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CHAPTER 6

SUMMARY AND GENERAL DISCUSSION
SUMMARY

Allogeneic hematopoietic stem cell transplantation (alloSCT) is a potentially curative treatment for various hematological malignancies. The beneficial Graft-versus-Leukemia (GvL) effect of alloSCT is mediated by donor-derived allo-reactive T cells targeting the malignant cells of the patient. Unfortunately, detrimental Graft-versus-Host-Disease (GvHD) often co-develops due to recognition of allo-antigens by donor-derived T cells on non-hematopoietic tissues. To prevent the development of GvHD, donor T cells can be depleted from the alloSCT graft. Although the risk and severity of GvHD are effectively reduced by T cell depleted (TCD) alloSCT, the absence of donor T cells in the stem cell graft also leads to an increased risk of relapses of malignancies. Early intervention with unmanipulated donor lymphocyte infusion (DLI) may effectively prevent or treat post-transplant relapses, but is frequently associated with re-introduction of GvHD. Although post-transplant relapses of chronic myeloid leukemia (CML) in chronic phase can be effectively treated with DLI, patients with relapsed acute leukemia often fail to respond to DLI and their prognosis remains poor. This disease-specific difference in efficacy of DLI may be explained by the poor capacity of acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and CML in blast crisis (CML-BC) to function as professional antigen presenting cells (APC) and induce primary T cell responses. In addition, acute leukemias are generally rapidly proliferating aggressive malignancies that may outpace the donor T cell responses as induced after DLI. Therefore, novel T cell based immunotherapeutic strategies with potent efficacy, but limited treatment-related toxicity are highly relevant to improve the clinical outcome for patients with aggressive acute malignancies.

In contrast to constitutive expression of HLA-class I molecules on all nucleated cells, HLA-class II molecules are only constitutively expressed on normal hematopoietic cells as well as most B-lineage and myeloid malignancies, while most non-hematopoietic cells only express HLA-class II under inflammatory conditions. Therefore, CD4+ T cells recognizing allo-antigens in the context of HLA-class II molecules under non-inflammatory circumstances are likely to mediate selective GvL reactivity without GvHD. Clinical application of HLA-identical DLI depleted of CD8+ T cells has demonstrated to induce conversion to donor hematopoiesis and disease remissions in patients with relapsed malignancies, in the absence of induction of severe GvHD. In addition, it has been demonstrated that allo-HLA-DPB1 specific CD4+ T cells can mediate profound GvL reactivity in the absence of clinically significant GvHD after DLI following HLA-DPB1 mismatched TCD alloSCT, indicating that allo-HLA-class II molecules can also serve as malignancy-specific targets. Therefore, a possible strategy to induce profound and selective GvL immunity against acute leukemia without causing GvHD may be achieved by targeting (disparate) HLA-class II molecules by CD4+ T cell based immunotherapy following TCD alloSCT. This thesis explored the benefits and threats of this approach in a preclinical mouse model and in patients.
In **chapter 2** we investigated and compared the capacity of fully HLA-matched (12/12 match) and HLA-class II mismatched, but HLA-class I matched, DLI to induce GvL responses against established human HLA-class II positive ALL and CML-BC in NOD/scid mice. In mice engrafted with ALL or CML-BP, treatment with HLA-matched DLI induced expansion of human CD8+ and CD4+ T cells in peripheral blood, but leukemic cells were only delayed in growth, and not eliminated. In contrast, after HLA-class II mismatched DLI, leukemic cells rapidly disappeared upon emergence of human CD8+ and CD4+ T cells in peripheral blood. Analysis of the clonally isolated CD4+ and CD8+ T cells from treated mice demonstrated that none of the isolated CD4+ and CD8+ T cell clones from mice treated with HLA-matched DLI were allo-reactive, as defined by the absence of recognition of patient malignant and non-malignant hematopoietic cells, whereas the majority of CD4+, but none of the CD8+, T cell clones from mice treated with HLA-class II mismatched DLI were allo-reactive and restricted by the mismatched HLA-DRB3, HLA-DQB1 and HLA-DPB1 alleles of the patient. The results in this study show impairment of HLA-matched DLI to mediate GvL reactivity against established ALL and CML-BC, whereas the *in vivo* immunogenicity of the leukemic cells was sufficiently high to generate effective T cell responses across HLA-class II barriers. The data therefore emphasize the relevance of HLA-class II mismatched DLI as treatment modality that is necessary and sufficient to combat poorly immunogenic HLA-class II positive aggressive malignancies.

As the allo-HLA reactive T cell responses arise from a donor T cell repertoire that has not been selected based on tolerance for allo-HLA molecules, they may contain T cells with recognition of multiple disparate allo-HLA molecules. This broad allo-HLA reactivity may confer a risk for detrimental off-target reactivity in patients and thus hamper clinical application of allo-HLA restricted T cells with beneficial reactivities. In **chapter 3** we investigated the potential risk for off-target toxicity in an allo-HLA-class II directed T cell response induced in human CML-BC engrafted NOD/scid mice after treatment with HLA-class II mismatched DLI. As GvL effects in leukemia-engrafted NOD/scid mice treated with DLI can develop in the absence or presence of xenogeneic GvHD, we investigated whether xeno-reactivity occurred as a result of off-target cross-reactivity of allo-HLA-class II directed T cells or whether GvL reactivity and xeno-reactivity were mediated by separate T cells. Analysis of the clonally isolated CD4+ and CD8+ T cells from treated mice during GvL effect and xenogeneic GvHD demonstrated that T cell responses induced *in vivo* after HLA-class II mismatched DLI consisted of allo-HLA-class II restricted leukemia-reactive CD4+ T cells and different H-2-class I or H-2-class II restricted xeno-reactive CD4+ and CD8+ T cells, demonstrating that GvL reactivity and xenogeneic GvHD are mediated by different T cells with distinct specificities. The results in this study show that allo-reactive T cells isolated during an *in vivo* allo-HLA-class II directed immune response did not exert broad allo-MHC cross-reactivity. The data show that the NOD/scid mouse model of ALL and CML-BC may be suitable for analysis of *in vitro* generated and selected leukemia-reactive allo-HLA-class II directed T cells.
in a diversity of HLA-mismatched situations for their beneficial on-target efficacy and potential to exhibit broad cross-reactive off-target toxicity.

Immunotherapy across HLA-class II barriers with CD4+ T cell selected DLI as compared to unmanipulated DLI may confer less risk for development of GvHD due to the absence of CD8+ T cells recognizing allo-antigens in the context of ubiquitously expressed HLA-class I molecules. Whether CD4+ T cells, in the absence of any CD8+ T cells, were capable of acting as T-effector cells in GvL immunity was not clear. In chapter 4 we investigated and compared the capacity of CD4+ T cell selected and unmanipulated DLI in three HLA-class II mismatched settings to mediate GvL responses against established human ALL and CML-BC in NOD/scid mice. Highly purified CD4+ DLI, in the absence of any CD8+ T cells, efficiently eradicated engrafted leukemic cells, and induced leukemic cells to acquire an APC phenotype in vivo on treatment, similarly as unselected DLI. Analysis of the clonally isolated CD4+ T cells from CD4+ DLI treated mice demonstrated that allo-reactive CD4+ T cells were restricted by the allo-HLA-class II molecules, and exerted T-effector functions by direct cytolytic activity against leukemic cells and T-helper functions by producing cytokines and activating leukemic cells to acquire an APC phenotype. The results in this study show the capacity of CD4+ T cells directed to allo-HLA-class II molecules to act as potent mediators of GvL immunity. The data therefore emphasize the clinical relevance of targeting allo-HLA-class II molecules by CD4+ T cell based immunotherapy for HLA-class II positive aggressive malignancies after alloSCT.

Clinical application of CD8+ T cell depleted DLI following HLA-matched related and unrelated alloSCT induced conversion to donor hematopoiesis and disease remissions in patients with relapsed malignancies in the absence of induction of severe GvHD, providing a rationale for exploration of CD4+ T cell based immunotherapy as treatment modality for selective stimulation of GvL reactivity after TCD alloSCT. To explore the clinical impact of CD4+ DLI in patients, a clinical study was initiated at the department investigating the efficacy and toxicity of prophylactic CD4+ T cell selected DLI administered early after TCD alloSCT. In chapter 5, we analyzed the specificity of T cell responses in two patients with AML who converted to donor chimerism but developed severe colonic GvHD after prophylactic CD4+ DLI administered 3 months after 10/10 HLA-matched, but HLA-DPB1 mismatched TCD unrelated alloSCT. Clonal analysis of activated CD4+ and CD8+ T cells isolated during GvHD demonstrated allo-reactivity exerted only by CD4+ T cells, which were directed against patient mismatched HLA-DPB1 molecules and recognized patient hematopoietic cells and skin-derived fibroblasts cultured under inflammatory conditions to induce HLA-class II expression. Analysis of the clinical course of the patients suggested that the inflammatory conditions in patients were likely to be caused by cytomegalovirus (CMV) reactivations prior to CD4+ DLI and concurrent patient-derived anti-CMV T cell responses, since we demonstrated that patient samples at the time of CD4+ DLI contained residual patient-derived T cells with an activated phenotype and significant numbers of CMV specific T cells. As we demonstrated that CMV infection of non-hematopoietic skin fibroblasts itself does not induce
HLA-class II expression, activated CMV specific T cells are likely to have induced a particularly vigorous local pro-inflammatory response in CMV infected colonic tissues, leading to upregulated HLA-class II expression on non-hematopoietic cells, and therefore predisposing them to attack by HLA-DPB1 specific CD4+ T cells. The results in this study show that GvHD following HLA-DPB1 mismatched CD4+ DLI can be mediated by allo-HLA-DPB1 directed CD4+ T cells, and provide relevant insight into clinical circumstances that may trigger development of GvHD after immunotherapy with CD4+ DLI.
GENERAL DISCUSSION

Benefits and limitations of the NOD/scid mouse model of human ALL and CML-BC

The NOD/scid mouse model of human ALL and CML-BC allows *in vivo* study of clinically relevant questions with regard to various aspects of leukemia biology as well as therapeutic efficacy of treatment modalities, including T cell based immunotherapies. NOD/scid mice can be reproducibly engrafted with human ALL and CML-BC, and progression of the malignancy can be quantitatively monitored in the course of time\(^\text{221}\). Previously, this model has been used to investigate the *in vivo* specificity and GvL efficacy of *in vitro* generated and selected leukemia-reactive T cells\(^\text{219}\) and MiHA specific T cell clones\(^\text{218}\). The kinetics of the T cell therapy can be assessed in this model by quantitatively analyzing the expansion and persistence of T cells in time\(^\text{218-220}\). We have used the model in chapter 4 to investigate the efficacy of CD4+ T cells to act as sole mediators of GvL immunity, in the absence of any contaminating or residual CD8+ T cells. This relevant question cannot conclusively be addressed in the clinical transplantation setting due to the presence of donor-derived CD8+ T cells in the patients. In addition, this model allowed us to disclose *in vivo* cross-talk between human leukemic cells and human T cells resulting in induction of human leukemic cells with a leukemic APC phenotype (leukemic APC). Although we did not study the relevance of this phenomenon in this thesis, it may be possible that the efficacy and overall magnitude of the GvL response is dependent on the ability of leukemic cells to acquire a leukemic APC phenotype *in vivo* upon interaction with human T cells. Furthermore, the NOD/scid mouse model of human ALL and CML-BC allowed us to compare the efficacy of HLA-disparate versus fully matched cellular therapy to mediate GvL immunity against these malignancies, as described in chapter 2, which obviously cannot be adequately addressed in a clinical setting within a single patient.

A limitation of the NOD/scid mouse model of human ALL and CML-BC is the development of lethal xeno-reactive responses in these mice upon infusion of a broad repertoire of human T cells. This phenomenon reduces the follow up time of GvL reactivity, and prohibits long-term analysis of remissions induced by sustained T cell responses. Infusion of leukemia-reactive T cells with a restricted T cell repertoire has previously been demonstrated to prevent development of xenogeneic GvHD\(^\text{218,219}\), thus allowing long-term analysis of the efficacy of these cellular therapies in leukemia-engrafted NOD/scid mice. In chapter 3, we investigated and demonstrated that GvL reactivity and xeno-reactivity are mediated by different human T cells with distinct specificities. Many investigators have proposed the use of xenogeneic GvHD developing in immunodeficient mice after infusion of human T cells as a model for human GvHD\(^\text{193-196}\). It is obvious that xenogeneic GvHD cannot easily be extrapolated to human GvHD, but characterization of the similarities and differences between both types of GvHD may help to mimick and investigate certain aspects of clinical GvHD in an *in vivo* pre-clinical setting. Whether xeno-reactivity as mediated by human T cells resembles HLA restricted allo-reactivity in humans has not been investigated in much
detail. We demonstrated in chapter 3 that xeno-reactive human T cells resemble allo-reactive T cells specific for mismatched HLA alleles in that they recognize target cells in a MHC-restricted fashion. Although xeno-reactive T cells failed to exert direct cytolytic activity against murine target cells, what may be explained by a species barrier in accessory molecules, human cytokines were produced at pathological levels that are likely to cause lethal xeno-reactivity in mice. Others have shown that infiltration of human T cells into murine tissues is variable in xenogeneic GvHD in different immunodeficient models, and that the typical pathohistology of xenogeneic GvHD can be distinguished from human GvHD. In addition, recognized allo-antigens by human T cells in xenogeneic and human GvHD are not similar, and therefore, the inability of human T cells to exert xeno-reactivity in NOD/scid mice does not preclude reactivity of these T cells against non-hematopoietic tissues in human GvHD. Therefore, to assess whether allo-reactive T cells may cause GvHD upon adoptive transfer to patients, reactivity of these T cells should also be analyzed in vitro against a panel of human non-hematopoietic target cells. However, the value of xenogeneic GvHD models has been demonstrated for evaluation of specific therapeutic interventions directed at suppressing engrafted human T cells or cytokines produced by engrafted human T cells, and as such, the models can improve our understanding of mechanisms of GvHD development. In this thesis, the NOD/scid mouse model of human ALL and CML-BC has been used to analyze the in vivo efficacy of human leukemia-reactive T cell therapies as well as their potential to exert broad allo-MHC reactivity.

Benefits of targeting HLA-class II molecules in GvL immunity

Targeting donor-derived allo-reactive CD4+ T cells in GvL immunity to HLA-class II molecules on hematological malignancies has several potential benefits. Traditionally, protective anti-tumor immunity has been ascribed to CD8+ T cells with direct cytotoxic activity. As CD8+ T cells recognize antigens in the context of ubiquitously expressed HLA-class I molecules, immunotherapy with unmanipulated CD8+ T cells is likely to induce GvL reactivity as well as GvHD. In contrast, CD4+ T cells recognize antigens in the context of HLA-class II molecules, which are under non-inflammatory conditions predominantly expressed on normal and malignant hematopoietic cells. Therefore, under non-inflammatory circumstances, CD4+ T cell responses directed to minor histocompatibility antigens (MiHA) presented in the context of HLA-class II and CD4+ T cell responses directed to disparate HLA-class II molecules are both expected to selectively induce GvL effects with no or limited GvHD. In an inflammatory environment, however, non-hematopoietic cells may acquire expression of HLA-class II, and allo-reactive CD4+ T cells may therefore induce GvHD under these conditions.

Although the important role of CD4+ T cells as T-helper cells in anti-tumor immunity is generally appreciated, their relevance role as T-effector cells has been largely unexplored. MiHA specific and allo-HLA-class II restricted CD4+ T cells with direct cytolytic activity against hematological malignancies have been isolated from patients with profound GvL responses
after HLA-identical and HLA-class II mismatched DLI, respectively\textsuperscript{103-108}. Furthermore, clinical studies have shown that administration of CD4+ selected or CD8+ T cell depleted DLI can induce conversion to donor hematopoiesis and disease remissions in patients with relapsed malignancies after alloSCT without severe GvHD, suggesting that CD4+ T cells may be potent and relevant T-effector cells in inducing GvL immunity in the absence of detrimental GvHD\textsuperscript{152-154}. In the above clinical studies, however, it proved to be difficult to demonstrate that anti-tumor immunity was solely mediated by CD4+ T-effector cells, as illustrated by the finding that donor-derived CD8+ T cells were the prominent T-effector cells mediating GvL reactivity and GvHD in a CML patient who responded to CD8+ T cell depleted DLI\textsuperscript{155}. In chapter 4, we provided final evidence that CD4+ T cells, in the absence of any CD8+ T cells, can mediate efficient GvL immunity \textit{in vivo} in NOD/scid mice engrafted with HLA-class II positive malignancies. Collectively, these findings justify exploration of CD4+ T cell based immunotherapy to provide effective GvL immunity with a low risk of GvHD.

Allo-reactive donor T cell responses after HLA-identical or fully HLA-matched alloSCT and DLI are directed against MiHA, and MiHA specific T cells are known to reside within the naïve donor T cell compartment in low frequencies\textsuperscript{71;124}. In contrast, allo-reactive donor T cells targeting disparate HLA molecules can be derived from the naïve as well as memory T cell compartment\textsuperscript{122}, and their frequencies have been estimated to be a thousand fold higher than MiHA-specific T cells in HLA-identical settings\textsuperscript{123;124}. In chapter 2, we investigated and compared the efficacy of fully HLA-matched and HLA-class II mismatched DLI to elicit GvL responses against poorly immunogenic HLA-class II positive ALL and CML-BC in NOD/scid mice. We demonstrated that HLA-matched DLI failed to induce effective GvL reactivity against ALL and CML-BC, whereas DLI across HLA-class II barriers effectively mediated GvL responses. This difference in efficacy may be explained by the differences in frequencies of allo-reactive T cells between HLA-matched and HLA-class II mismatched DLI. In addition, impairment of HLA-matched DLI to induce GvL reactivity may be explained by the failure of ALL and CML-BC to acquire a professional APC phenotype\textsuperscript{75-77}, since MiHA-specific T cells derived from the naïve T cell repertoire require priming by activated APC\textsuperscript{71-74;122}. In contrast, in HLA-class II mismatched DLI, GvL responses can be initiated by memory T cells that are cross-reactive against mismatched HLA-class II molecules and do not require professional APC in order to be activated. In chapter 4, we demonstrated that some allo-HLA-class II restricted CD4+ T cell clones recognized unmodified primary ALL and CML-BC, whereas other allo-HLA-class II restricted CD4+ T cell clones recognized ALL and CML-BC only upon their \textit{in vitro} modification into leukemic APC displaying a phenotype with increased expression of HLA-class II, adhesion and co-stimulatory molecules. In addition, we demonstrated that ALL and CML-BC cells acquired a leukemic APC phenotype \textit{in vivo} upon treatment with DLI, and that a leukemic APC phenotype could be induced \textit{in vitro} upon co-culture with allo-HLA-class II restricted CD4+ T cells that are capable of recognizing unmodified ALL and CML-BC. Based on these data, we postulate that the avidity of only a fraction of allo-HLA-class II reactive CD4+
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T cells is sufficiently high to be activated in vivo by unmodified ALL and CML-BC, and that the leukemic cells acquire an APC phenotype upon interaction with these T cells, thereby further broadening the repertoire of allo-HLA-class II reactive CD4+ T cells and resulting into a profound GvL response.

HLA-identical sibling donors or fully HLA-matched (12/12 alleles) unrelated donors (URD) are regarded as the best donors for patients undergoing alloSCT. However, our data described in chapter 2 provide evidence that immunotherapy with DLI across HLA-class II barriers may be preferred to fully HLA-matched DLI for treatment of poorly immunogenic malignancies. Since under non-inflammatory conditions HLA-class II expression is mainly confined to cells of hematopoietic origin, alloSCT across HLA-class II barriers may induce a beneficial allo-HLA-class II directed CD4+ T cell response preferentially targeting patient-derived malignant (and normal) hematopoietic cells, but not non-hematopoietic cells. Moreover, alloSCT across HLA-class II barriers may also be a broadly applicable strategy as the probability of finding an URD matched at HLA-A, -B, -C and -DRB1 (8/8 match) is 60-70% for Caucasian patients226. Matching at 8/8 HLA alleles is considered the minimum requirement, as a single mismatch at any of these loci is associated with poor survival, treatment-related mortality and GvHD113;115-118. In the 8/8 HLA-matched group, genetic disparity for HLA-DQB1 is observed in 9%227 and for HLA-DPB1 in 70-80%113;119 of the cases. HLA-DQB1 and HLA-DPB1 have shown to function as classical transplantation antigens, since mismatching for these loci was associated with both GvHD and GvL effect160;228. Studies in HLA-DPB1 mismatched alloSCT have demonstrated that in contrast to non-TCD alloSCT118;160;161, however, mismatching for HLA-DPB1 in TCD alloSCT was also associated with GvL reactivity but without an effect on the incidence of GvHD159. In non-TCD alloSCT, abundant presence of pro-inflammatory soluble factors produced at significant levels in the early posttransplantation period as a result of the transplant conditioning procedure can upregulate expression of HLA-class II molecules on non-hematopoietic tissues, thereby making them targets for CD4+ T cells. When donor T cells are administered late after TCD alloSCT after the acute inflammatory processes have resolved, HLA-class II expression on non-hematopoietic cells is expected to no longer be upregulated, and consequently, allo-HLA-class II directed CD4+ T cells may confer selective GvL effect with no or limited risk of GvHD. This is supported by a study from Rutten et al.106 who demonstrated that allo-HLA-DPB1 specific CD4+ T cells after postponed infusion of DLI following HLA-DPB1 mismatched TCD alloSCT mediated profound GvL reactivity in the absence of clinically significant GvHD, indicating that disparate HLA-class II molecules can serve as malignancy-specific targets for allo-reactive CD4+ T cells administered by DLI after TCD HLA-class II mismatched alloSCT. Recent reports suggest that HLA-DQB1 mismatches may also confer limited risk of GvHD117;118. Thus immunotherapy with CD4+ DLI targeting patient mismatched HLA-DQB1 and/or HLA-DPB1 alleles under non-inflammatory conditions after TCD alloSCT may be the treatment of choice to efficiently attack poorly
immunogenic HLA-class II positive hematopoietic malignancies, while sparing non-hematopoietic tissues.

**Threats of targeting HLA-class II molecules in GvL immunity**

Therapies targeting donor-derived CD4+ T cells to HLA-class II molecules for treatment of HLA-class II positive hematological malignancies are, however, limited by several potential threats. As inflammatory conditions can induce expression of HLA-class II on non-hematopoietic cells, selective reactivity of HLA-class II restricted CD4+ T cells to hematopoietic cells requires non-inflammatory circumstances. Several lines of evidence indeed suggest that the clinical effect of CD4+ T cell responses is not only determined by the specificity of the T cells, but also by clinical circumstances that influence the activation state of GvHD target tissues. The observations that CD4+ T cell mediated GvL effects in non-TCD alloSCT can be associated with severe GvHD118;160;228;229 can be explained, as mentioned above, by the upregulated expression of HLA-class II molecules on non-hematopoietic tissues due to pro-inflammatory soluble factors released by cells of the immune system, including T cells, as the result of tissue damage induced by the conditioning regimen and upon encountering activated residual tissue-resident patient-derived APC. In addition, homeostatic proliferation of donor T cells due to lymphopenic conditions induced by the conditioning49;50 as well as danger signals provided by pathogens128;134 are likely to amplify the allo-immune response. Evidence for involvement of CD4+ T cells in GvHD early after partial TCD alloSCT has been provided by the detection and isolation of MiHA specific CD4+ T cells at the onset of GvHD after HLA-identical alloSCT230 and allo-HLA-DPB1 specific CD4+ T cells from skin biopsies of patients at the onset of GvHD after HLA-DPB1 mismatched alloSCT231.

Postponed administration of DLI 3-6 months after TCD alloSCT is associated with a reduced incidence and severity of GvHD, most likely due to largely restored tissue damage, absence of HLA-class II expression on non-hematopoietic tissues, and gradual replacement of tissue-resident APC from patient to donor origin. However, recently Rutten et al.163 demonstrated in a large group of patients that high frequencies of allo-HLA-DPB1 specific CD4+ T cells were detected in patients who responded to postponed infusion of DLI after HLA-DPB1 mismatched TCD alloSCT with and without development of GvHD. In this thesis, we provide two possible explanations for development of GvHD after postponed administration of DLI after HLA-DPB1 mismatched TCD alloSCT. In chapter 4, we demonstrated that primary ALL and CML-BC can be modulated into professional APC expressing high levels of HLA-class II, adhesion and costimulatory molecules *in vivo* in mice and *in vitro* by pro-inflammatory soluble factors produced by allo-HLA-class II restricted CD4+ T cells upon interaction with leukemic cells. This finding suggests that CD4+ DLI administered late after TCD alloSCT during overt leukemic relapse may lead to vigorous and profound GvL responses, and that pro-inflammatory cytokines released by allo-reactive donor CD4+ T cells upon interaction with leukemic cells may trigger development of GvHD by increasing HLA-class II expression on non-hematopoietic cells. In chapter 5, we
provide evidence that non-hematopoietic tissues in patients after TCD alloSCT may also become targets for GvHD as a result of infection with viral pathogens. Pro-inflammatory factors produced by virus-specific T cells as a consequence of an ongoing viral infection may upregulate HLA-class II expression on non-hematopoietic tissues, thereby making them targets for allo-reactive CD4+ T cells.

Patients undergoing TCD alloSCT are exposed to a high risk of opportunistic infections, including CMV infection, due to depletion of pathogen-specific T cells. Treatment of CMV reactivations or infections with anti-viral therapy reduces the risk of CMV disease development\textsuperscript{232}. In the two patients described in chapter 5, treatment of CMV reactivations after alloSCT with anti-viral agents coincided with expansion of patient-derived CMV specific T cells, resulting in disappearance of CMV DNA viral load from peripheral blood. Approximately one month later, at the time of CD4+ DLI, the CMV DNA viral load in peripheral blood was still undetectable, but both patients contained significant numbers of patient-derived CMV specific T cells. These patient-derived T cells directly after \textit{ex vivo} isolation displayed modest expression of HLA-DP and failed to activate allo-reactive HLA-DPB1 specific CD4+ T cells. \textit{In vitro} isolated and non-activated patient-derived CMV specific T cells also expressed low levels of HLA-DP and adhesion molecules, and were not capable of stimulating HLA-DPB1 specific CD4+ T cells. However, \textit{in vitro} stimulation of these patient-derived CMV specific T cells with CMV antigens upregulated their expression of HLA-DP and adhesion molecules, and they acquired the capacity to stimulate allo-reactive HLA-DPB1 specific CD4+ T cells. These data indicate that residual patient-derived T cells activated by CMV reactivation \textit{in vivo} can become targets for HLA-DPB1 specific CD4+ T cells and are also expected to have played a role in the induction or amplification of allo-reactive HLA-DPB1 specific CD4+ T cell responses in both patients. In addition, anti-CMV immune responses in colonic tissues probably resulted in local upregulation of HLA-class II on colonic epithelial cells. As a consequence, this local inflammatory state may have amplified the donor-derived HLA-DPB1 specific CD4+ T cell responses against HLA-class II expressing colonic epithelial cells, resulting in local exacerbation of GvHD. Recent studies by Van der Zouwen \textit{et al.}\textsuperscript{233} demonstrated that expression of MiHA and HLA molecules on non-hematopoietic cells was not sufficient for attack by MiHA specific or allo-HLA specific T cells, and that full T-effector functions required expression of adhesion molecule ICAM-1 to enable formation of high-avidity interactions with non-hematopoietic cells. ICAM-1 is only expressed on non-hematopoietic cells under inflammatory conditions, and this study therefore underlines the relevance of the local activation state of GvHD target tissues in the delicate balance between GvL effect and GvHD.

In chapter 5, we described two patients who converted to full donor chimerism and developed severe acute GvHD after prophylactic CD4+ DLI following HLA-DPB1 mismatched TCD alloSCT. In both patients, CMV reactivation and concurrent patient-derived anti-CMV T cell responses were demonstrated prior to CD4+ DLI. Other patients who participated in this
clinical study were treated with prophylactic CD4+ DLI from HLA-identical donors, and these patients showed a decrease in patient chimerism with no or only limited GvHD. The difference in development of GvHD between patients treated with HLA-DPB1 mismatched as compared to HLA-identical CD4+ DLI may be explained by the CD4+ T cell repertoire, since the two HLA-DPB1 mismatched donors probably contained significant numbers of allo-HLA-DPB1 reactive CD4+ T cells in their memory compartment which may have easily been activated by patient HLA-DP expressing non-hematopoietic cells. The same clinical circumstances and factors that have been shown to contribute to development of GvHD after HLA-DPB1 mismatched CD4+ DLI are expected to be relevant in patients treated with HLA-identical CD4+ DLI. Since the activation state of GvHD tissues, numbers of residual activated patient-derived APC as well as frequency of T cells specific for ubiquitous allo-antigens within the donor T cell repertoire are known to influence development of GvHD after DLI, more insight into these clinical circumstances and factors is strongly recommended for accurate prediction of clinical outcome of HLA-class II directed cellular immunotherapies.

**Alternative T cell based therapeutic strategies to separate GvL and GvHD**

Although studies presented in this thesis encourage targeting of disparate HLA-class II molecules by CD4+ DLI with the aim to induce efficient GvL immunity against poorly immunogenic and aggressive HLA-class II positive malignancies, clinical application of this therapy confers a risk for severe GvHD. Thus, the question remains whether the therapeutic option of CD4+ DLI to prevent or treat relapses after HLA-class II mismatched alloSCT can be refined and what alternative therapeutic strategies are available to improve the benefit to risk profile for patients.

A proposed strategy to lower the morbidity and mortality of GvHD and improve clinical outcome of patients transplanted with 10/10 HLA-matched but HLA-DPB1 mismatched unrelated alloSCT, involves targeting of specific HLA-DPB1 mismatches for which no adverse risks of alloSCT have been found (permissive mismatches) and avoiding HLA-DPB1 mismatches that have been shown to associate with adverse risks (non-permissive mismatches)234-236. This classification of HLA-DPB1 alleles is based on T cell epitope similarity rather than HLA-DPB1 allele identity, and assigns HLA-DPB1 alleles to specific groups according to their predicted immunogenicity based on the presence or absence of specific amino acid sequences within the hypervariable regions of the HLA-DPB1 chain234;236. Individuals carrying highly similar HLA-DPB1 alleles presenting shared T cell epitopes are expected to have deleted allo-reactive T cells for these epitopes, and these so-called permissive HLA-DPB1 mismatches are predicted to be tolerated. In contrast, individuals expressing highly divergent HLA-DPB1 alleles lacking shared T cell epitopes are expected to have retained allo-reactive T cells for these epitopes, and these so-called non-permissive HLA-DPB1 mismatches are predicted to confer adverse effects to alloSCT. Recently, Fleischhauer et al.237 compared in a large cohort of patients the clinical outcome of patients treated with 12/12 HLA-matched unrelated alloSCT with clinical outcome of patients
treated with HLA-DPB1 mismatched unrelated alloSCT either for permissive or non-permissive HLA-DPB1 alleles. Both permissive and non-permissive HLA-DPB1 mismatches were found to be associated with a decreased risk of relapse and an increased risk of GvHD when compared to the 12/12 HLA-matched cohort. These balanced effects resulted in a similar mortality rate in patients with permissive HLA-DPB1 mismatches and patients treated with 12/12 HLA-matched alloSCT, whereas non-permissive HLA-DPB1 mismatches were associated with an increased mortality rate as a result of an increased risk of severe GvHD. However, the relapse rate was also reduced in patients with non-permissive HLA-DPB1 mismatches as compared to patients with permissive HLA-DPB1 mismatches, suggesting that more profound allo-reactive CD4+ T cell responses may have been induced in patients with non-permissive HLA-DPB1 mismatches. Sizzano et al.\textsuperscript{238} have indeed demonstrated up to 10-fold higher T cell frequencies against non-permissive as compared to permissive HLA-DPB1 mismatches \textit{in vitro}. Rutten et al.\textsuperscript{239,240} confirmed these results both \textit{in vitro} and \textit{in vivo}, but also demonstrated that both permissive and non-permissive HLA-DPB1 mismatches can result in strong allo-HLA-DPB1 specific CD4+ T cell responses. We confirmed this finding in \textit{chapter 5} by demonstrating that T cell clones directed against both permissive (HLA-DPB1*0101) and non-permissive (HLA-DPB1*0301) HLA-DPB1 alleles could be isolated from patient 1 during \textit{in vivo} immune responses mediating GvL effect and GvHD. Similarly, others isolated HLA-DPB1 specific CD4+ T cells from skin biopsies of patients at the onset of acute GvHD after both permissive and non-permissive HLA-DPB1 mismatched alloSCT\textsuperscript{241}. Collectively, these findings suggest that HLA-DPB1 disparities cannot simply be distinguished into permissive and non-permissive mismatches. However, the differences in frequencies of allo-reactive T cells for permissive and non-permissive HLA-DPB1 mismatches allows manipulation of the cell dose, recipient conditions and timing of administration of CD4+ DLI to achieve optimal GvL reactivity with a low risk of GvHD. Lowering the cell dose of CD4+ DLI in case of non-permissive HLA-DPB1 mismatches, for example, may preserve the GvL effect with a reduced risk of GvHD. In addition, anti-viral prophylaxis to prevent systemic inflammatory infections as well as increasing the interval between alloSCT and CD4+ DLI may reduce the risk of GvHD after HLA-DPB1 mismatched alloSCT, what may be particularly relevant for non-permissive mismatches.

Donor T cells with beneficial GvL and detrimental GvHD reactivities have shown to be retained within polyclonal allo-reactive T cell responses\textsuperscript{242-246}. Therefore, adoptive transfer of donor CD4+ T cells with a restricted T cell repertoire, devoid of detrimental specificities, may be another approach to effectively separate GvL from GvHD. Several strategies for selective depletion of allo-reactive T cells have been developed, and the majority of these strategies are based on \textit{ex vivo} stimulation of donor T cells with patient hematopoietic APC as stimulator cells, followed by selective elimination of allo-reactive T cells by virtue of their activation status\textsuperscript{247-249}. Adoptive transfer of allo-depleted T cells was associated with less GvHD in clinical trials, but lack of pathogen-specific and in particular leukemia-specific immunity was the major cause of treatment failure\textsuperscript{250}. The use of hematopoietic APC as stimulator cells most likely activates allo-
reactive T cells against MiHA with ubiquitous and hematopoiesis-restricted expression profiles, and depletion of activated T cells thus results in reduced frequencies of allo-reactive T cells with detrimental and beneficial reactivities. In chapter 5, we demonstrated that recognition of cytokine-treated patient-derived fibroblasts was restricted to a proportion of allo-HLA-DPB1 specific CD4+ T cell clones isolated from patient 1 (9 out of 19) and patient 2 (10 out of 16), whereas patient-derived hematopoietic cells were strongly recognized by all allo-HLA-DPB1 specific CD4+ T cell clones. These data suggest that the CD4+ T cells recognize cell type specific antigens in allo-HLA-DPB1 molecules, and depletion of activated CD4+ T cells upon stimulation with cytokine-treated patient-derived non-hematopoietic cells, instead of patient-derived hematopoietic APC, may therefore be a more selective strategy to manipulate CD4+ DLI for induction of GvL reactivity with a reduced risk of GvHD in HLA-class II mismatched alloSCT. As non-hematopoietic cells, primary skin fibroblasts and keratinocytes can be obtained from minimally invasive skin punch biopsies from patients prior to alloSCT, but these cells subsequently need to be expanded in vitro to sufficient numbers within a relatively limited time span. Nonn et al. have investigated the feasibility of this approach and showed that skin fibroblasts, but not keratinocytes, could be reliably expanded to large cell numbers. In addition, they showed that cytokine pre-treated fibroblasts can be used for substantial depletion of allo-reactive T cells for allo-HLA molecules and ubiquitous MiHA from donor-derived T cell lines stimulated with patient-derived leukemic cells based on surface expression of activation induced antigen CD137. Although this approach is technically and logistically challenging, it may allow selective depletion of CD4+ T cell reactivities against ubiquitously expressed allo-HLA-class II restricted antigens, while preserving CD4+ T cell reactivities targeting allo-HLA-class II restricted antigens with restricted expression to malignant (and normal) hematopoietic cells of the patient, thereby reducing the risk of GvHD after adoptive transfer.

Detrimental T cell reactivities against non-hematopoietic tissues of the patient may also be bypassed by adoptive transfer of donor T cells selectively targeting MiHA with hematopoiesis restricted expression. A limited number of hematopoiesis-specific MiHA presented in different HLA-class I and HLA-class II molecules have been identified thus far, allowing treatment of only a subset of patients who are mismatched with their donors for the MiHA of interest. Broad application of this T cell therapy therefore requires identification of multiple therapeutically relevant MiHA with balanced population frequencies in common HLA-class I and HLA-class II alleles. Moreover, adoptive transfer to patients with relapsed leukemia after alloSCT requires robust methods for in vitro generation and expansion of MiHA specific T cells to cell numbers that are sufficient for large-scale clinical application, but these methods are currently largely unavailable. Recently, Warren et al. demonstrated for the first time in a clinical phase I/II study the feasibility of adoptive transfer of in vitro generated, selected and expanded MiHA specific CD8+ T cells to patients with acute leukemia who relapsed after alloSCT. Adoptive transfer of MiHA specific CD8+ T cells resulted in transient clinical remissions in several patients, but in vitro
selection criteria of MiHA specific CD8+ T cells based on recognition of patient hematopoietic cells and lack of reactivity against patient-derived fibroblasts (only in the absence of cytokines) did not prevent development of GvHD. This study suggests that more stringent selection of MiHA is necessary to exclude all MiHA that are expressed on non-hematopoietic cells under normal as well as inflammatory conditions.

An alternative group of HLA-class II associated MiHA that can be targeted by T cell therapies with the aim to induce GvL reactivity without GvHD does not require selection based on their tissue distribution pattern, but on differences in processing and presentation on hematopoietic and non-hematopoietic cells. Recently, Kremer et al. identified a group of ubiquitously expressed HLA-class II associated MiHA (HLA-DM sensitive antigens) that are efficiently presented on HLA-class II positive malignant (and normal) hematopoietic cells in particular of B cell origin, but not on (cytokine-treated) non-hematopoietic cells. Non-hematopoietic cells failed to present HLA-DM sensitive antigens even under inflammatory conditions due to their failure to express HLA-DO, a relevant molecule in HLA-class II antigen processing and presentation. HLA-DM sensitive antigens represent an immunogenic class of antigens, since they have been identified as targets for allo-reactive CD4+ T cells during an in vivo immune response in a patient successfully treated with unmanipulated DLI after HLA-identical alloSCT. Selective processing and presentation of this category of antigens on HLA-class II positive malignant (and normal) hematopoietic cells opens possibilities for selective targeting of HLA-class II positive malignancies without inducing GvHD.

Based on the finding that expression of HLA-DQ on non-hematopoietic cells was only moderately upregulated under inflammatory conditions as compared to HLA-DR and HLA-DP, and that levels of expression were not sufficient for recognition by HLA-DQB1 restricted MiHA-specific CD4+ T cells with broad tissue distribution, whereas these T cells efficiently recognized HLA-DQ positive primary malignant cells, Griffioen et al. speculated that allo-reactive CD4+ T cells recognizing antigens in the context of HLA-DQ may exert more selective GvL reactivity without GvHD as compared to CD4+ T cells specific for HLA-DR or HLA-DP restricted allo-antigens. Interestingly, studies analyzing the impact of mismatching for HLA-DQB1 on the outcome of alloSCT demonstrated no adverse risks of HLA-DQB1 mismatches. Others have reported, however, that expression of HLA-DQ on ALL and AML cells may be inferior as compared to HLA-DR and HLA-DP. In chapter 4, we confirmed that HLA-DQ was expressed at lower levels on primary leukemic cells as compared to HLA-DR and HLA-DP. Allo-reactive HLA-DQB1 restricted CD4+ T cells may therefore also have a reduced capacity to exert direct GvL reactivity. These findings emphasize the relevance of more knowledge of the expression patterns of HLA-DR, HLA-DQ and HLA-DP on malignant (and normal) hematopoietic cells of different origins and on non-hematopoietic cells under normal and inflammatory circumstances for optimal design of CD4+ T cell based immunotherapies after alloSCT.
Concluding remarks

In conclusion, restricted expression of HLA-class II molecules on cells of hematopoietic origin under non-inflammatory conditions is the fundamental basis for selective targeting of HLA-class II positive hematological malignancies by CD4+ T cells without or with a low risk of GvHD. In this thesis, we demonstrated that HLA-class II directed CD4+ T cell responses can also lead to development of GvHD due to increased expression of HLA-class II molecules on non-hematopoietic tissues under inflammatory circumstances. A diversity of possibilities, however, can still be considered to manipulate HLA-class II directed CD4+ T cell responses in favor of GvL reactivity. The dose of CD4+ T cells can be adjusted to control the magnitude of the immune responses, the timing of adoptive transfer of CD4+ T cells can be based on concomitant specific clinical circumstances and CD4+ T cells for adoptive transfer can be selected based on their antigen specificity to control the risk for antigen recognition on non-hematopoietic tissues. All these strategies may be followed to exploit the therapeutic potential of targeting HLA-class II molecules on hematological malignancies by CD4+ T cells in order to induce an optimal balance between beneficial GvL reactivity and detrimental GvHD.