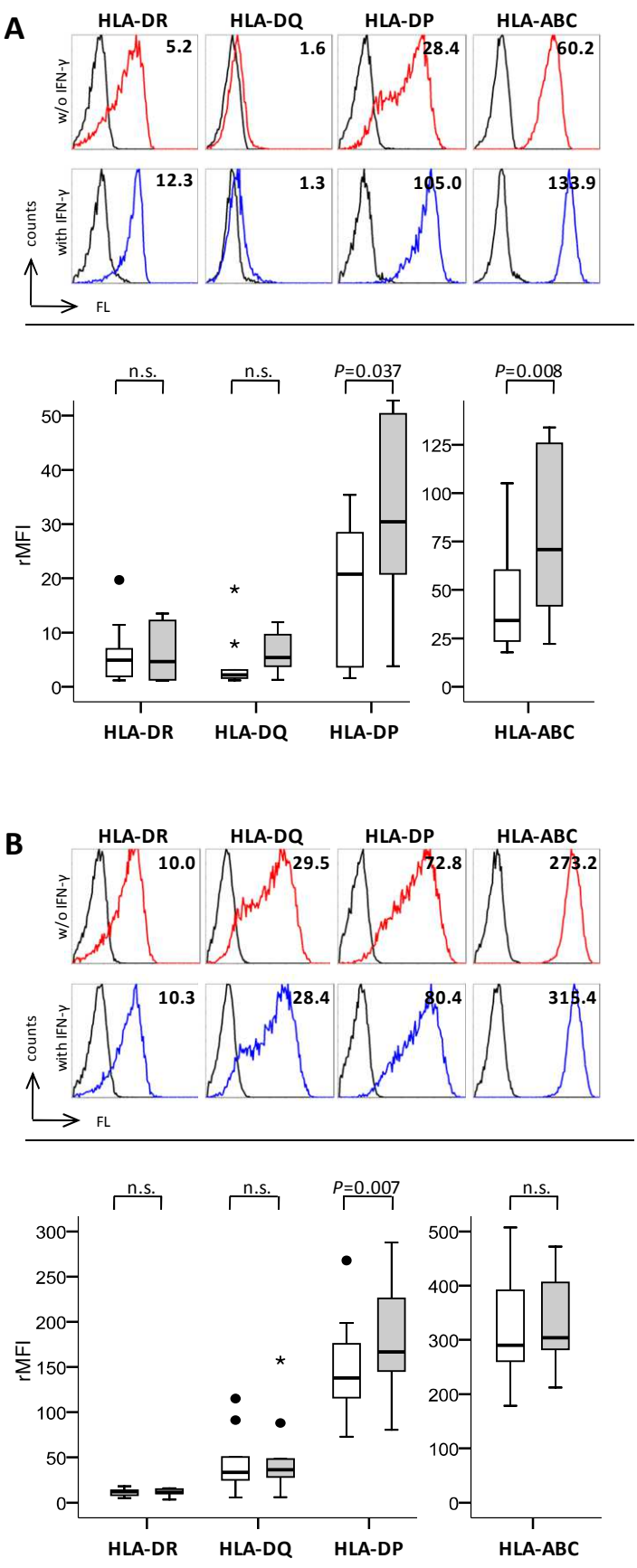


Figure 21. HLA expression pattern on hematopoietic cells. (A) Analysis of HLA molecule expression on AML blasts. Upper panel: Representative example of HLA surface expression on untreated MZ667-AML. Shown are stainings with mAbs binding to HLA-DR, HLA-DQ, HLA-DP, and HLA-A/B/C on untreated AML blasts (red lines) or binding to AML blasts pre-incubated for four days with IFN-γ (blue lines). Isotype matched IgG mAbs served as controls (black lines). Given numbers are relative medians of the fluorescence intensities (rMFI) calculated by division of the median fluorescence intensity (MFI) of the antigen staining by the MFI of the isotypic control staining. Lower panel: Statistical analysis of HLA expression on AML blasts of ten patients (MZ109-, MZ114-, MZ237-, MZ251-, MZ308-, MZ506-, MZ561-, MZ653-, MZ667-, MZ888-AML). Data are shown for untreated AML blasts (white boxes) and AML blasts, which were pre-incubated with IFN-γ for four days (grey boxes). Box plots with median (line), 25th to 75th percentile (box), and minimum and maximum values (error bars) are indicated. Outliers are indicated by dots and stars. The Wilcoxon signed-rank test was used to calculate *P*-values. **(B)** Upper Panel: Representative analysis of HLA molecule expression on B-LCL of donor MZ3603-NKC. Lower panel: Statistical analysis of HLA marker expression on B-LCL of ten donors (MZ114-, MZ237-, MZ251-, MZ308-, MZ667-, MZ683-, MZ888-AML and MZ3114-, MZ3212-, MZ3603-NKC). Data are presented as described in **(A)**. FL, fluorescence; n.s., not significant; w/o, without.

20.8 (range, 1.6 - 35.6) followed by HLA-DR (rMFI = 4.9; range, 1.2 - 19.7) and HLA-DQ (rMFI = 2.2; range, 1.2 - 18.0). After pre-treatment with IFN-γ the expression of HLA-DP was upregulated 1.5-fold with statistical significance (rMFI = 30.4; range, 3.8 - 105.0; *P* = 0.037). The expression levels for HLA-DR and HLA-DQ did not change significantly. HLA-class I molecules were expressed at high levels on untreated AML blasts (rMFI = 34.2; range, 17.8 - 105.0). The level of expression did also increase 2.1-fold after IFN-γ treatment significantly (rMFI = 70.8; range, 22.1 - 409.4). If the leukemia cells were incubated with the cytokine cocktail the expression pattern did only increase marginally (data not shown).

B-LCL. Similar results were observed for B-LCL of ten different donors (Figure 21 B). Overall the rMFI for B-LCL were much higher than the rMFI that were obtained for AML blasts. All three HLA class II molecules were detected on the cell surface in high amounts. Very strong expression was found for HLA-DP with rMFI of 137.9 (range, 72.8 - 267.8) followed by HLA-DQ (rMFI = 33.4; range, 5.5 - 115.0) and HLA-DR (rMFI = 11.8; range, 5.0 - 18.0). IFN-γ pre-incubation significantly increased the amount of HLA-DP molecules on the cell surface 1.2-fold (rMFI = 166.6; range, 80.4 - 287.8; *P* = 0.007) while the expression pattern for the HLA-DQ/-DP proteins did not change at significant levels. The expression of HLA class I molecules was already very strong under non-inflammatory conditions (rMFI = 290.0; range, 178.3 - 507.0) since B lymphocytes are professional APC. The class I expression did not rise with IFN-γ incubation significantly, which might be due to the already existing strong protein level. The addition of the cytokine cocktail to B-LCL cultures did not increase the HLA expression substantially (data not shown).

5.4.4 Reactivity of allo-HLA-DQ/-DP specific CD4 T cells against non-hematopoietic cells

5.4.4.1 Allo-HLA-DQ/-DP specific CD4 T cells do only recognize IFN-γ-pre-treated primary FB & KC

In order to investigate if allo-HLA-DQ/-DP specific CD4 T cells were able to recognize non-hematopoietic tissues a broad panel of primary fibroblasts (FB) and keratinocytes (KC) isolated from juvenile foreskin of healthy donor origin was screened as target cells in IFN-γ ELISpot assays. Analysis were performed at d26 of culture five days after the third T-cell restimulation. The cells were either utilized after pre-incubation with medium containing IFN-γ for four days or after incubation in medium without addition of the inflammatory cytokine. Thereby it was possible to study if allo-HLA-DQ/-DP reactive T cells recognize FB and KC under inflammatory and non-inflammatory conditions.

Fibroblasts. Figure 22 shows representative original IFN-γ ELISpot data obtained for two allo-HLA-DQ (donor 302 and 492) and two allo-HLA-DP (donor 914 and 927) specific T-cell populations, which were tested against fourth to twelfth passage FB cultures of six donors. The identity of fibroblasts

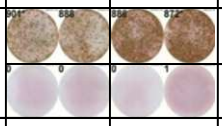
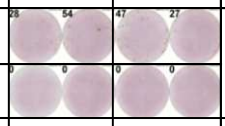
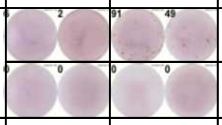
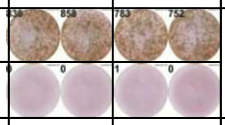
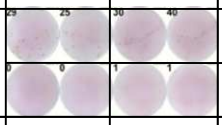
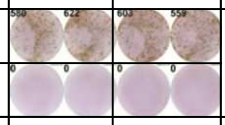
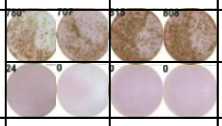
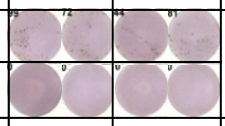
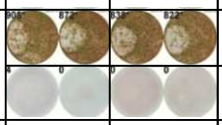
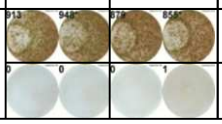
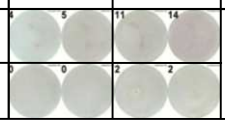
FB (n=21)	DQB1*	allo-DQB1*06:02 Don302	allo-DQB1*06:02 Don492	IFN-γ	allo-DPB1*02:01 Don914	allo-DPB1*02:01 Don927	DPB1*
#09	06:02 / 06:09			+ / Ø			04:01 / 13:01
#13	03:01 / 03:03			+ / Ø			02:01 / 04:01
#15	03:01 / 05:01			+ / Ø			02:01 / 03:01
#19	06:02 / 03:02			+ / Ø			03:01 / 04:01
#46	06:02 / 06:04			+ / Ø			03:01 / 04:01
#47	03:01 / 06:02			+ / Ø			04:01

Figure 22. Recognition of primary fibroblasts by allo-HLA-DQ/-DP specific CD4 T cells. Shown are results for CD4 T cells of donor 302 and 492 specific to allo-HLA-DQB1*06:02 and donor 914 and 927 specific to allo-HLA-DPB1*02:01. MLR populations ($2x10^4$/well) were analyzed in a 18h IFN-γ ELISpot assay on d26 (i.e. 5 days after the third allo-HLA restimulation on d21) for reactivity against FB ($1x10^4$/well). Original IFN-γ ELISpot data are displayed for six representative FB cultures. HLA-DQ and HLA-DP typings of FB cultures are indicated. FB were either pre-incubated over four days with or without the addition of IFN-γ. For further data see Supplementary Table 2.

was verified in flow cytometry by the characteristic fibroblast phenotype D7-Fib^{pos}, CD90^{pos}, HLA-A/B/C^{pos} while contaminations with CD45^{pos}, CD33^{pos}, and CD14^{pos} cells were rare (data not shown). In total a panel of 21 diverse FB cultures was explored (Supplementary Table 2). Under non inflammatory conditions primary FB were not recognized by any of the tested T-cell populations. This observation was independent from the HLA status of the FB meaning that also FB, which expressed the HLA-DQB1*06:02 or HLA-DPB1*02:01 alleles that were used for the T-cell priming, did not stimulate significant spot production. However, the HLA-DQ/-DP specific T cells mediated strong IFN-γ spot production (>580 SFC) if FB cultures, which were pre-treated with IFN-γ and carried the HLA alleles used for the T-cell priming, were utilized as stimulators. This was the case for all five FB cultures (e.g.FB culture #09) that expressed the DQB1*06:02 allele and all seven cultures (e.g. FB culture #13), which carried the DPB1*02:01 allele. In a few cases T-cell populations showed minor reactivity to FB that did not express the HLA allele used for the T-cell priming (e.g. HLA-DP specific T cells of donor 927 to FB culture #19). This spot production could be explained by residual mismatch reactivity since the T-cell populations were in an early stage of culture and probably polyclonal when the analysis was performed.

Keratinocytes. The results obtained for IFN-γ ELISpot analysis performed with primary KC as target cells were similar to that for FB. Representative original IFN-γ ELISpot data of allo-HLA-DQ (donor 492) and allo-HLA-DP (donor 927) specific T-cell populations, which were tested against first passage KC cultures of six donors is displayed in Figure 23. In total a panel of 14 KC cultures was explored for recognition by two HLA-DQ and two HLA-DP mismatch reactive T-cell populations (Supplementary Table 3). Under non-inflammatory conditions no significant spot production was observed against all tested KC cultures independent of their HLA expression status. After pre-incubation with IFN-γ significant reactivity to KC, which expressed the HLA-DQ/-DP alleles used for T-cell priming, could be observed. For example KC culture #58 expressed the DPB1*02:01 but not the DQB1*06:02 allele and was only recognized by HLA-DP reactive T cells. HLA-DQ specific T cells did also recognize the DQB1*06:03 mismatch suballele (e.g. KC culture #68), while the DQB1*06:01 and DQB1*06:04 mismatch suballeles did not lead to significant spot production (e.g. KC culture #61 or #55). The overall reactivity to KC was much lower than to FB (e.g. maximum 288 SFC for donor 927 to KC culture #60 compared to maximum >1000 SFC for donor 492 to FB culture #47). Residual reactivity to irrelevant HLA mismatches was not observed for KC.

These results are in strong accordance with the flow cytometric analysis of the HLA expression pattern on non-hematopoietic cells under inflammatory and non-inflammatory conditions (chapter 5.4.4.3). The analysis illustrated that primary FB and KC do not carry HLA-class II alleles on their cell surface under non-inflammatory condition, but upregulated the expression after IFN-γ pre-

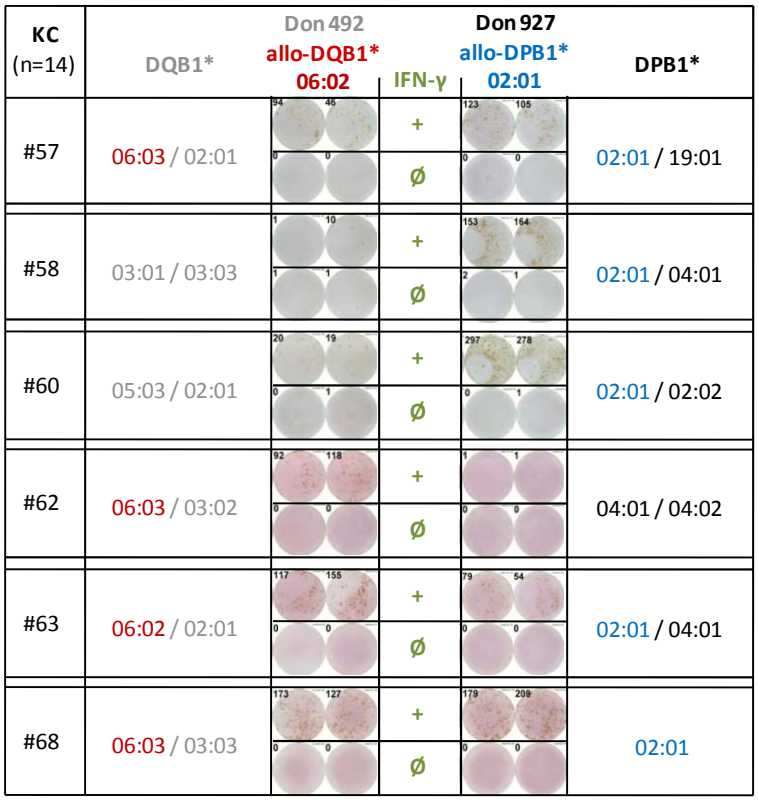


Figure 23. Recognition of primary keratinocytes by allo-HLA-DQ/-DP specific CD4 T cells. Shown are results for CD4 T cells of donor 492 specific to allo-HLA-DQB1*06:02 and donor 927 specific to allo-HLA-DPB1*02:01. MLR populations ($2x10^4$/well) were analyzed in a 18h IFN-γ ELISpot assay on d26 (i.e. 5 days after the third allo-HLA restimulation on d21) for reactivity against KC ($2x10^4$/ well). Original IFN-γ ELISpot data are displayed for six representative KC cultures. HLA-DQ and HLA-DP typings of KC cultures are indicated. KC were either pre-incubated over four days with or without the addition of IFN-γ. For further data see Supplementary Table 3.

treatment. For that reason the lacking reactivity of the HLA-DQ/-DP mismatch reactive T cells to untreated mesenchymal and epithelial cells was most likely caused by missing HLA class II molecules on the cell surface.

5.4.4.2 Cytolysis of fibroblasts requires IFN-γ pre-treatment

In the following experiments we analyzed if the allo-HLA specific CD4 T cells would be able to mediate cell lysis of FB in ^{51}chromium-release assays. The FB were either pre-incubated with IFN-γ or without addition of the cytokine as it has been described for the ELISpot assays in the previous section. The cytotoxicity assays were performed at d26 of culture five days after the third T-cell restimulation. The same experiments were performed with KC but could not be evaluated. Since primary KC are a very sensitive cell type the cells died during the time course of the experiment.

Figure 24 A shows the results for FB culture #13, which was positive for the HLA-DPB1*02:01 allele but negative for the HLA-DQB1*06:02 allele. HLA-DQ/-DP mismatch reactive CD4 T cells of four donors were used as responder cells in a 5h ^{51}chromium-release assay. CD4 T cells of donors 914 and 927 were primed against the allo-HLA-DPB1*02:01 allele, the other two T-cell populations derived from donor 302 and 569 and were stimulated against the HLA-DQB1*06:02 allele. FB that did not undergo pre-treatment with IFN-γ were not lysed by the allo-HLA reactive T cells of all four donors independently if the T-cells were primed for the HLA-DP allele that is expressed by the FB or not. If the stimulator cells were pre-incubated with the inflammatory cytokine cell lysis was mediated in low to intermediate levels by HLA-DP specific T cells of donor 914 and 927, which were primed against the distinct HLA-DP protein on the FB, but not by the HLA-DQ reactive T cells of the donors 302 and 569, that were primed against the HLA-DQB1*06:02 allele. Additionally, FB that express the

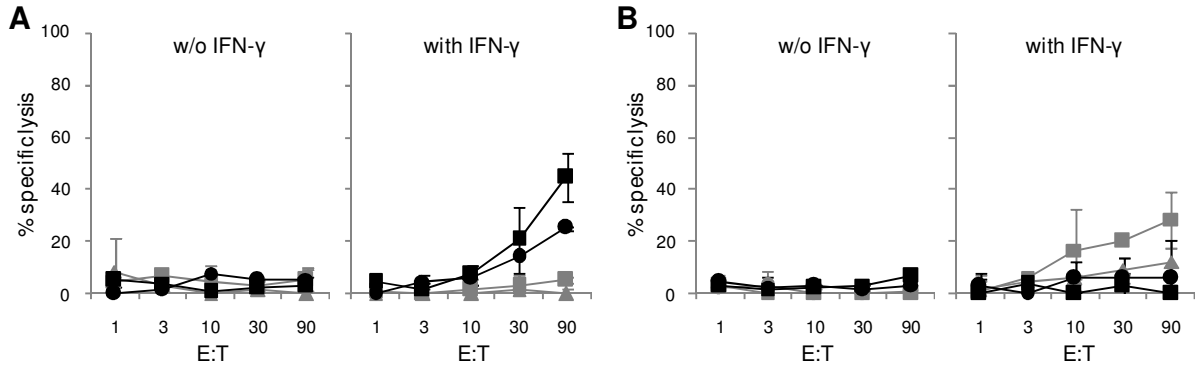


Figure 24. Cytolytic activity of HLA-DQ/-DP mismatch specific CD4 T cells against fibroblasts. HLA-DQB1*06:02 specific CD4^{pos} T-cell populations of Don 302 (▲) and Don 569 (■) as well as HLA-DPB1*02:01 specific CD4^{pos} T cells of Don 914 (●), and Don 927 (■) were analyzed for cytolytic activity in ^{51}chromium release assays on d26 of culture (5 days after the third restimulation) against fibroblast culture #13 (DPB1*02:01^{pos}, DQB1*06:02^{neg}) **(A)** and culture #19 (DQB1*06:02^{pos}, DPB1*02:01^{neg}) **(B)**. Cytotoxicity was analyzed with or without pre-incubation of the fibroblasts for four days with IFN-γ. E:T, effector-to-target ratio; w/o, without.

HLA-DQB1*06:02 allele on their cell surface were analyzed for killing by T cells of the same four donors described above. The results are exemplarily shown for FB culture #19 in Figure 24 B. Cytolytic activity could only be obtained after pre-incubation with IFN-γ and solely for the CD4 T cells primed against the distinct HLA-DQ allele.

Overall the strong HLA-restriction and specificity of the cytolytic activity of DQ-/DP-specific CD4 T cells was demonstrated for IFN-γ pre-treated FB from ten different donors. Experiments with and without IFN-γ incubation were performed with five FB cultures and always showed that IFN-γ pre-treatment is required for the cytolysis of the FB. Hence the observations for cell cytotoxicity against FB were in strong accordance with the results for IFN-γ cytokine secretion (chapter 5.4.4.1).

5.4.4.3 Characterization of the HLA-expression pattern on non-hematopoietic cells

Additionally to hematopoietic cells the expression pattern of the three respective HLA class II molecules and as a control of total HLA class I proteins on non-hematopoietic cells under inflammatory and non-inflammatory conditions was determined by flow cytometry analysis. In this way it was investigated which cell types would be recognized by the newly generated HLA-DQ/-DP specific CD4 T cells under different inflammatory conditions. Primary FB and primary KC served as mesenchymal and epithelial cells, respectively, and were newly generated for the experiments from human foreskin (chapter 4.2.1.1). Primary non-malignant kidney cells (NKC) were also used as representatives of epithelial cells. The different experimental arms and their treatment with inflammatory cytokines were described in chapter 5.4.3.3.

Fibroblasts. All three HLA class II molecules could not be detected or only in very slight amounts on primary FB of ten healthy donors (Figure 25 A) without incubation with inflammatory cytokines. If IFN-γ was given to the cell culture the rMFI of the HLA-DP expression was upregulated 6.6-fold from 1.4 (range, 1.2-1.7) to 9.2 (range, 1.0-2.0) and for HLA-DR 2.1-fold from 1.0 (range, 0.9-1.0) to 2.1 (range, 1.6-3.4) with statistical significance (HLA-DP: P=0.005; HLA-DR: P=0.005) while the HLA-DQ expression on FB did not increase: rMFI without IFN-γ = 1.9 (range, 1.4-3.0); rMFI with IFN-γ = 1.6 (range, 1.2-2.4). HLA class I proteins were already strongly expressed under non-inflammatory conditions (rMFI = 16.2 (range, 8.2-22.3)) and were significantly upregulated 1.9-fold by IFN-γ (rMFI = 30.2 (range, 20.6-83.8; P = 0.005)).

Keratinocytes. The screening of the HLA expression pattern of primary KC (Figure 25 B) of eleven healthy donors gave similar results than the analysis of primary FB. HLA class II proteins were not expressed or only very slightly without cytokine pre-incubation. Under inflammatory conditions HLA-DP expression increased 14.0-fold from a rMFI of 1.7 (range, 1.3-3.0) to 23.8 (range, 9.8-62.3) and HLA-DR expression 12.9-fold from a rMFI of 0.8 (range, 0.6-1.0) to 10.3 (range, 1.5-32.7) with

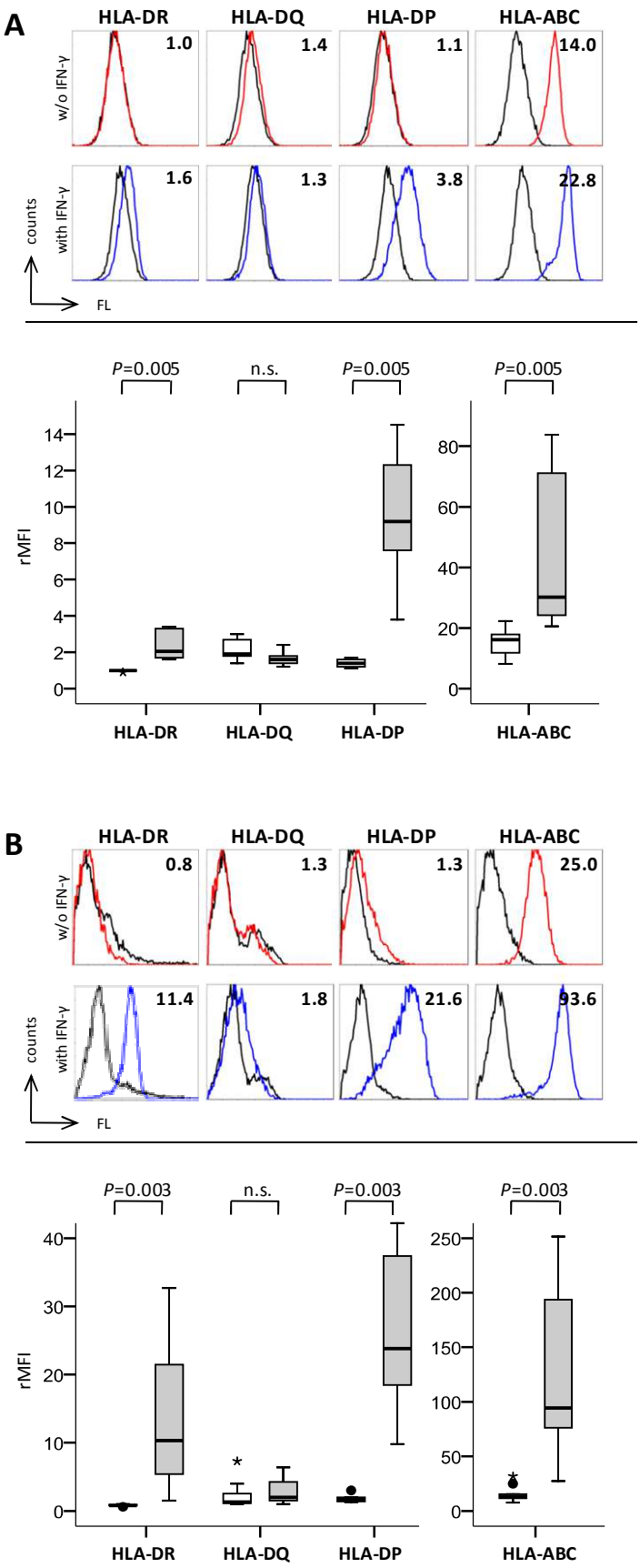
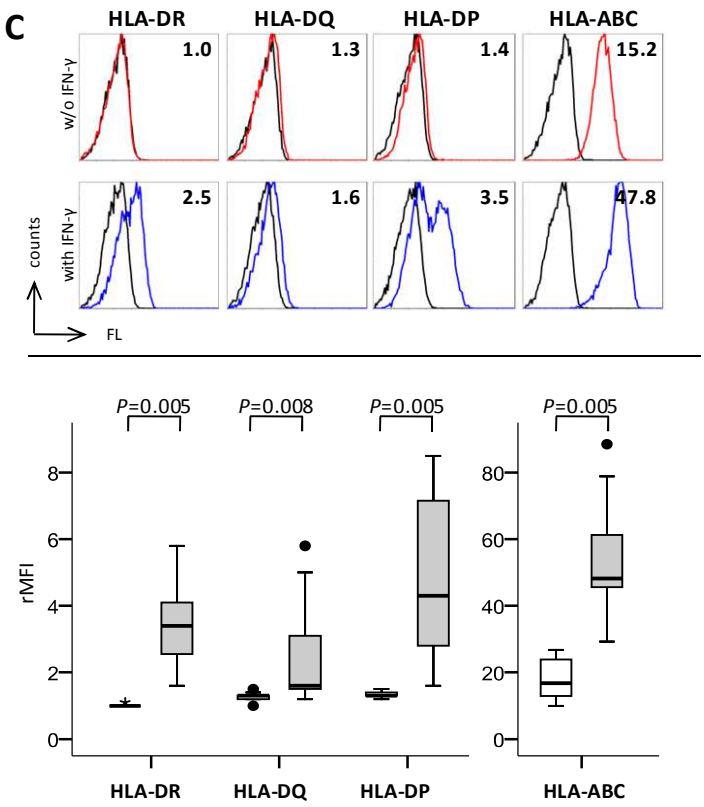


Figure 25. HLA expression pattern on non-hematopoietic cells. Analysis of HLA molecule expression on primary FB **(A)**, KC **(B)**, and NKC **(C)**. **(A)** Upper panel: Representative analysis of HLA surface expression on FB culture #14. FB were either pre-incubated with or without the addition of IFN-γ for four days. Shown are stainings with mAbs binding to HLA-DR, HLA-DQ, HLA-DP, and HLA-A/B/C on untreated FB (red lines) or binding to FB pre-incubated for four days with IFN-γ (blue lines). isotype matched IgG mAbs served as controls (black lines). Given numbers are relative medians of the fluorescence intensities (rMFI) calculated by division of the median fluorescence intensity (MFI) of the antigen staining by the MFI of the isotypic control staining. Lower panel: Statistical analysis of HLA expression on FB cultures of ten donors (FB culture #01, #02, #04, #05, #06, #07, #14, #15, #16, #17). Data are shown for untreated FB cultures (white boxes) and FB cultures, which were pre-incubated with IFN-γ for four days (grey boxes). Box plots with median (line), 25th to 75th percentile (box), and minimum and maximum values (error bars) are indicated. Outliers are indicated by dots and stars. The Wilcoxon signed-rank test was used to calculate *P*-values.
(B) Upper Panel: Representative analysis of HLA molecule expression on KC culture #39. Lower panel: Statistical analysis of HLA marker expression on KC cultures of eleven donors (KC culture #35, #36, #38, #39, #40, #41, #42, #43, #44, #45, #46). Data are presented as described in **(A)**. FL, fluorescence; n.s., not significant; w/o, without.



(C) Upper Panel: Representative analysis of HLA molecule expression on NKC culture #3811. Lower panel: Statistical analysis of HLA marker expression on NKC cultures of eleven donors (NKC culture #3099, #3102, #3114, #3418, #3603, #3639, #3763, #3799, #3811, #3823, #3908). Data are presented as described in **(A)**. FL, fluorescence; w/o, without.

statistical significance (HLA-DP: *P*=0.003; HLA-DR: *P*=0.003) while the HLA-DQ molecules on KC were not upregulated in significant manners: rMFI without IFN-γ = 1.3 (range, 1.0-4.0); rMFI with IFN-γ = 2.0 (range, 1.0-6.4). Surface expression of HLA class I molecules did increase 7.1-fold after IFN-γ pre-treatment with statistical significance (rMFI without IFN-γ = 13.3 (range, 7.5-25.0); rMFI with IFN-γ = 94.3 (range, 27.5-251.5), *P*=0.005).

Non-malignant kidney cells. The observations made for NKC (Figure 25 C) of eleven healthy donors were in accordance with the results for the FB and KC. Under non-inflammatory conditions HLA class II molecules were not or only in very slight amounts detected on the cell surface, while HLA class I proteins were already strongly expressed. The addition of IFN-γ led to a significant increase of all HLA molecules. The upregulation for HLA-DP was 3.3-fold (rMFI without IFN-γ = 1.3 (range, 1.2-1.5); rMFI with IFN-γ = 4.3 (range, 1.6-8.5), *P*=0.005), for HLA-DR 3.4-fold (rMFI without IFN-γ = 1.0 (range, 1.0-1.1); rMFI with IFN-γ = 3.4 (range, 1.6-5.8), *P*=0.005), and for HLA-A/B/C 2.7-fold (rMFI without IFN-γ = 16.8 (range, 10.0-26.8); rMFI with IFN-γ = 48.2 (range, 29.3-88.5), *P*=0.005), while HLA-DQ expression only increased 1.2-fold: rMFI without IFN-γ = 1.3 (range, 1.0-1.5); rMFI with IFN-γ = 1.6 (range, 1.2-5.8), *P*=0.008.

If the non-hematopoietic cells were incubated with the cytokine cocktail the expression pattern did only change marginally for all three non-hematopoietic cell types (data not shown). For KC the

analysis was only performed with IFN-γ additionally to the experimental arm without cytokines but not with the cytokine cocktail due to the low number of first passage KC.

5.4.5 Phenotypic characterization of allo-HLA-DQ/-DP specific CD4 T-cells

In order to investigate if the HLA-DQ/-DP reactive CD4 T cells might be able to survive, expand, and accomplish effector functions *in vivo* after adoptive transfer into the patient, we performed flow cytometric analysis of distinct cell surface markers. CD45RA and CD45RO were chosen as differentiation markers as well as the costimulatory molecules CD27 and CD28. CD62L and CCR7 served as markers for T-cell homing, and CD25 and CD127 as markers for the responsiveness to cytokines.

Figure 26 displays the results for the phenotypic screening of six donor / allo-DQ/-DP combinations (HLA-DQ = 3, HLA-DP = 3) at day 28 of culture exemplarily for donor 927. High expression levels were observed for CD45RO (rMFI = 133.8; range, 25.5-258.5) as well as CD28 (rMFI = 82.0; range, 38.7-87.0), while CD45RA, CD25, and CD127 were expressed on the T-cell surface at intermediate levels (CD45RA: rMFI = 9.8; range, 5.0-16.0; CD25: rMFI = 3.1; range, 2.2-5.3; range, 1.7-3.5; CD127: rMFI = 4.3; range, 1.7-47.0). CD27 as well as CD62L were only expressed at low levels (CD27: rMFI = 2.5; range, 1.7-3.5; CD62L: rMFI = 2.0; range, 1.3-3.0) while CCR7 (rMFI = 1.0; range, 1.0-1.0) could not be found on the cell surface anymore. This phenotype is typical for effector memory T cells. The intermediate expression of the naive T-cell marker CD45RA is comparably high at this time of culture. It might be due to the initial flow cytometric sorting for the T-cell subset with very strong CD45RA expression or might result from significant numbers of late differentiated (end stage) effector cells. In addition, the CD4 T-cell populations were screened for contaminations with NK cells as it is previously described (chapter 5.3.5). All six tested T-cell lines were free of NK-cell contaminations (median percentage of NK cells 0.0 (range, 0.0-0.8) %).

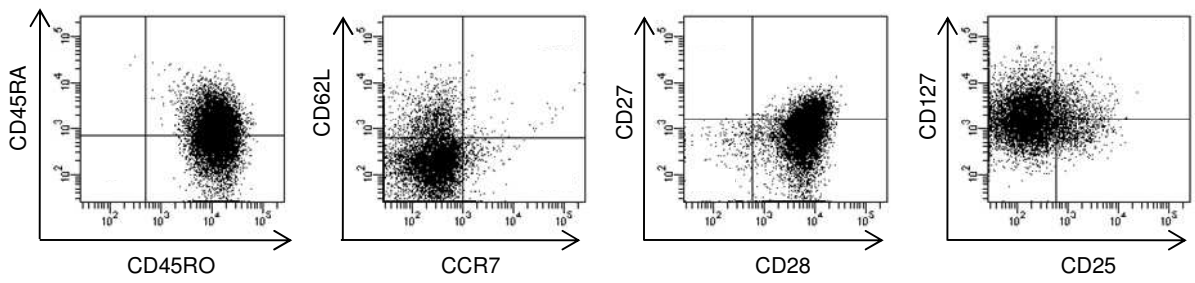


Figure 26. Phenotypic characterization of allo-HLA-DQ/-DP specific CD4 T-cells by flow cytometry. CD4 T-cell populations of 6 donors were stained at d28 of culture with 7AAD and mAb for CD3 and CD4 along with mAb for T-cell differentiation (CD45RA, CD45RO, CD27, CD28), homing (CD62L, CCR7) as well as for markers indicating the responsiveness to cytokines (CD25, CD127). Viable CD3 CD4 T cells of the lymphocyte population were investigated for co-expression of further T-cell marker. Shown are representative dot plot analysis of Don 927 stimulated against mDC-DP.

6 Discussion

The goal of this PhD thesis was the development of two methodical protocols, which might be of potential use in the adoptive immunotherapy of leukemia.

The alloreactive potential of naive- & memory-enriched allo-HLA-DR/-DQ specific T-cell subsets

The first part of this PhD thesis compassed the development of a novel screening protocol for the detection of CD4 T-cell reactivity of donors to single HLA class II alloantigens. It is based on the *in vitro* stimulation of naive- or memory-enriched as well as entire CD4 T-cell subsets of healthy donor origin against single allo-HLA molecules in short-term MLR followed by the determination of allorecognition in a sensitive IFN-γ readout assay and of alloproliferation by cell counting (Figure 7). The data have already been published in "*Haematologica*" in 2011 (Distler, Bloetz, *et al* 2011).

Allostimulation with K562 cells as standard APC. Since HLA molecules are the major targets of alloreactive T cells (Felix and Allen 2007), the HLA class II molecules HLA-DRB1 and HLA-DQB1, which are currently considered for donor selection in allo-HSCT, were chosen as surrogate alloantigens in this study. As stimulator cells for presentation of allo-HLA molecules in MLR, the human cell line K562 was utilized as a fast and unlimited available source of standard artificial APC (Turtle and Riddell 2010). This cell line expresses several molecules that enhance the ability to serve as APC for the efficient activation and expansion of T-cells *in vitro* (Britten*, et al* 2002, Van Tendeloo*, et al* 2001, Wehler*, et al* 2007). In addition, genetically engineered K562 cells are already used in good manufacturing practice (GMP) quality in clinical trials (Butler*, et al* 2007, Smith*, et al* 2010). K562 cells were transfected with "off-the-shelf" HLA-DR or HLA-DQ IVT mRNA by electroporation resulting in transient expression of the HLA molecules (Figure 5). This mRNA electroporation approach is particularly attractive due to its speed, simplicity and high efficiency. Since K562 cells do not express endogenous HLA molecules under non-inflammatory conditions they are advantageous for the detection of alloreactivity solely attributed to an individual HLA mismatch allele and to minimize the interference by other T-cell specificities. By this means, the level of reactivity to allogeneic HLA molecules could be compared between distinct T-cell subsets and different donors, respectively.

MLR cultures were supplemented with cytokines in a sequential protocol in order to provide the T cells with optimal conditions for priming, survival and proliferation. In the first week of culture IL-12 was administered, which plays an important role for priming of naive T cells and promotes T_H1 cell generation (Trinchieri 1995). IL-2, IL-7 and IL-15 are known to promote survival and proliferation of naive as well as memory T cells (Tan*, et al* 2001, Waldmann 2006). Since IL-2 additionally triggers activation and proliferation of T_{reg}, induces apoptosis in activated T cells, and might lead to an unspecific T cell growth it was not applied during the first week of culture (Thornton*, et al* 2004, Zheng*, et al* 1998).

HLA-DR/-DQ specific allorecognition of CD4 T-cell subsets. Allo-HLA class II reactivity of MLR cultures was analyzed in IFN-γ ELISpot assays. The results demonstrated the strong specificity and HLA-restriction of our approach (Figure 8) since significant alloresponses were only observed if K562-HLA-DR/-DQ transfectant cells were applied as stimulator cells which were used for preceding T-cell priming. Furthermore, HLA class II reactivity could be abrogated in blocking studies with monoclonal antibodies against the respective HLA class II molecules used for T-cell stimulation. Most important, allo-HLA-DR/-DQ reactive T cells were mainly found in CD4 T-cell populations enriched for naive T cells compared to their memory counterpart fractions for all differentiation markers tested (Figure 9). Altogether, these results provide the rationale for the selective *in vitro* depletion of naive T-cells from the allograft in order to minimize alloreactive precursors. Stronger alloreactivity in naive T-cell populations might be due to the enormous TCR repertoire of naive T cells, which should contain most alloreactive precursors increasing the ability to recognize allogeneic antigens. In contrast, the memory T-cell pool contains a more limited repertoire of antigens against the host was previously exposed to. It consequently has a diminished TCR variability leading to reduced allorecognition (Chandok and Farber 2004, Nikolich-Zugich*, et al* 2004).

Our data are in accordance with a report by Foster and colleagues, who found stronger *in vitro* alloreactivity in human $CD62L^{pos}$ compared to $CD62L^{neg}$ T-cells (Foster*, et al* 2004). In this previous study T-cell populations isolated by flow cytometric cell sorting were expanded for up to three weeks by unspecific OKT3/IL-2 stimulation. In a following MLR EBV-transformed B-LCL with multiple HLA-mismatches were applied as stimulator cells. The screening protocol developed in this PhD thesis has several advantages towards Foster´s approach. First, the unspecific stimulation step at the beginning of the culture period is not necessary thereby avoiding *in vitro* bias. Second, alloreactivity is detected to single HLA class II mismatch alleles using K562-HLA transfectant cells, which is not possible after stimulation with B-LCL with multiple HLA-mismatches. Additionally, the test system excludes the occurrence of residual matched HLA molecules between donor and recipient B-LCL stimulator cells, which would consequently lead to responses against recipient antigens. These reactivity, for instance against EBV, would most probably not be equally distributed between donor-derived naive and memory T cells but it would compete with alloreactive T-cell responses. For that reason, the use of stimulator cells expressing the full set of HLA molecules as well as viral antigens may impede an accurate comparison of allo-HLA reactivity between distinct T-cell subsets and individuals, respectively.

Best phenotypic marker for allo-depletion. One objective of this thesis was the determination of the T-cell differentiation marker, which might be most suitable for the separation of alloreactivity in naive- and memory-enriched CD4 T-cell subsets. Once identified the marker can be utilized for the depletion of CD4 T-cells with strong alloreactive potential from the allograft. A combination of

several differentiation markers for the depletion procedure might provide a more precise definition of naive and memory T cells rather than the description by a single marker. Nevertheless, in order to facilitate the future establishment of a clinical grade depletion protocol under GMP conditions CD4 T-cell subsets were defined by a single marker. All four differentiation markers analyzed in this work represented a unique T-cell population (Figure 3). CD45RA and CD45RO are traditionally used to define naive ($CD45RA^{pos}$, $CD45RO^{neg}$) and memory ($CD45RA^{neg}$, $CD45RO^{pos}$) T-cell compartments, while $CCR7^{pos}$ as well as $CD62L^{pos}$ populations comprise naive and central memory T cells (Appay*, et al* 2008, Sallusto*, et al* 2004). In order to isolate very pure CD4 T-cell subsets (> 99% purity) defined by the absence or strong expression of the respective differentiation marker flow cytometric cell sorting of healthy donor PBMC was used (Figure 4). During the culture period subsets enriched for naive T cells retained expression of early differentiation markers in a higher proportion of MLR responders compared to their memory counterparts (Figure 13).

IFN-γ ELISpot results obtained from MLR cultures demonstrated that all naive-enriched CD4 T-cell fractions were more responsive in MLR against single allo-HLA-DR as well as allo-HLA-DQ alleles than their memory counterpart fractions (Figure 9). Statistical significant differences in the strength of alloreactivity between naive- and memory-enriched T-cell populations combined for stimulations with allo-HLA-DR and allo-HLA-DQ were observed for CD45RA (P=0.011) and CD62L (P=0.008) (Figure 10). Furthermore, a comparison of the alloresponse of memory-enriched fractions with entire CD4 T cells revealed that the median numbers of allo-HLA-DR/-DQ reactive T cells were 16.6-fold lower in $CD45RA^{neg}$ and 3.5-fold lower in $CD62L^{neg}$ memory fractions than in entire CD4 T cells. Unfortunately, for all four differentiation markers the memory-enriched subsets showed no significant reduction in allorecognition compared to entire CD4 T cells (data not shown). This might be explained by the comparable strong allorecognition in distinct memory subsets of individual donors, which is discussed in detail in one of the following paragraphs. The threshold for the selective depletion of alloreactive T cells in order to achieve biological relevance concerning GvHD prophylaxis is proposed to be at least one order of magnitude. Currently used selective allo-depletion approaches specifically eliminate donor-*versus*-recipient reactive T cells from all T-cell compartments, which means the naive as well as the memory T-cell subsets, but they require the *in vitro* culture of donor T cells for several days. Additionally, hematopoietic recipient cells are utilized as alloantigen-presenting cells, which might not be sufficient to deplete clinically relevant alloreactivity to epithelial organs of the patient (Amrolia*, et al* 2006, Andre-Schmutz*, et al* 2002, Bonini*, et al* 1997, Guinan*, et al* 1999, Solomon*, et al* 2005). For that reason, our data suggest the selective *in vitro* depletion of $CD45RA^{pos}$ CD4 naive T cells from DLI or CD8-depleted DLI products as alternative and comparably easy procedure for a substantial reduction of alloreactivity.

This observation was in accordance to results obtained with CD8 T cells using the same K562-HLA-based MLR test system, in which naive T cells were also found to be the main producers of alloreactive T-cell responses. The experiments were performed by the members of our research group Eva Distler and Saliha Asdufan. The summary of data on alloreactivity of CD4 and CD8 T-cell subsets were recently published in the journal "*Haematologica*" in 2011 (Distler, Bloetz*, et al* 2011). The combination of allo-HLA recognition data for CD4 and CD8 T cells to identify which subset marker would allow for most efficient separation of alloreactivity in entire T cells resulted with statistical significance in stronger allorecognition in naive compared to memory T cells for all markers analyzed. Additionally, $CD45RA^{neg}$ as well as $CD45RO^{pos}$ memory fractions showed significantly lower allorecognition than entire T cells (e.g. for $CD45RA^{neg}$ *versus* entire T cells, p=0.016; median reduction in IFN-γ spots, 7.4-fold). However, since only data for CD45RA naive and memory T-cell populations were significantly different for both CD4 and CD8 T cells the CD45RA allo-depletion approach appears optimally suited for entire CD3 T cells.

Influence of distinct T-cell populations on allorecognition. To investigate the influence of central memory cells on alloreactivity we compared the alloresponses in fractions containing and lacking T cells with this differentiation stage. If central memory T cells were additionally depleted to naive T lymphocytes as it was represented by $CD62L^{neg}$ and $CCR7^{neg}$ T-cell subsets, alloreactivity was reduced to a comparable level as in $CD45RO^{pos}$ as well as in $CD45RA^{neg}$ populations, which both contained central and effector memory T cells. Additionally, the median number of alloreactive precursors in $CCR7^{pos}$ T-cell subsets was higher than in $CD45RA^{pos}$ fractions and comparable to $CD45RO^{neg}$ naive T-cell populations while $CD62L^{pos}$ populations were less reactive than $CD45RO^{neg}$ and $CD45RA^{pos}$ fractions. Altogether, these data suggested that the central memory T-cell compartment played only a minor role in the alloreactive CD4 T-cell response.

Since naturally occurring T_{reg} are known to suppress T-cell reactivity and therefore might contribute to the detected alloreactivity pattern between the individual fractions (Vignali*, et al* 2008) we analyzed the ratio of T_{reg} exemplarily in the naive-enriched $CD45RA^{pos}$ and memory-enriched $CD45RA^{neg}$ CD4 T-cell subsets (Figure 14). The data showed that a higher fraction of T_{reg} was present in $CD45RA^{neg}$ populations compared to $CD45RA^{pos}$ T-cell subsets confirming data by Seddiki *et al* (Seddiki*, et al* 2006). Therefore, the impact of T_{reg} on reduced alloreactivity in the memory T-cell compartment could not be excluded.

Moreover, it need to be considered that CD45RA is not only a marker found on naive T cells but is reexpressed by highly differentiated memory T cells. This T-cell population is able to perform rapid effector functions after encounter with specific antigen. Nevertheless, the impact of CD45RA end-stage effectors on allorecognition in our screening approach could be neglected due to decreased

proliferative capacity and a greater susceptibility to apoptosis of this subset *in vitro* but it cannot be excluded for individual donors (Henson*, et al* 2012).

HLA-DR/-DQ specific alloproliferation of CD4 T-cell subsets. Additionally to allorecognition the alloproliferative *in vitro* response of CD4 T-lymphocytes to mismatched HLA-DR/-DQ alleles were investigated as a further parameter of T-cell activation. The observed alloproliferation was comparable strong in all CD4 T-cell subsets analyzed (Figure 11 & 12). Therefore, statistical significance in alloproliferation between the naive- and memory-enriched populations could not be observed (Figure 12). This finding was not in accordance with the IFN-γ ELISpot results, which demonstrated stronger alloresponses in naive CD4 T-cell populations. TNF-α ELISpot assays and IL-4 ELISA showed also a tendency of stronger cytokine production in MLR derived from naive-enriched subsets confirming the IFN-γ ELISpot results (data not shown). One reason for the different results obtained for allorecognition and alloproliferation could be the increased maturation development from naive to memory T cells between the IFN-γ ELISpot assays at d12 and the analysis of alloproliferation at d21. Nevertheless, T-cell proliferation was not only determined at d21 but additionally during the first week of MLR by cell counting as well as CFSE staining as further readout assay (data not shown). Significant differences in proliferation between the naive- and memory-enriched CD4 T-cell fractions could also not be found at this early culture time. Hence, the prolonged culture period could be excluded as the explanation for the lacking correlation of alloreactivity. Another reason for strong alloproliferation of all CD4 T-cell populations might be due to the addition of surplus cytokines to MLR cultures, which of course not exactly match the *in vivo* situation and promoted optimal cell survival, growth, and proliferation to all T-cell subsets thereby most likely leading to *in vitro* bias.

Memory T-cell therapy. Immunotherapy with memory T cells might be advantageous and promising in terms of GvHD prophylaxis, but several issues need to be taken into account. One of the problems after allo-HSCT is the weak immune reconstitution of naive T cells, which cannot be restored by pure memory T cells (Seggewiss and Einsele 2010). Furthermore, since effector memory cells show a mature differentiation phenotype, thereby failing to express molecules for T cell homing into lymphoid organs and bone marrow, their ability to survive, expand, migrate to end organs, and function *in vivo* might be limited (Beilhack*, et al* 2005, Sallusto*, et al* 2004). This might contribute to the desired reduction of alloresponses occurring in memory T cells of unprimed donors (Chen*, et al* 2004) but would also impair beneficial T-cell responses to pathogens and leukemic cells.

Furthermore, for individual donors stronger allorecognition could be detected in distinct memory subsets (Figure 9, Don 053 and 073 for subsets $CD45RO^{pos}$ and $CCR7^{neg}$). This alloreactivity might be attributed to previous priming against alloantigens *in vivo* by heterologous immunity (Amir*, et al*

2010, Burrows*, et al* 1994). After allo-HSCT pathogen-specific memory T cells cross-reacting with allogeneic HLA molecules or alloreactive memory stem cells (Zhang*, et al* 2005a, Zhang*, et al* 2005b) could be reactivated by infectious agents. Since memory T cells rapidly expand and produce cytokines after secondary antigen encounter alloreactive memory T cells might have serious consequences in terms of GvHD (Chandok and Farber 2004). Until now, the prediction if individual donor/recipient pairs will develop GvHD is not possible considering the interindividual heterogeneity of previous and current infections along with the development of heterologous immunity. For that reason the test system developed in this work might help to identify HLA-mismatched donors with the lowest level of reactivity towards allogeneic HLA molecules of the patient prior transplantation.

Leukemia reactivity. In order to investigate the capability of memory T cell subsets to provoke leukemia reactivity Jana Albrecht, a member of our research group, analyzed primary mixed lymphocyte leukemia cultures performed with naive- or memory-enriched CD8 T-cell subsets of HLA-matched sibling and unrelated donor/patient pairs for alloreactivity. CD8 T-cell populations were isolated from healthy donors using the same strategy as previously described for CD4 T-cells. Thus, all memory CD8 T-cell subsets tested ($CCR7^{neg}$, $CD45RO^{pos}$) lacked significant anti-leukemia CTL responses *in vitro* (Distler, Bloetz*, et al* 2011). Additionally, it has already been demonstrated by our group and others that CD8 T-cells reactive to leukemia cells mainly derive from phenotypically naive precursors in healthy unprimed individuals and recognize polymorphic minor histocompatibility antigens (mHAg) and nonpolymorphic leukemia-associated antigens (LAA) (Albrecht*, et al* 2011, Bleakley*, et al* 2010, Distler*, et al* 2008, Quintarelli*, et al* 2008). Nevertheless, memory $CD62L^{neg}$ CD8 T cells of healthy individuals regularly contain low numbers of AML-reactive CTL (Distler*, et al* 2008). Similar experiments need to be performed with leukemia-reactive CD4 T-cell subsets in order to confirm if the distribution of leukemia reactivity in individual CD4 T-cell subsets is in congruence with the data for CD8 T cells showing lower numbers in memory subsets compared to their naive counterparts. Altogether, the transplantation of memory T cells from donors who were previously primed against hematopoietically expressed mHAg and LAA by natural immunization or vaccination seems as an optimal strategy to address this problem.

Therapeutic strategies. Today, in donor lymphocyte infusion protocols usually the entire donor T-cell pool is transfused in order to induce GvL effects (Kolb 2008). Additionally, CD8-depleted DLI were introduced and successfully reduced GvHD reactions while antileukemic reactivity was maintained at significant levels (Alyea*, et al* 1998, Champlin*, et al* 1990, Meyer*, et al* 2007). The current study suggests the depletion of the naive T-cell compartment from DLI products. Thereby the adoptive transfer of both memory CD4 and CD8 T-cell subpopulations as well as the transfer of memory CD4 T cells alone could be considered. This approach will potentially decrease the incidence and severity

of GvHD in most patients. Furthermore, it may enhance important post-transplant immune functions, particularly against infectious agents. The adoptive immunotherapy with memory T cells will be most suitable for HLA-mismatched leukemia patients at high-risk of GvHD and low-risk of disease relapse, as well as for patients with non-malignant diseases or severe repeated infections. The reduced GvL potential might be strengthened by the co-transfer of leukemia-reactive T cells previously isolated from the naive T-cell repertoire of donors by primary *in vitro* stimulation protocols (Albrecht*, et al* 2011, Bleakley*, et al* 2010, Distler*, et al* 2008).

The K562-HLA platform might also be useful as screening protocol for donor T-cell reactivity to individual HLA class II mismatch alleles in situations, in which a donor with full HLA match is lacking. HLA disparities, particularly at all HLA class I loci and the HLA class II locus HLA-DR, are known to increase the risk of graft rejection and severe GvHD, and may even decrease survival after allogeneic HSCT (Bray*, et al* 2008, Flomenberg*, et al* 2004). For that reason, the K562-HLA based *in vitro* MLR screening approach might help to select a HLA-mismatched donor (e.g. in haploidentical HSCT), who carries the lowest level of reactivity against non-shared recipient HLA antigens.

Allo-HLA-DQ/-DP specific CD4 T cells for the adoptive immunotherapy of leukemia

The second part of this PhD thesis is based on interesting results observed during the first part of this work. The K562-HLA MLR test system demonstrated that T cells analyzed for reactivity against HLA-DQ mismatch alleles provoked much lower allorecognition compared to T cells tested against allo-HLA-DR molecules. This finding is in accordance with clinical studies revealing that disparities at the HLA-DQ locus are much better tolerated and do not influence overall mortality in allo-HSCT whereby mismatches in the HLA-DR as well as all HLA class I loci might have tremendous consequences on the outcome of a transplantation (Bray*, et al* 2008, Flomenberg*, et al* 2004, Lee*, et al* 2007). Disparities in the third HLA class II molecule HLA-DP, which is until now not taken into consideration for donor selection, were retrospectively studied. It turned out that there are so-called “permissive” HLA-DP mismatch constellations that have no impact on overall survival of the patient and may even reduce the risk of leukemia relapse following allo-HSCT (Crocchiolo*, et al* 2009, Shaw*, et al* 2007). There is also evidence from *in vitro* experiments that HLA-DP mismatch antigens can be specific targets of cellular immunotherapy in HLA class II-expressing leukemias (Ibisch*, et al* 1999, Rutten*, et al* 2008, Rutten*, et al* 2012, Rutten*, et al* 2010b). Furthermore, Stevanovic and colleagues could already demonstrate in a humanized murine model that CD4 T cells efficiently eradicated HLA class II mismatched leukemic cells and that HLA-DR, -DQ as well as -DP specific T cells contributed to the GvL effect. This indicated that CD4 T cells can act as sole mediators of anti-leukemia reactivity (Stevanovic*, et al* 2011). Overall, these observations gave rise to the second part of this PhD thesis dealing with the *in vitro* generation of CD4 T-cell lines recognizing HLA-DQ or HLA-DP mismatch alleles for the adoptive immunotherapy of leukemias. These T cells might be applied in clinical

settings, in which patient and donor are matched in all HLA molecules except one single HLA-DQ or permissive HLA-DP allele. The allo-HLA-DQ/-DP specific CD4 T cells may primarily target leukemia cells of the patients while presumably sparing non-hematopoietic recipient tissues under non inflammatory conditions. In these donor/patient constellations HLA-DQ/-DP reactive CD4 T lymphocytes might also be applied as a supplement during memory T-cell therapy in order to increase GvL reactivity (see above).

Allostimulation with autologous mDC as APC stocks. For the *in vitro* generation protocol of allo-HLA-DQ/-DP specific T cells naive CD4 T-cell populations of healthy donor origin were stimulated against single HLA-DQ or HLA-DP alleles of the patient in MLR. The protocol can be devided into two steps (Figure 15). In the first step APC stocks expressing single HLA-DQ or HLA-DP mismatch alleles were produced. As APC we aimed for mature dendritc cells (mDC) of healthy donor origin. mDC are the most potent APC for the activation of T lymphocytes. They highly express molecules involved in antigen presentation like MHC molecules as well as costimulatory molecules and further synthesize cytokines, which for their part attract and activate T cells (Banchereau and Steinman 1998). Additionally, the *in vitro* generation and maturation of mDC under GMP quality is extensively studied and mDC were already used in clinical trials (Palucka and Banchereau 2012). mDC were transfected with “off-the-shelf” IVT mRNA of the respective allogeneic HLA-DQ or HLA-DP allele of the patient by electroporation, resulting in transient expression of the HLA molecules. Since mRNA has a short half-life and lacks the potential to integrate into the host genome mRNA-transfected cells are safe for clinical use in contrast to those generated by viral transduction methods. Thereby, the use of mRNA electroporated mDC generated via a “FAST-DC” protocol in 48h (Dauer*, et al* 2003) is not only simple, fast and efficient in terms of the capability to present the exogenously introduced antigen but it can furthermore be applied in the clinic (Markovic*, et al* 2006, Van Driessche*, et al* 2009). Resulting transfectant allo-HLA-DQ/-DP mDC could be used as a stock of APC expressing a single HLA mismatch allele (Figure 6). Moreover, PBMC, PHA blasts, or B-cells could replace mDC and function as allo-DQ/-DP APC stocks. Nevertheless, the alloresponse using PBMC as well as PHA blasts as APC, which were also analyzed in this work, was much lower compared to mDC since they lack main features of professional APC (data not shown).

In the second step of the manufacturing protocol the allo-HLA-DQ/-DP mDC served as stimulator cells in MLR with T cells of the same donor. Since we could prove in the first part of this study that alloreactive precursors mainly exist in the naive T-cell compartment we chose the $CD4^{pos}CD45RA^{pos}$ T-cell subset for the stimulation protocol in order to obtain highly alloreactive T-cell lines. Pure $CD4^{pos}CD45RA^{pos}$ T-cell fractions (> 99% purity) were isolated by flow cytometry cell sorting according to the sorting strategy described in the first part of this work (Figure 4). Cytokines were administered to MLR cultures as described above.

One of the main advantages of this protocol is that we generated allo-HLA-DQ/-DP specific CD4 T cells, which were reactive against the mismatch allele of the patient, without the necessity of patient cells. Basically, we worked in an entirely autologous system, in which mDC carrying single HLA-DQ/-DP mismatch alleles were stimulated in MLR with autologous naive T cells. In this way the emergence of indirect allorecognition of donor-derived peptides on MHC molecules of the patient, which is described to contribute to graft rejection (Gould and Auchincloss 1999), could be eliminated. Furthermore, the identification of mismatched HLA-antigens between donor and recipient by HLA typing is routinely performed during the donor selection procedure (Choo 2007). Hence, the HLA status is easy and fast available, while the identification of mismatched minor histocompatibility antigens (mHAg) as well as leukemia-associated antigens (LAA) between donor/patient pairs is a time consuming and laborious process (Bleakley and Riddell 2004). In addition, mHAg and LAA are restricted to distinct HLA-molecules, thereby reducing their therapeutic benefit to a limited number of patients.

Leukemia reactivity. MLR cultures were analyzed in sensitive IFN-γ ELISpot assays for allo-HLA reactivity demonstrating the strong specificity and HLA-restriction of this approach (Figure 16). Significant alloresponses were only observed if mDC-HLA transfectant cells were applied as stimulator cells that had been also used during initial T-cell priming. In contrast, autologous untransfected mDC did not provoke any significant spot production. Furthermore, allo-HLA class II reactivity could be blocked by monoclonal antibodies binding to the respective HLA molecules used for the initial T-cell stimulation.

In order to investigate if the allo HLA-DQ/-DP specific T cells would also recognize acute myeloid leukemia (AML) blasts a broad panel of untreated primary leukemic cells was screened for allorecognition in IFN-γ ELISpot assays (Figure 17, Supplementary Table 1). Significant alloresponses were only observed if the AML blasts carried the respective HLA-DQ/-DP allele, which was used for the initial T-cell stimulation. These results were very consistent with one exception. MZ308-AML carried the distinct HLA-DP allele used for the initial stimulation but was not recognized by all HLA-DP reactive T-cell populations tested. One explanation might be the low expression of HLA-DP molecules on the cell surface of MZ308-AML (rMFI=1.6, data not shown). Another reason might be based in the complex nature of alloreactivity. It is known that in allorecognition the TCR interacts with the allogeneic MHC molecule as well as the bound peptide. Thereby the interaction can either be peptide or MHC specific (Felix and Allen 2007, Nagy 2012). If MZ308-AML lacked the endogenous peptide, which would be necessary for allorecognition, e.g. because the T-cells were previously primed against a different allopeptide epitope, the leukemic cells could not be recognized.

Furthermore, we detected donor-specific minor reactivity to single leukemic samples that did not expressed the HLA allele used for T-cell priming (Supplementary Table 1). Since the T-cell populations

were in an early stage of culture and therefore not clonal when the analysis was performed at d19 the IFN-γ spot production could be attributed to residual reactivity to other HLA mismatch alleles. During prolonged *in vitro* culture this residual mismatch reactivity gradually decreased since T cells analyzed on d33 (Supplementary Table 1) showed overall less alloreactivity compared to cultures examined at an earlier time point.

We also investigated if the allo-HLA specific T cells would recognize leukemia cells expressing HLA-mismatch suballeles. This was exemplarily examined for HLA-DQ specific T-cells tested against AML blasts of five different patients expressing a mismatched suballele (Supplementary Table 1). Leukemic samples of two patients were not significantly recognized, the blasts of two further patients were slightly recognized, while one AML sample caused strong alloresponses. This again reflects the complexity of allorecognition and indicates that HLA-DQ/-DP suballele mismatches of the donor in combination with the allogeneic HLA allele of the patient used for the T-cell generation should be carefully considered during donor selection.

Cytolytic activity of allo-HLA-DQ/-DP reactive CD4 T-cells against primary AML blasts was very specific since only leukemic samples, which expressed the HLA allele used for the primary *in vitro* stimulation were lysed (Figure 18 & 19). Additionally, HLA-restriction was demonstrated in blocking studies with mAb binding to the HLA class II molecule utilized for T-cell priming. In contrast to allorecognition in IFN-γ ELISpot assays cytotoxicity of CD4 T-cells could only be observed towards a part of untreated AML blasts expressing the respective HLA allele, while IFN-γ pre-treated leukemic cells were always lysed.

Failing cytotoxicity towards untreated leukemia blasts might be attributed to both tumor escape mechanisms and to the fact that CD4 T cells are not the main cytotoxic effector cells:

CD4 T cells mainly mediate helper functions but they may also be able to lyse target cells. The pure allo-DQ/-DP mismatch reactive CD4 T-cell populations lysed the leukemia samples in low to intermediate levels. Blocking of cytotoxicity by mAb binding to CD4 confirmed that cytolytic activity was CD4-dependent (Figure 19 B). Cytolysis was most probably granule-mediated, which is one of the main and most rapid mechanisms of lymphocyte-mediated cytotoxicity (Barry and Bleackley 2002, MacLeod*, et al* 2009). We found strong expression of the serine proteases granzyme A and B, but only low levels of the pore-forming protein perforin in intracellular granules of the allo-HLA-DQ/-DP specific T cells reflecting the medium strength of target cell killing (Figure 20). If cell lysis is mediated by this mechanism should further be confirmed by inhibition assays.

Phenotyping of hematopoietic cells. Leukemia cells may aquire characteristics, with which they overcome immune surveillance. They may escape T-cell recognition and cytolysis e.g. by down-regulation of costimulatory molecules, adhesion molecules, or even HLA molecules whereas the expression of inhibitory costimulatory molecules might be upregulated and inhibitory cytokines

secreted (Barrett and Le Blanc 2010, Kolb*, et al* 2004). We analyzed the expression pattern of all three HLA class II molecules on a panel of primary AML blasts under inflammatory and non-inflammatory conditions in order to investigate if HLA-molecule expression contributes to lacking cytotoxicity towards single untreated AML blasts. All three HLA class II molecules were significantly expressed on leukemia samples (Figure 21). The strongest expression was found for HLA-DP followed by HLA-DR and HLA-DQ. Most important, cell surface expression of distinct HLA-molecules varied considerably between individual donors. After pre-treatment with IFN-γ significant up-regulation of HLA-DP molecules was observed while the expression of HLA-DQ and HLA-DR did not change significantly. The expression of costimulatory and adhesion molecules on leukemia blasts was not analyzed in this study but was already described without (Brouwer*, et al* 2000, Graf*, et al* 2005) and after IFN-γ pre-treatment (Berthon*, et al* 2010, Munker and Andreeff 1996).

Our data are in accordance with a previous report by Ibisch *et al*, who investigated direct cell lysis of leukemia cells by an allo-HLA-DPB1 specific T-cell clone. They also found that leukemic blasts expressing the respective HLA-DP allele were only in part accessible to direct lysis by the HLA-DP specific T cells. Interestingly, they did not detect a correlation between the variability in mean fluorescence intensities of HLA-DP expression on untreated leukemic blasts and the alteration in cytotoxicity scores (Ibisch*, et al* 1999). The T-cell clone applied in the previous study was derived from MLR between two healthy donors matched for all HLA molecules but the HLA-DP alleles. This generation protocol is disadvantageous compared to our approach since it requires cells of two donors thereby enhancing the probability for the occurance of GvHD and graft rejection. Furthermore, T-cell cloning demands long culture periods compared to the rapid generation of T-cell lines in our protocol.

Moreover, screening of B-LCL for HLA class II expression under inflammatory and non-inflammatory conditions revealed the same expression pattern than obtained for AML blasts with significant HLA expression of all three class II molecules following the hierarchy: HLA-DP > HLA-DQ > HLA-DR. This indicated that HLA-DQ/-DP specific T cells can not only recognize hematopoietic cells of myeloid origin but also cells derived from the lymphoid lineage. Not surprisingly, the overall HLA expression levels on B-LCL were higher than on AML blasts since the B-LCL cell lines derived from B lymphocytes, which are professional APC that endogenously express high amounts of HLA class II molecules.

In summary, these data demonstrated that allo-HLA-DQ/-DP specific CD4 T-cells might be capable to recognize and cytolyse most leukemic blasts carrying the respective HLA-DQ/-DP molecules. It is still very difficult to predict if the leukemia cells of the patient would be well-provided with sufficient amounts of HLA- and costimulatory molecules on their cell surface as well as the respective endogenous peptides necessary for T-cell allorecognition and cytotoxicity also in the *in vivo* situation. This point needs careful consideration and additional testing in preclinical humanized animal models

for adoptive immunotherapy (Distler*, et al* 2008). In addition, each donor/patient pair should be tested individually prior transplantation for recognition and lysis of primary patient AML blasts.

Reactivity against non-hematopoietic cells. We could further demonstrate in this work that non-hematopoietic recipient tissues would most probably be spared by the allo-HLA-DQ/-DP specific CD4 T cells because allorecognition of primary fibroblasts (FB) and keratinocytes (KC) was not observed in IFN-γ ELISpot assays under non-inflammatory conditions, meaning without pre-incubation of target cells with proinflammatory cytokines (Figure 22 & 23, Supplementary Table 2 & 3). This observation was independent from the HLA status meaning that also FB or KC, which were genotypically positive for the respective HLA alleles used for the T-cell priming, did not stimulate T cells for any significant spot production. However, the HLA-DQ/-DP specific T cells mediated IFN-γ spot production if target cell cultures were pre-treated with IFN-γ and carried the HLA alleles used for T-cell priming. The observations for allorecognition of FB were in strong accordance with the results for cell cytotoxicity in ^{51}chromium-release assays (Figure 24). Cytolytic activity could only be obtained after pre-incubation with IFN-γ and solely for the CD4 T-cell lines primed against the respective HLA allele.

Phenotyping of non-hematopoietic cells. The allorecognition results are in strong accordance with the HLA class II expression data on non-hematopoietic cells under inflammatory and non-inflammatory conditions (Figure 25). The analysis revealed that primary FB, KC as well as non-malignant kidney cells (NKC) did not or only in very slight amounts carry HLA-class II molecules on their cell surface under non-inflammatory conditions, but upregulated significantly the HLA-DR and HLA-DP expression and on NKC additionally also the HLA-DQ expression after IFN-γ pre-treatment. For that reason lacking reactivity of the HLA-DQ/-DP mismatch reactive T cells to untreated mesenchymal and epithelial cells was most probably caused by lacking HLA class II molecules on the cell surface. The overall reactivity of the CD4 T cells was much stronger towards FB compared to KC. Since the HLA-molecule expression on pre-treated KC was similar (HLA-DQ) or even stronger (HLA-DR, HLA-DP) than on FB, the reason for diminished allorecognition of KC might also relate to the huge diversity of alloreactivity as described above.

Furthermore, pre-incubation of hematopoietic as well as non-hematopoietic tissues with TNF-α, IL-1β, and IL-6 did not lead to significant changes in the HLA expression pattern (data not shown). This observation indicated that these three cytokines, which contribute substantially to the pathophysiology of GvHD (Antin and Ferrara 1992, Sun*, et al* 2007), play only a minor role in the upregulation of HLA-molecule expression.

Therapeutic strategies. Since the HLA-DQ/-DP mismatch reactive T cells expanded rapidly they could be adoptively transferred at an early time of culture. At d28 the pure CD4 T-cell populations can phenotypically be categorized as effector memory cells (Figure 26). They express costimulatory

molecules ($CD27^{low}$, $CD28^{high}$), cytokine receptors ($CD127^{int}$, $CD25^{int}$), and markers for T-cell homing ($CD62L^{low}$) but already lost chemokine receptors ($CCR7^{neg}$). The main question remains if the cells are able to survive and proliferate *in vivo,* migrate to lymphoid organs and accomplish effector functions after the adoptive transfer (Appay*, et al* 2008, Sallusto*, et al* 2004). This needs further investigation in a suitable therapeutic mouse model that would pave the way for prospective clinical trials (Distler*, et al* 2008).

Overall, our data suggest that the allo-HLA-DQ/-DP specific CD4 T-cells will most probably recognize and lyse hematopoietic cells thus eliminating the leukemia cells of the patient, while they would spare non-hematopoietic tissues like epithelial cells, which are mainly affected by GvHD, and mesenchymal cells. Such a cellular product might be most suitable for patients with advanced stage AML after reduced-intensity conditioning and T-cell depletion for the replenishment of anti-leukemic reactivity if the risk for a disease relapse is high. We propose to administer these T cells using a delayed add-back protocol after vaccination or together with virus specific T cells (Sellar and Peggs 2012). Furthermore, the combination of HLA-DQ/DP mismatch reactive CD4 T cells with memory T-cell therapy, which was discussed in the first part of this thesis, is supposed to increase important post-transplant immune functions and therefore seems very promising.

7 Conclusions & Outlook

In this PhD thesis we developed two methodical protocols, which might be of potential use in the adoptive immunotherapy of allogeneic hematopoietic stem cell transplantation (allo-HSCT) patients.

In the first part of this thesis we designed a novel screening system for the detection of CD4 T-cell reactivity of individual donors to single allogeneic human leukocyte antigen (HLA) class II alleles. The approach is based on K562-HLA-DR/-DQ transfectant cells as standard antigen-presenting cells in mixed lymphocyte reactions (MLR). CD4 T-cell populations were defined by a single marker to facilitate translation into a clinical-grade allo-depletion procedure. We could demonstrate that allo-HLA class II mismatch reactivity preferentially derived from naive compared to memory CD4 T-cell populations in most healthy individuals. *In vitro* strategies for depleting naive CD4 T cells seem to be most efficient if CD45RA is used as differentiation marker to develop a suitable sorting strategy. Combined data for CD4 as well as CD8 T cells revealed that CD45RA is also the most efficient marker for allo-depletion protocols of entire T cells. Since memory T-cell responses against leukemia antigens are usually low we propose the application of donor allografts, which contain leukemia-reactive T cells in the memory compartment due to previous priming against minor histocompatibility antigens (mHag) and leukemia-associated antigens (LAA) during natural immunity or vaccination. Alternatively, memory T cells could be replenished with leukemia-reactive T cells previously isolated from the naive T-cell subset of donors by primary *in vitro* stimulation. The establishment of a good manufacturing practice (GMP) procedure for depleting naive T cells or selecting memory T cells *in vitro* appears relatively easy compared to more complex selective allo-depletion approaches. Ongoing work of our group analyzes the impact of a CD45RA allo-depletion protocol on healthy donor leukapheresis *in vitro* under GMP conditions. This would pave the way for clinical trials that investigate whether depletion of naive T cells from donor-derived stem-cell grafts or lymphocyte products will indeed reduce graft-*versus*-host disease (GvHD) in allo-HSCT patients, while beneficial memory reactivity to pathogens will be preserved. Most suitable study populations are HLA-mismatched patients at high-risk of GvHD and low-risk of leukemia relapse, as well as patients with diseases other than leukemia. Furthermore, the test system might be applied for screening purposes to identify HLA-mismatched donors with the lowest level of reactivity against non-shared HLA molecules of the patient if a donor with a complete match in all HLA alleles is missing. Thereby, the risk of graft rejection and severe GvHD might be diminished and overall survival after allo-HSCT might be increased.

In the second part of this PhD thesis we developed an innovative protocol for the generation of allo-HLA-DQ/-DP specific CD4 T cells, which will be applicable if donor and recipient are matched in all HLA alleles but one single HLA-DQ or permissive HLA-DP allele. Compared to the identification of

mHAg or LAA, which are additionally restricted to specific HLA alleles, the HLA status of donor and patient is easy and fast available. The generation procedure was designed in an entirely autologous system without the requirement of patient material. Briefly, naive CD45RA^{pos} CD4 T cells isolated from healthy donor peripheral blood mononuclear cells by flow cytometric cell sorting were stimulated in MLR against single allo-HLA-DQ/-DP alleles transfected into autologous mature dendritic cells by mRNA electroporation. Our data demonstrated that the HLA-DQ/-DP mismatch reactive CD4 T-cells specifically recognized and lysed most acute myeloid leukemia (AML) blasts, while they spared non-hematopoietic epithelial and mesenchymal cells, which are mainly affected by GvHD, under non-inflammatory conditions. For that reason the cells might be capable to provide anti-leukemic reactivity for patients with advanced stage AML after reduced-intensity conditioning and T-cell depletion if the risks for a disease relapse are high. Although we could show that AML blasts express HLA-DP as well as HLA-DQ molecules it is very difficult to predict if individual AML blasts are able to provoke direct recognition as well as leukemia cell lysis. Therefore, each donor/patient pair should be tested individually prior transplantation. In order to minimize the risks for infections we propose to administer the allo-DQ/-DP specific T cells using a delayed add-back protocol after vaccination or together with virus specific T cells. Additionally, the application of HLA-DQ/DP mismatch reactive CD4 T cells together with memory T-cell therapy, which is supposed to increase post-transplant immune functions, might be advantageous. The potential of the HLA-DQ/-DP mismatch reactive CD4 T cells to survive, proliferate, and accomplish effector functions *in vivo* thereby eradicating the leukemia cells needs to be confirmed in therapeutic murine models. In this regard NOD/SCID IL2Rγnull mice, which lack T-, B- and NK-cell functions (Shultz*, et al* 2007), appear feasible. The mice should be engrafted with human AML blasts to establish leukemic hematopoiesis in mice prior to the adoptive T-cell transfer. These experiments would pave the way for prospective clinical studies with allo-HLA-DQ/-DP specific CD4 T cells generated in a donor/patient specific manner.

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9 Abbreviations

7-AAD	7-amino-actinomycin D
AEC	3-amino-9-ethylcarbazole
ALL	acute lymphatic leukemia
allo	allogeneic
AML	acute myeloid leukemia
APC	antigen-presenting cell
APC	allophycocyanin
B-LCL	B-lymphoblastoid cell line
BM	bone marrow
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFSE	carboxyfluorescein succinimidyl ester
CIP	alkaline phosphatase calf intestinal
CLL	chronic lymphatic leukemia
CML	chronic myeloid leukemia
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen-4
DC	dendritic cell
DLI	donor lymphocyte infusion
DNA	deoxyribonucleic acid
DMF	dimethylformamide
DMSO	dimethyle sulfoxide
Don	donor
EBV	Epstein-Barr Virus
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immunosorbent Spot assay
E:T	effector-to-target
FAB	French American British Cooperative Group
FACS	fluorescence-activated cell sorting
FB	fibroblast
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	fluorescence
FLT3	fibroblast-macrophage stimulating factor receptor-like tyrosine kinase receptor 3
FSC	forward scatter
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
GvHD	graft-*versus*-host disease
GvL	graft-*versus*-leukemia
GvT	graft-*versus*-tumor
HLA	human leukocyte antigen
HS	human serum
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
IFN	interferon
Ig	immunglobulin

IL	interleukine
IU	international unit
IVT	*in vitro* transcription
KC	keratinocyte
KIR	killer cell immunoglobulin like receptor
K-SFM	Keratinocyte Serum Free Medium
LAA	leukemia-associated antigen
LPS	lipopolysaccharide
MACS	magnetic cell sorting
mAb	monoclonal antibody
mDC	mature dendritic cell
MFI	median fluorescence intensity
mHAg	minor histocopatibility antigen
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
mRNA	messenger ribonucleic acid
n.d.	not determined
NK	natural killer
NKC	non-malignant kidney cell
NOD/SCID	nonobese diabetic / severe combined immune deficient
NPM1	nucleophosmin 1
n.s.	not significant
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PE	phycoerythrin
PE-Cy7	phycoerythrin cyanine dye 7 tandem conjugate system
PerCp	peridinin chlorophyll protein complex
PGE_2	prostaglandin E_2
PHA	phytohemagglutinin
P/S	penicillin/streptomycin
rMFI	relative median fluorescent intensity
RT	room temperature
SD	standard deviation
SFC	spot forming conlonies
SSC	sideward scatter
TAP	transporter associated with antigen processing
T_{CM}	central memory T cell
T_{EFF}	effector T cell
T_{EM}	effector memory T cell
T_H	T helper cell
T_{reg}	regulatory T cell
TCD	T-cell depletion
TCE	T-cell epitope
TCR	T-cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
WHO	World Health Organisation
w/o	without

10 Appendix

10.1 Publication derived from this Thesis

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

Eva Distler*, Andrea Bloetz*, Jana Albrecht, Saliha Asdufan, Alexander Hohberger, Michaela Frey, Elke Schnürer, Simone Thomas, Matthias Theobald, Udo F. Hartwig and Wolfgang Herr
* contributed equally to this work

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Abstract

Background HLA mismatch antigens are major targets of alloreactive T cells in HLA-incompatible stem-cell transplantation, which can trigger severe graft-*versus*-host disease and reduce survival in transplant recipients. Our objective was to identify T-cell subsets with reduced *in vitro* reactivity to allogeneic HLA antigens.

Design and Methods We sorted CD4 and CD8 T-cell subsets from peripheral blood by flow cytometry according to their expression of naive and memory markers CD45RA, CD45RO, CD62L, and CCR7. Subsets were defined by a single marker to facilitate future establishment of a clinical-grade procedure for reducing alloreactive T-cell precursors and graft-*versus*-host disease. T cells were stimulated in mixed lymphocyte reactions against HLA-deficient K562 cells transfected with single HLA-A/-B/-C/-DR/-DQ mismatch alleles. Alloreactivity was measured by interferon-γ spot production and cell proliferation.

Results We observed that allogeneic HLA-reactivity was preferentially derived from subsets enriched for naïve T cells rather than memory T cells in healthy donors, irrespective of the HLA mismatch allele. This separation was most efficient if CD45RA (*versus* other markers) was used for sorting. The numbers of allogeneic HLA-reactive effector cells were in median 7.2-fold and 16.6-fold lower in $CD45RA^{neg}$ memory CD8 and CD4 T cells than in entire CD8 and CD4 T cells, respectively. In contrast, proliferation of memory T cells in response to allogeneic HLA was more variably reduced (CD8) or equivalent (CD4) when compared to that of naïve T cells. We also demonstrated in HLA-matched donor-patient pairs that leukemia-reactive CD8 cytotoxic T-lymphocytes were mainly derived from subsets enriched for naïve T cells compared to memory T cells.

Conclusions Memory T-cell subsets of most healthy individuals showed decreased allogeneic HLA-reactivity, but lacked significant anti-leukemia responses *in vitro*. The clinical use of memory or naïve-depleted T cells might be beneficial for HLA-mismatched patients at high risk of graft-*versus*-host disease and low risk of leukemia relapse. Preferred allografts are those which contain leukemia-reactive memory T cells. Alternatively, replenishment with leukemia-reactive T cells isolated from naïve subsets is desirable.

10.2 Supplementary Tables

Supplementary Table 1. Recognition of primary AML blasts by allo-HLA-DQ/-DP specific CD4 T cells. Shown are results for CD4 T cells of donor 302, 409, and 569 specific to allo-HLA-DQB1*06:02 and donor 905, 914, and 927 specific to allo-HLA-DPB1*02:01. MLR populations ($2x10^4$/well) were analyzed in a 18h IFN-γ ELISpot assay on d19 or d33 (i.e. 5 days after the second or fourth allo-HLA restimulation on d14 or d27) for reactivity against AML blasts ($5x10^4$/well) of 24 patients. Reactivity to the original mDC-HLA stimulator cells ($1x10^4$/well) is also displayed. HLA-DQ and HLA-DP typings of the respective donors and AML patients are indicated. AML blasts were pre-incubated overnight in cytokine-free medium. Original IFN-γ ELISpot data are exemplarily displayed in Figure 17. AML, acute myeloid leukemia; Don, donor; n.d., not determined; *, determined at d12 (i.e. 5 days after the first allo-HLA restimulation on d7).

allo-HLA-DQB1*06:02			MZ AML			allo-HLA-DPB1*02:01		
Don 302	Don 409	Don 569				Don 905	Don 914	Don 927
02:01/03:01	03:01/05:01	03:01 or 03:19/04:02	HLA-DQB1*		HLA-DPB1*	03:01/04:01	04:01	03:01/04:01
313	158	255	03:01 / 05:02	**114**	05:01	98	27	248
20	35	26	03:01 / 06:03	**131**	04:01 / 05:01	1	1	1
450	450	400	03:01 / 06:02	**237**	02:01 / 04:01	659	64	350
99	90	193	03:01 / 03:03	**251**	04:02	1	27	291
n.d.	n.d.	n.d.	03:02 / 04:02	**285**	03:01 / 04:02	3	11	n.d.
3	3	0	03:02 / 05:01	**294**	04:01 / 04:02	0	2	1
144	90	92	05:03 / 06:03	**308**	02:01 / 05:01	21	6	26
79	50	148	03:01 / 05:02	**370**	04:02 / 14:01	20	10	75
2	5	1	03:02 / 04:02	**458**	03:01 / 04:01	1	0	0
31	25	38	03:01 / 05:01	**470**	04:01	11	9	33
62	60	153	02:01 / 03:03	**558**	04:01 / 13:01	16	44	238
41	15	54	03:05	**561**	04:01 / 10:01	157	149	75
297	110	314	05:03 / 06:04	**632**	03:01 / 04:01	5	23	71
1000	1210	600	02:02 / 06:02	**653**	04:01 / 04:02	4	12	60
16	10	42	03:01	**667**	04:01 / 04:02	6	3	20
2	40	5	03:01 / 03:02	**683**	04:01 / 13:01	n.d.	n.d.	5
2000	1550	2000	06:02	**728**	04:01	8	4	30
23	40	54	02:01 / 02:02	**770**	01:01	3	19	53
2	13	3	02:01 / 03:01	**784**	01:01 / 03:01	1	1	1
10	20	54	03:01	**849**	02:01 / 04:01	n.d.	394	>2000
523	348	311	03:02 / 06:02	**874**	03:01 / 04:01	0	2	8
>2000	1550	>2000	02:02 / 06:02	**888**	02:01 / 14:01	4000	284	>2000
>2000	418	2000	02:01 / 06:03	**903**	02:01 / 04:01	1003	164	2000
2	10	6	03:02 / 06:03	**911**	04:01	0	0	1
>2000	106 *	>2000		**mDC-HLA**		2000	>1000	>2000
0	13	2		**T cells only**		0	0	0
d19	d19	d19		**day of culture**		d33	d19	d19

Supplementary Table 2. Recognition of primary fibroblasts by allo-HLA-DQ/-DP specific CD4 T cells. Shown are results for CD4 T cells of donor 302 and 492 specific to allo-HLA-DQB1*06:02 and donor 914 and 927 specific to allo-HLA-DPB1*02:01 stimulated in two different experiments. MLR populations (2x10^4/well) were analyzed in a 18h IFN-γ ELISpot assay on d26 (i.e. 5 days after the third allo-HLA restimulation on d21) for reactivity against FB (1x10^4/well) of 21 donors. Reactivity to the original mDC-HLA stimulator cells (1x10^4/well) is also displayed. HLA-DQ and HLA-DP typings of the respective donors and FB cultures are indicated. FB were either pre-incubated over four days with or without the addition of IFN-γ. Original IFN-γ ELISpot data are exemplarily displayed in Figure 8. FB, fibroblasts; Don, donor; n.d., not determined; *, experiment one; **, experiment two.

FB #	allo-HLA-DQB1*06:02			IFN-γ		allo-HLA-DPB1*02:01	
	Don 302	Don 492				Don 914	Don 927
	02:01/03:01	02:02	HLA-DQB1*		HLA-DPB1*	04:01	03:01/04:01
06*	>1000 0	>1000 1	05:02 / 06:02	+ -	02:01 / 04:01	620 0	>1000 2
07**	3 0	31 1	03:01	+ -	04:01 / 04:02	4 0	54 1
08**	4 0	37 0	02:02	+ -	04:01 / 15:01	10 0	20 0
09**	895 0	>1000 1	06:02 / 06:09	+ -	04:01 / 13:01	41 0	37 0
10**	315 0	128 2	04:02 / 03:01	+ -	02:01 / 03:01	918 0	868 0
11**	5 0	14 0	02:02 / 03:01	+ -	03:01 / 13:01	25 0	14 0
12**	1 0	52 1	03:01 / 03:03	+ -	04:02 / 05:01	18 0	28 0
13**	4 0	70 0	03:01 / 03:03	+ -	02:01 / 04:01	847 0	768 1
14**	2 0	14 1	02:02 / 03:01	+ -	03:01	56 0	5 0
15**	27 0	35 1	05:01 / 03:01	+ -	02:01 / 03:01	601 0	581 0
16*	71 1	95 1	03:01 / 05:03	+ -	02:01 / 04:02	340 1	882 2
17*	12 2	48 1	03:01 / 03:02 or 03:19	+ -	02:01 / 03:01	254 1	863 2
19**	744 12	812 0	06:02 / 03:02	+ -	03:01 / 04:01	86 0	63 0
20**	12 0	3 1	n.d.	+ -	n.d.	124 0	338 0
22*	457 1	95 0	n.d.	+ -	n.d.	13 1	28 2
27*	25 0	60 1	n.d.	+ -	n.d.	288 1	>800 4
28*	54 0	36 1	n.d.	+ -	n.d.	126 3	103 8
29*	19 0	70 0	n.d.	+ -	n.d.	26 0	12 1
45*	23 0	64 1	03:03 / 03:21	+ -	04:01 / 03:01	64 1	64 1
46*	>1000 2	>1000 0	06:02 / 06:04	+ -	03:01 / 04:01	48 1	16 1
47*	>1000 0	>1000 1	06:02 / 03:01	+ -	04:01	5 0	13 2
mDC-HLA	* > 2000 ** > 2000	* > 600 ** > 2000		-		* 425 ** > 2000	* > 1000 ** > 2000
T cells only	1	1		-		1	1

Supplementary Table 3. Recognition of primary keratinocytes by allo-HLA-DQ/-DP specific CD4 T cells. Shown are results for CD4 T cells of donor 302 and 492 specific to allo-HLA-DQB1*06:02 and donor 914 and 927 specific to allo-HLA-DPB1*02:01 stimulated in two different experiments. MLR populations ($2x10^4$/well) were analyzed in a 18h IFN-γ ELISpot assay on d26 (i.e. 5 days after the third allo-HLA restimulation on d21) for reactivity against KC ($2x10^4$/well) of 14 donors. Reactivity to the original mDC-HLA stimulator cells ($1x10^4$/well) is also displayed. HLA-DQ and HLA-DP typings of the respective donors and KC cultures are indicated. KC were either pre-incubated over four days with or without the addition of IFN-γ. Original IFN-γ ELISpot data are exemplarily displayed in Figure 23. KC, keratinocytes; Don, donor; n.d., not determined; *, experiment one; **, experiment two.

KC #	allo-HLA-DQB1*06:02			IFN-γ		allo-HLA-DPB1*02:01	
	Don 302	Don 492				Don 914	Don 927
	02:01/03:01	02:02	HLA-DQB1*		HLA-DPB1*	04:01	03:01/04:01
55*	3 1	0 1	03:02 / 06:04	+ -	03:01 / 04:01	0 0	1 2
56*	40 1	5 0	03:01 / 05:01	+ -	03:01 / 15:01	2 0	6 1
57*	138 1	70 0	02:01 / 06:03	+ -	02:01 / 19:01	11 0	114 0
58*	1 0	6 1	03:01 / 03:03	+ -	02:01 / 04:01	9 0	159 2
59*	29 n.d.	66 n.d.	n.d.	+ -	n.d.	2 n.d.	200 n.d.
60*	11 1	20 1	02:01 / 05:03	+ -	02:01 / 02:02	26 0	288 1
61*	17 0	9 0	03:01 / 06:01	+ -	04:02	1 0	4 2
62**	67 0	105 0	03:02 / 06:03	+ -	04:01 / 04:02	1 0	1 0
63**	129 0	137 0	02:01 / 06:02	+ -	02:01 / 04:01	29 0	67 0
64**	13 0	17 1	03:03 / 05:01	+ -	04:01 / 14:01	2 0	4 0
65**	0 0	6 1	02:02 / 03:01	+ -	04:01	0 0	2 0
66**	0 0	2 1	02:02 / 03:01		06:01 / 17:01	7 0	62 0
68**	94 1	150 0	03:03 / 06:03	+ -	02:01	122 0	194 0
69**	6 0	30 1	02:01 / 05:01	+ -	03:01 / 04:01	6 0	4 0
mDC-HLA	* > 2000 ** > 2000	* > 600 ** > 2000		-		* 425 ** > 2000	* > 1000 ** > 2000
T cells only	1	1		-		1	1