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CHAPTER 1

INTRODUCTION
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ACUTE LEUKEMIA

Acute leukemias are a group of hematological disorders characterized by aggressive and uncontrolled proliferation of immature hematopoietic progenitor cells in the bone marrow and peripheral blood\(^1\). Accumulation of these rapidly growing malignant blast cells in the bone marrow and peripheral blood leads to replacement and inhibition of normal hematopoiesis, eventually resulting in bone marrow failure if left untreated. Acute leukemias can be divided into two types, namely acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), on the basis of the lymphoid or myeloid lineage commitment of the malignant clone. Chronic myeloid leukemia (CML) is a myeloproliferative disorder of the hematopoietic stem cell characterized by the Philadelphia (Ph) chromosome, a chromosomal abnormality resulting from the t(9;22) reciprocal translocation\(^2\). CML inevitably evolves into a blast crisis (CML-BC) as the result of additional transformations of the malignant cells, resulting in a disease resembling and behaving clinically like an acute leukemia with aggressive proliferation of malignant blast cells of lymphoid or myeloid lineage. Acute leukemias can occur at any age, but ALL is most common in childhood and young adulthood (<20 years of age), whereas AML and CML-BC are rare in childhood, but common in adults and elderly (>65 years of age). In the United States, the annual incidence of ALL in childhood and young adulthood is 3 per 100,000 and 1 per 100,000 in adults and elderly\(^3\). For AML, the annual incidence is 3 per 100,000 in adults and increasing to 16 per 100,000 in the elderly. The annual incidence of CML is 1.5 per 100,000 in adults and increasing to 7 per 100,000 in the elderly. In the Netherlands, similar age-specific incidence rates are observed with approximately 200 new ALL, 600 new AML and 500 new CML cases diagnosed each year\(^4\).

ALL and AML are classified into different subtypes according to the World Health Organization classification system that combines morphological, cytochemical, immunophenotypic, genetic and clinical features of the diseases\(^5\). Based on these characteristics, patients are also stratified into risk categories correlating with unfavourable, intermediate or favourable prognosis, which is defined by the responsiveness of different ALL and AML subtypes to conventional chemotherapy. Treatment regimens for acute leukemia generally include the administration of multiple chemotherapeutic agents during the induction and post-remission period\(^6\)\(^-\)\(^8\). The cure rates of ALL and AML with chemotherapy differ among various subtypes. For instance, the Ph chromosome is observed in approximately 3% of pediatric ALL\(^9\)\(^,\)\(^10\) and in 20-44% of ALL in adults and elderly\(^11\)\(^,\)\(^12\), and its presence is an indicator of unfavorable prognosis reflected by high relapse risk after chemotherapy. In pediatric ALL, the 5-year survival rates with chemotherapy are approximately 80%\(^13\)\(^-\)\(^15\). However, in adults with ALL and in patients with AML, the 5-year survival rates with chemotherapy are 15-50%\(^16\)\(^-\)\(^18\) and 4-69%\(^19\)\(^-\)\(^23\), respectively. The response rates
of patients with CML-BC to chemotherapy are approximately 22-61% with 1-year survival rates for responders of 20-50%, but most patients eventually relapse\textsuperscript{24-28}.

Despite intensive chemotherapy, the majority of patients with acute leukemia cannot be cured with chemotherapy alone and require additional treatment modalities. Novel treatment modalities have emerged with targeted and subtype-specific mode of action, such as tyrosine kinase inhibitors\textsuperscript{29} and monoclonal antibodies\textsuperscript{30}. Tyrosine kinase inhibitors have been explored in patients with Ph positive ALL and CML-BC as these agents have shown efficacy in controlling the progression of Ph positive chronic phase CML and are currently its first line therapy\textsuperscript{31,32}. Although tyrosine kinase inhibitors can induce clinical responses in patients with Ph positive ALL and CML-BC, long-term outcome has not improved significantly mainly due to unresponsiveness or resistance to therapy\textsuperscript{33-36}. Application of different monoclonal antibodies targeting a variety of cell surface molecules expressed by different subtypes of acute leukemias in combination with chemotherapy is currently explored and may improve prognosis\textsuperscript{30,37,38}. A common treatment modality that may benefit all patients with acute leukemia who relapse or carry a high-risk for relapse after chemotherapy is allogeneic hematopoietic stem cell transplantation (alloSCT) due to its additional therapeutic effect.

\textbf{ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION}

AlloSCT is a potentially curative treatment modality for a range of malignant hematological disorders\textsuperscript{39-41}. The alloSCT procedure involves pre-transplantation treatment of patients with irradiation, chemotherapy and/or immune suppression as part of the conditioning regimen to eradicate malignant cells and to reduce and suppress the hematopoietic system of the patient allowing engraftment of a donor-derived hematopoietic system. Following conditioning, a healthy donor-derived graft containing hematopoietic stem and progenitor cells and immune cells, including T, B and NK cells, is infused that can fully replace the patient hematopoietic system. Traditionally, myeloablative conditioning regimens have been used with profound myeloablative and immunosuppressive effects aiming at complete eradication of the malignant cells and prevention of graft rejection by patient immune cells. Irreversible damage to patient bone marrow caused by these regimens requires engraftment of donor-derived hematopoietic cells to prevent hematopoietic failure\textsuperscript{42}. In addition, the beneficial effects of myeloablative conditioning regimens are frequently offset by the significant regimen-related toxicity, making this treatment unsuitable for elderly and patients with compromised organ function or co-morbidities. Development of non-myeloablative and reduced intensity conditioning regimens that are not fully myeloablative, but are sufficiently myelo- and immunosuppressive to allow engraftment of donor stem cells and limit graft rejection, has decreased the toxicity of alloSCT, thereby making the treatment applicable for patients considered ineligible for myeloablative conditioning\textsuperscript{42-44}. Since these regimens do not eliminate all malignant cells, they rely on the beneficial Graft-versus-Leukemia (GvL) effect
mediated by donor immune cells present in the alloSCT graft to fully eradicate residual malignant cells.

The initial indirect evidence for the beneficial GvL effect in clinical transplantation came from studies reporting that patients who developed Graft-versus-Host Disease (GvHD) after alloSCT had a lower probability of leukemic relapse\textsuperscript{45,46}. GvHD is a potentially life-threatening complication in which donor immune cells attack healthy tissues of the patient, causing significant morbidity and mortality\textsuperscript{47,48}. GvHD is clinically divided as acute and chronic GvHD based on the time of onset and distinct clinical presentations. Acute GvHD usually manifests within 100 days after alloSCT and is characterized by damage to the skin, liver and/or gut. The severity of acute GvHD is clinically divided into four grades (I-IV) depending on the stage of involvement of these organs. Chronic GvHD typically presents later after alloSCT and frequently follows acute GvHD (further referred as GvHD). In chronic GvHD, multiple organs are affected causing autoimmune like symptoms. In the early posttransplant period, lymphodepletion caused by the conditioning regimen induces compensatory proliferation of donor immune cells, called homeostatic proliferation, which is likely to increase detrimental reactivity against healthy tissues of the patient\textsuperscript{49,50}. Depletion of T cells from the alloSCT graft is effective at reducing the incidence and severity of GvHD, but also associated with an increased risk of relapse of malignancies\textsuperscript{51,52}. Large retrospective analysis has indeed demonstrated a low rate of leukemic relapses in patients with GvHD, whereas this rate is higher in patients without clinically evident GvHD and most significantly increased in patients receiving T cell depleted (TCD) alloSCT grafts\textsuperscript{53}. These studies provided compelling evidence that donor T cells present in the alloSCT graft play a crucial role in development of both GvHD and GvL effect and illustrated the shared biology between these clinical entities. In addition, clinical observations that the incidence of leukemic relapse was higher after autologous and syngeneic SCT as compared to alloSCT indicated that the mere presence of T cells was not sufficient to mediate GvL effect, but that genetic disparity between patient and donor was required for the beneficial GvL effect\textsuperscript{54,55}.

**DONOR LYMPHOCYTE INFUSION**

Relapses of hematological malignancies are a major cause of mortality after alloSCT\textsuperscript{56,57}. First direct evidence for the beneficial GvL effect by allogeneic T cells without additional chemotherapy was reported in patients with relapsed CML after alloSCT who achieved sustained complete remissions after treatment with donor lymphocyte infusion (DLI) from the transplant donor\textsuperscript{48}. This report has led to the development of DLI as most common cellular intervention for treatment and prevention of relapses of various hematological malignancies after alloSCT. Although profound GvL responses mediated by DLI are observed in various malignancies, the susceptibility of different types of hematological malignancies to respond to DLI varies\textsuperscript{57,59,60}. The best responses to DLI are observed in patients with chronic phase CML, with approximately 75% of patients
achieving complete long-lasting remissions. Patients with CML-BC are less responsive to DLI, with complete remissions in 12-33% of patients, and a poor two-year overall survival (12-16%). Overall remission rates in patients with AML are 15-63%, with a two-year overall survival between 15-31%. ALL has demonstrated to be the least susceptible to DLI, with 0-25% complete remissions and 5-25% of patients achieving survival longer than one year.

The lack of responses in ALL, AML and CML-BC are believed to be multifactorial. Clinically evident GvL effects of DLI are documented to require weeks to months to become apparent, suggesting that DLI may often fail to control these malignancies due to their rapid growth. In support of this, it has been demonstrated that reduction of tumor burden by chemotherapy prior to DLI can improve response rates in ALL and AML. In addition to a reduction in tumor burden, chemotherapy prior to DLI may induce lymphodepletion, thereby allowing homeostatic proliferation of the infused donor T cells. Furthermore, it is generally appreciated that for induction of efficient primary GvL responses in vivo, priming of allogeneic T cells by professional antigen presenting cells (APC) is required. It has been illustrated that primary ALL, CML-BC and AML cells lack a professional APC phenotype and that expression of adhesion and co-stimulatory molecules which are relevant for proper activation of allogeneic T cells are low or absent. In vitro modification of these leukemic cells into leukemic APC displaying increased expression of HLA, adhesion and co-stimulator molecules leads to an enhanced capacity of these malignancies to stimulate allogeneic T cell responses. Therefore, it may be possible that the efficacy of DLI is dependent on the ability of leukemic cells to become leukemic APC in vivo or on the presence of residual patient-derived normal APC of hematopoietic origin.

As patients with ALL, AML and CML-BC are usually at high risk of relapse after alloSCT, administration of DLI prophylactically to prevent relapse or preemptively to correct incomplete donor chimerism has been explored. Although these treatments have proven to induce high responses and full donor chimerism, the increased risk of severe GvHD continues to be a significant cause of morbidity and mortality. Therefore, refining these strategies to improve the benefit to risk profile for these patients is warranted.

**BIOLOGY OF ALLOREACTIVITY**

**Histocompatibility antigens**

AlloSCT is accompanied by reciprocal immune reactions between patient and donor immune cells due to genetic disparity, leading to both detrimental and beneficial clinical responses. Graft rejection, GvHD and GvL effect are the main clinical manifestations of these allo-reactive immune responses. Graft rejection can occur when immunocompetent residual patient-derived T cells exert reactivity against donor cells within the alloSCT graft, but significant immunosuppression of the patient as a part of the pre-transplantation conditioning regimen efficiently minimizes this
phenomenon. Both GvHD and GvL effect arise from alloreactivity of immunocompetent donor T cells towards disparate antigens expressed by patient cells.

Human leukocyte antigen (HLA) molecules are cell surface molecules expressed on nucleated cells, which present potentially antigenic peptides to T cells and are therefore crucial for development of allo-immune responses. HLA molecules that are most important in immune responses are divided into HLA-class I (HLA-A, -B and -C) and HLA-class II (HLA-DR, -DQ and –DP) molecules. HLA-class I molecules are expressed on all nucleated cells and present intracellularly processed peptides (8-11 amino acids in length) of self or foreign origin to CD8+ T cells. HLA-class I molecules are heterodimers consisting of a membrane-spanning α-chain and a soluble β-chain (beta2-microglobulin). Segments of the α-chain form the peptide-binding domains (α1 and α2) that are important for interaction with the T cell receptor, and the immunoglobulin-like domain (α3) is relevant as docking site for the CD8 co-receptor. HLA-class II molecules are normally expressed on a restricted range of cells, including B cells, monocytes, macrophages, dendritic cells and thymic epithelial cells, but expression can be induced on various hematopoietic and non-hematopoietic cells under inflammatory circumstances. HLA-class II molecules are heterodimers consisting of a membrane-spanning α-chain and β-chain. Segments of the α-chain and β-chain form the peptide-binding domains (α1 and β1), and the immunoglobulin-like domain (β2) forms a docking site for the CD4 co-receptor. HLA-class II molecules present peptides (12-25 amino acids in length) to CD4+ T cells that are generally derived from extracellular proteins from self or foreign origin.

**T cells**

The ability of T cells to distinguish self from foreign antigens and establish immunological memory plays an important role in alloSCT. Antigenic peptides, called epitopes, are generated by the antigen processing machinery within the cell and presented to CD8+ and CD4+ T cells by HLA-class I and HLA-class II surface molecules, respectively. Tolerance to self-antigens presented by self-HLA molecules is a feature imprinted during thymic T cell development to prevent generalized autoimmunity. T cells are not tolerant towards foreign antigens, and therefore, cells expressing these foreign antigens are eliminated. Immunological memory allows long-term surveillance and initiation of a fast and efficient T cell response upon re-encounter with the foreign antigen, which is essential for control of latent or persistent viral infections.

T cells express T cell receptors (TCRs), which recognize antigenic peptides in the context of HLA molecules. The majority of mature peripheral T cells express a TCR consisting of a transmembrane heterodimer of a TCR α and β chain. In addition to the TCR, mature T cells express a complex of CD3 proteins necessary for TCR signaling. T cell precursors originate in the bone marrow and traffic to the thymus where they undergo several stages of T cell development before populating the periphery. In the thymus, rearrangement of TCR genes takes place resulting in the formation of a diversity of TCRs. Subsequent thymic selection is determined by the strength of the
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TCR to interact with self-peptide/HLA complexes expressed by thymic APC. During this process of positive selection in the thymic cortex, immature T cells expressing TCRs with adequate affinity for self-antigen/HLA complexes receive a survival signal, whereas T cells with TCRs with no or low affinity die by neglect. Furthermore, T cells interacting with HLA-class I molecules commit to the CD8 lineage, whereas T cells interacting with HLA-class II molecules mature into CD4+ T cells. Subsequently, negative selection occurs in the thymic medulla, and CD8+ and CD4+ T cells interacting with self-peptide/HLA complexes with high avidity are eliminated to prevent autoimmunity. Thymic education and selection processes ensure that functional mature naïve T cells leave the thymus equipped with a unique TCR that is tolerant towards self-antigen/HLA complexes.

**T cell activation and response**

Immune responses are initiated in secondary lymphoid organs where naïve CD4+ and CD8+ T cells are retained and activated by APC presenting foreign antigens taken up in the periphery. The signals that lead to T cell activation are generated at the contact area between T cells and APC, where TCRs, peptide/HLA complexes and adhesion and co-stimulatory molecules are clustered. Naïve T cells require two signals for activation, proliferation and differentiation into memory T cells. The first signal is provided by the TCR upon interaction with the peptide/HLA complex presented on the surface of the activated APC. For proper activation of naïve T cells, professional APC are required. Dendritic cells (DC) possess specific features for pathogen recognition, antigen capturing and presentation, adhesion and co-stimulatory molecules, and migratory capacity allowing them to act as professional APC. Activation of DC can be triggered through a variety of receptors for pathogens (e.g. Toll like receptors), soluble inflammatory factors (e.g. TNF-α, IL-1) and T cells (CD40 ligand), which lead to maturation of the DC. Maturation stimuli upregulate expression of HLA and co-stimulatory molecules to ensure a strong capacity to present antigens and induce TCR triggering and amplification of signal transduction pathways in naïve T cells. The second signal is provided by co-stimulation upon interaction of accessory cell-surface molecule CD28 expressed on the naïve T cells with CD80/CD86 molecules expressed on the activated APC. Co-stimulation is required to amplify the signaling process initiated by the TCR, and naïve T cells become anergic in the absence of co-stimulatory signals.

After proper activation, naïve CD4+ and CD8+ T cells undergo rapid clonal expansion, acquire unique phenotypic and functional characteristics that allow them to migrate to sites of inflammation or infection and exert as T effector cells. Upon removal of the antigen, a contraction phase is initiated, and a subset of T cells become memory T cells to enable long-term immunosurveillance to the antigen to which they have been primed. In contrast to naïve T cells, memory T cells can be activated in the absence of co-stimulation as the TCR upon triggering by peptide/HLA complexes is efficiently coupled to downstream signaling processes. As a
consequence, memory T cells can be activated by non-professional and non-activated APC, and upon interaction, memory T cells rapidly expand and efficiently exert effector functions.

Depending on the priming environment (e.g. type of antigen, type of APC, cytokine environment), naïve CD4+ and CD8+ T cells give rise to a heterogeneous population of antigen-experienced T cells with distinct phenotypic and functional properties. Regarding effector functions of antigen-experienced T cells, CD8+ T cells are traditionally regarded as T-effector cells exerting direct cytolytic function towards foreign antigen expressing target cells and producing effector cytokines, whereas CD4+ T cells have usually been considered as T-helper cells that license APC through CD40-CD40 ligand interaction and produce cytokines for generation and maintenance of memory CD8+ T cells. However, CD4+ T-effector cells capable of exerting direct cytolytic function and producing effector cytokines have been shown to exist as well, suggesting that CD4+ T cells also contribute to direct elimination of foreign antigen expressing target cells.

**ALLOREACTIVITY IN ALLOSCT**

**HLA matching in alloSCT**

Any pair of siblings have a 25% chance of inheriting an identical set of HLA molecules from their parents, and thus being HLA-identical. Because HLA genes are highly polymorphic, it is unlikely for two randomly selected individuals to express identical sets of HLA molecules. If patient and donor HLA molecules differ, the main targets for donor T cells will be the disparate HLA (allo-HLA) molecules as expressed by the patient. In fully HLA-matched alloSCT, however, the major targets for donor T cells are minor histocompatibility antigens (MiHA) that are immunogenic peptides derived from polymorphic proteins encoded by genes located outside the HLA loci, which are presented in HLA-class I or HLA-class II molecules that are shared between patient and donor and recognized as allo-antigens by donor CD8+ or CD4+ T cells, respectively.

Donor T cell responses against allo-antigens, either allo-HLA molecules or MiHA, are associated with development of GvL reactivity and GvHD. To minimize the risk for GvHD, patients are preferably transplanted with HLA-identical sibling donors. As HLA-identical sibling donors are frequently unavailable, the majority of alloSCT are performed with stem cell grafts from HLA-matched or HLA-mismatched unrelated donors (URD). Matching between patients and URD is traditionally performed for HLA-A, -B, -C, -DRB1 and -DQB1 alleles (10/10 match) as the risk of adverse clinical events has shown to be increased with disparities at these loci. Recent studies have demonstrated that mismatching for HLA-DQB1 does not confer additional adverse risks, and therefore, some transplantation centres no longer take HLA-DQB1 into consideration for URD selection. Moreover, matching for HLA-DPB1 is usually not performed, and consequently, 70-80% of patients transplanted with alloSCT grafts from 10/10 matched URD are mismatched for a single or both HLA-DPB1 loci. Thus, in URD alloSCT,
mismatching for HLA-class I is usually avoided, as it is associated with detrimental outcome, whereas disparity at HLA-DQβ1 and/or -DPβ1 molecules is generally accepted.

**Induction of GvL and GvHD after alloSCT and DLI**

After alloSCT, the hematopoietic system of the patient is replaced by donor hematopoiesis. Both donor and recipient hematopoietic cells may, however, also co-exist in the patient in a state called mixed hematopoietic chimerism. Donor T cell responses directed against allo-antigens expressed on residual normal hematopoietic cells from the patient can lead to elimination of patient hematopoiesis as well as GvL reactivity if the antigens are co-expressed on residual hematopoietic malignant cells. Co-development of GvHD occurs when the allo-antigens targeted by donor T cells are also expressed on non-hematopoietic tissues from the patient. Nevertheless, beneficial GvL responses are known to occur in the absence of GvHD, and GvHD can be occasionally observed in the absence of GvL reactivity, indicating that separation of GvL reactivity and GvHD may be feasible.

Several factors determine the likelihood for development of allo-reactive donor T cell responses leading to GvL reactivity and GvHD after alloSCT and DLI, including the heterogeneity of the donor T cell repertoire, HLA compatibility between patient and donor, tissue distribution of allo-antigens recognized by donor T cells, as well as clinical circumstances influencing the activation state of patient cells. The donor T cell repertoire as present in the infused alloSCT graft or DLI is heterogenous, containing naïve T cells as well as antigen-experienced effector and memory T cells\textsuperscript{120,121}. The latter cells consist mostly of T cells primed by exposure to viral and other pathogenic infections, but may also contain memory T cells induced through exposure to allo-antigens via blood transfusions and pregnancies. After HLA-matched alloSCT, MiHA presented in the context of self-HLA molecules are unlikely to have been previously encountered by donor T cells. As a consequence, MiHA specific T cells reside within the naïve donor T cell compartment. When alloSCT is performed over HLA barriers, donor T cells originally educated to recognize self-antigen/HLA complexes can exert cross-reactivity against antigens presented in the context of allo-HLA molecules\textsuperscript{110}. Therefore, allo-HLA reactive donor T cells reside within the naïve as well as memory T cell compartment\textsuperscript{122} and their frequencies are factors higher than frequencies of allo-reactive T cells in HLA-matched settings\textsuperscript{123,124}.

Investigations in mouse models have demonstrated that in the early posttransplantation period, host-derived APC that survived the conditioning regimen can become activated due to the presence of danger signals, such as pro-inflammatory soluble factors produced as a result of tissue damage induced by the conditioning and prime allogeneic T cell responses\textsuperscript{125-132}. Therefore, in the alloSCT setting, activated patient-derived APC may induce an effective GvL response if the targeted allo-antigens are co-expressed by the leukemic cells. An effective GvL response may also be induced by the residual leukemic cells themselves, as it has been demonstrated that leukemic cells be modified into professional APC in vitro and induce primary immune
responses. Co-development of GvHD can occur when the allo-antigens targeted by donor T cells are constitutively expressed on non-hematopoietic tissues or when expression on these tissues is induced by an inflammatory environment. In addition, various allo-antigens from damaged tissues can be presented by activated patient-derived as well as donor-derived APC, thereby contributing to GvHD. Furthermore, profound homeostatic proliferation of donor T cells due to lymphopenia induced by the conditioning as well as danger signals provided by pathogens are likely to amplify the allo-immune responses. Therefore, in the context of non-TCD alloSCT when donor T cells remain present in the stem cell graft, the abundant presence of danger signals and activated patient-derived APC early after alloSCT are likely to induce MiHA and allo-HLA specific T cell responses from the naïve donor repertoire, resulting in co-development of GvL reactivity and GvHD. As allo-HLA reactive T cells from the memory repertoire are also readily activated, particularly severe GvHD can be observed after HLA-mismatched alloSCT.

Profound T cell depletion from the alloSCT graft has demonstrated to prevent induction of GvHD, but at the expense of abrogating the GvL effect. In case of persisting mixed hematopoietic chimerism and residual or relapsed malignancy, postponed administration of donor T cells by DLI can re-introduce the GvL effect. Aggressive malignancies as ALL, AML and CML-BC carry a high risk of relapse after alloSCT, but early intervention with DLI is frequently associated with severe GvHD. The likelihood for development of severe GvHD is significantly reduced when DLI is administered late after alloSCT due to the fact that tissue damage induced by the conditioning regimen has been largely restored, profound inflammatory triggers and lymphopenia are generally absent and patient-derived APC have been largely replaced by APC from donor origin. However, if DLI is administered late after alloSCT when all APC are from donor origin, the relapsed leukemic cells have to serve directly as APC for induction of a GvL response. As ALL, AML and CML-BC cells lack a professional APC phenotype, it is unlikely that these cells will be capable of inducing efficient primary GvL responses in vivo, and therefore no GvL effect may be induced. Mouse studies have demonstrated that indirect presentation of allo-antigens by donor-derived APC can occur, which may lead to GvL immunity and GvHD if the indirectly presented allo-antigens are also directly presented on leukemic cells and non-hematopoietic tissues, respectively.

Selective infusion of CD4+ T cells to separate GvL and GvHD

Traditionally, CD8+ T cells are regarded as the major effector T cells in GvL reactivity, anti-pathogen immunity as well as GvHD. However, CD4+ T cells, however, in addition to their T-helper functions, have also been demonstrated to possess T-effector functions. In contrast to HLA-class I molecules which are constitutively expressed on all nucleated cells, constitutive expression of HLA-class II molecules is mainly confined to normal and malignant hematopoietic cells, and therefore, HLA-class II directed donor CD4+ T cells may induce selective GvL reactivity without GvHD. Several institutions have demonstrated that selective depletion of CD8+
T cells from the alloSCT graft\textsuperscript{147} or DLI\textsuperscript{148-152} reduces the incidence and severity of GvHD, while preserving beneficial GvL reactivity. Randomized studies of CD8+ T cell depleted HLA-identical sibling donor alloSCT\textsuperscript{153} and DLI\textsuperscript{154} confirmed these findings. In the first randomized study of CD8+ T cell depleted alloSCT, incidence of GvHD was reduced (4/19 patients with grade I/II GvHD and 1/19 with grade III GvHD) as compared to unmanipulated alloSCT (1/19 patients with grade I, 4/19 with grade 2 and 10/19 with grade III/IV GvHD), while relapse rates were comparable\textsuperscript{153}. Similarly, the first randomized trial of CD8+ T cell depleted DLI showed that patients receiving CD8+ T cell depleted DLI experienced no GvHD (9 patients), whereas the majority of patients receiving unmanipulated DLI (6 out of 9 patients) suffered from limited to severe GvHD\textsuperscript{154}. No differences in relapse rates were observed between the two groups. However, although the clinical efficacy of CD8+ T cell depletion of the alloSCT graft or DLI was demonstrated in these studies, it remained unclear whether clinical responses in these patients were directly mediated by CD4+ T cells, or could be attributed to residual donor-derived CD8+ T cells in the patients or contaminating CD8+ T cells in the DLI. One study investigated the specificity of T cell responses in a patient with relapsed CML who responded to CD8+ T cell depleted DLI after HLA-identical alloSCT with concomitant GvHD, and demonstrated that donor-derived MiHA specific CD8+ T cells were the prominent T-effector cells mediating these clinical immune responses\textsuperscript{155}.

\textbf{HLA-class II associated MiHA as target antigens for selective GvL reactivity}

HLA-class I restricted MiHA have been identified as targets for CD8+ T cells in patients successfully treated with unmanipulated DLI after HLA-identical alloSCT with concomitant limited or clinically significant GvHD\textsuperscript{111}. Simultaneous development of HLA-class II restricted MiHA specific CD4+ T cell responses could be demonstrated in some of these patients as well, indicating that HLA-class II restricted MiHA can be targeted by CD4+ T cells and are therefore likely to have contributed to the clinical immune responses in these patients\textsuperscript{111}. While the majority of MiHA discovered so far are expressed ubiquitously, a minority of MiHA showed expression restricted to cells of hematopoietic origin\textsuperscript{111}. Donor T cells targeting ubiquitously expressed MiHA are expected to induce GvL reactivity and well as GvHD, whereas donor T cells targeting hematopoiesis-specific MiHA may selectively mediate GvL reactivity. In our laboratory, we identified various HLA-class I and HLA-class II restricted MiHA as targets for CD8+ and CD4+ T cells, respectively, from a patient who responded to DLI after HLA-identical alloSCT without clinically significant GvHD\textsuperscript{104;107;156}. The HLA-class II restricted MiHA were all encoded by genes that were ubiquitously expressed in hematopoietic and non-hematopoietic tissues, suggesting that MiHA-specific CD4+ T cells may exert selective GvL reactivity irrespective of the tissue distribution of the targeted MiHA due to hematopoietic restricted expression of HLA-class II under non-inflammatory conditions. This is also supported by recent findings in our laboratory demonstrating induction of CD4+ T cells for MiHA encoded by ubiquitously expressed genes in a patient with CML-BC who converted to donor chimerism without any signs of GvHD after
prophylactic CD4+ selected DLI administered in the absence of clinically apparent inflammation after HLA-identical TCD alloSCT (*P. van Balen, personal communication*).

**Disparate HLA-class II molecules as target antigens for selective GvL reactivity**
Since allo-reactive T cells recognizing allo-HLA molecules are present in high frequencies in the donor T cell repertoire, strong allo-immune responses are expected after HLA mismatched alloSCT\(^{157,158}\). Investigations whether HLA-class II disparity may lead to selective GvL reactivity without GvHD were most extensively performed with patients that are mismatched with their donors at the HLA-DPB1 locus, as the majority of 10/10 URD alloSCT are mismatched for HLA-DPB1\(^{113,119}\). In TCD alloSCT, mismatching for HLA-DPB1 has been associated with a decreased risk of relapse without an increased incidence of severe GvHD\(^{159}\). In contrast, mismatching for HLA-DPB1 in non-TCD alloSCT has been associated with an increased risk for GvHD, whereas the risk of relapse was decreased\(^{118,160,161}\). These findings may be explained by different clinical circumstances in TCD vs non-TCD alloSCT that strongly influence the activation state of GvHD target tissues. Non-hematopoietic tissues can become targets for allo-HLA reactive donor CD4+ T cells when HLA-class II expression on non-hematopoietic tissues is upregulated by pro-inflammatory cytokines\(^{104,107,162}\), which are produced at significant levels in the early posttransplantation period as a result of the transplant conditioning procedure and abundance of inflammatory triggers\(^{125,132}\). When DLI is performed late after TCD alloSCT, when HLA-class II expression on non-hematopoietic tissues is no longer expected to be upregulated, allo-HLA-class II reactive donor CD4+ T cells are likely to be skewed towards HLA-class II expressing malignant (and normal) hematopoietic cells, thereby mediating selective GvL reactivity. However, in our laboratory, high frequencies of allo-HLA-DPB1 specific CD4+ T cells have been found in patients who responded to DLI administered late after HLA-DPB1 mismatched TCD alloSCT with and without clinically significant GvHD\(^{106,163}\), suggesting that certain clinical circumstances strongly influence the delicate balance between GvL effect and GvHD.

**Brief synopsis**
Relapses of acute leukemia are a major cause of treatment failure after TCD alloSCT. Early intervention with unmanipulated DLI is frequently associated with severe GvHD. Based on the limited tissue distribution of HLA-class II molecules under non-inflammatory conditions, treatment with CD4+ selected DLI may result in GvL reactivity without severe GvHD. For optimal design of CD4+ T cell based immunotherapies after TCD alloSCT, more insight into the contribution of HLA-class II directed CD4+ T cell responses to GvL effect and GvHD is warranted. Some questions are remaining, which cannot all easily be addressed in clinical transplantation studies due to ethical and logistical reasons as well as complicated clinical settings and heterogeneous patient populations. For example, clinical transplantation studies failed to show definite evidence that CD4+ T cells are capable of mediating GvL reactivity as T-effector cells in the absence of
any residual donor-derived CD8+ T cells in the patient or contaminating CD8+ T cells in the DLI. It also remains unknown whether MiHA specific CD4+ T cell responses as induced after fully HLA-matched DLI or allo-HLA-class II restricted CD4+ T cell responses as induced after HLA-class II mismatched DLI are capable of preventing relapse of HLA-class II positive malignancies. Moreover, whether CD4+ T cell responses are sufficiently strong to combat relapsed aggressive HLA-class II positive acute leukemia has not yet been studied, and whether the risk and severity of GvHD after (early) intervention with highly purified CD4+ selected DLI is decreased after both HLA-matched and HLA-class II mismatched TCD alloSCT is unknown. Preclinical mouse models that allow in vivo analysis of human T cell function and reactivity against human acute malignancies in a controlled setting may aid in obtaining answers to some of these clinically relevant issues.

**HUMANIZED MOUSE MODELS**

**Immunodeficient mice**

There are several murine strains of natural immunodeficiency available, including gene deficient and human gene transgenic models, that can be successfully engrafted with various types of human malignant and normal cells and tissue transplants. These humanized mouse models function as surrogate models to study human biological processes under physiological and pathological conditions in vivo and are valuable for in vivo pre-clinical testing of various therapeutic modalities. The choice of the immunodeficient mouse model is strongly dependent on the specific scientific questions and parameters to be studied, since each model has its advantages and limitations.

The discovery of severe combined immunodeficiency (scid) mutation was pivotal in the development of humanized mouse models. The genetic basis for this immunodeficiency is caused by the autosomal recessive mutation within the gene coding for protein kinase DNA-activated catalytic polypeptide (Prkdc) on chromosome 16 (scid mutation) in C.B-17 mice, which contain chromosome 17 from C57BL origin and all other chromosomes from BALB/c origin. This mutation prevents activation of the DNA-dependent protein kinase, which is indispensable for double-stranded DNA break repair and rearrangement of lymphocyte antigen receptors. As a consequence of defects in T and B cell receptor rearrangement, C.B-17-scid mice lack mature functional T and B cells, and are therefore incapable of mounting T cell and humoral immune responses. As a result, C.B-17-scid mice support engraftment of human hematopoietic stem cells, PBMC, fetal thymus and liver and human leukemic cells. However, engraftment rates were often low and irreproducible due to spontaneous development of mature T and B cells during aging (a phenomenon known as leakiness) and a fully functional murine innate immunity, particularly NK cell activity. In addition, the scid mutation causes an enhanced sensitivity to irradiation (used to improve human cell engraftment) due to a defective DNA repair machinery, thereby reducing the lifespan of irradiated mice.
Attempts to develop a mouse model deficient in adaptive and innate immunity included genetic crossing of C.B-17-scid mice with other strains of mice with defects in innate immunity. As a result, mice harbouring the scid mutation on the non-obese diabetic (NOD) background were found to be superior hosts for human cells. The NOD mouse is a well characterized model for spontaneous autoimmune diabetes. The unique H-2\textsuperscript{*}\textsuperscript{b} MHC haplotype of NOD mice (murine MHC is named H-2 and located on chromosome 17) encodes expression of MHC-class I molecules H-2K\textsuperscript{d} and H-2D\textsuperscript{b} and the MHC-class II molecule I-A\textsuperscript{d7} (I-A\textalpha\textsuperscript{d}, I-A\textbeta\textsuperscript{d7})\textsuperscript{180,181}, but surface expression of the MHC-class II molecule I-E\textsuperscript{d7} is absent due to a deletion in the promoter region of the I-E\textalpha gene\textsuperscript{180,182}. NOD/scid mice are protected from autoimmunity due to lymphocyte deficiency. In addition, NOD/scid mice have multiple innate immune defects owing to their NOD background including defects in macrophage development and function due to mutations in the promoter regions of Fc\gamma receptor 1 and 2\textsuperscript{183,184}, lack of complement dependent lysis due to lack of C5a component\textsuperscript{185} and decreased NK cell activity presumably due to mutations in the interleukin 2 (IL-2) gene\textsuperscript{178,186}. Engraftment rates of human cells are high in NOD/scid mice due to their immunological multidysfunctional phenotype, which designated them as the gold standard for human cell engraftment studies. Leakiness with aging, radiosensitivity and presence of NK cell activity, however, are still major limitations of NOD/scid mice as well as their relatively short lifespan due to development of thymic lymphomas caused by an endogenous ecotropic murine leukemia provirus (Emv30) unique to the NOD background\textsuperscript{175,187}.

**Humanized mouse models of GvHD**

Transplantation of human PBMC into C.B-17-scid mice was performed for the first time in 1988 by Mosier et al.\textsuperscript{170}, who demonstrated that engraftment of human T and B cells can be associated with the induction of allogeneic GvHD-like symptoms in mice. By selectively transplanting purified human T cells, it was demonstrated that the engrafted human T cells were the primary mediators of xenogeneic GvHD. Clinical appearance (ruffled fur, reduced mobility, sudden death) and measurable parameters, including weight loss (>20%) and pancytopenia (particularly anemia), are commonly used to diagnose xenogeneic GvHD. Failure to consistently and reproducibly induce xenogeneic GvHD in human PBMC engrafted mice was due to murine endogenous adaptive and innate immunity, as mentioned earlier. Multiple strategies have been explored to increase human T cell engraftment and incidence of xenogeneic GvHD, including pre-transplant irradiation\textsuperscript{188,189}, in vivo depletion of murine NK cells by NK cell specific antibodies\textsuperscript{188-190}, in vivo depletion of murine macrophages by clodronate-containing liposomes\textsuperscript{191} and use of newborn mice lacking a fully developed immune system\textsuperscript{192}. Reproducible models of xenogeneic GvHD were ultimately obtained by generating mice with increased immunodeficiency\textsuperscript{193-196}, and these models are currently used as a surrogate model for human GvHD\textsuperscript{197}.

The pathophysiology of human GvHD as proposed by Ferrara et al.\textsuperscript{48} based on mouse-to-mouse models, is considered to be a three-step process. In xenogeneic GvHD models, pre-
transplant conditioning by sublethal irradiation was shown to be essential for consistent induction of xenogeneic GvHD\textsuperscript{193,195,196,198}, suggesting that activation of host APC by the conditioning regimen enables and enhances human T cell activation in xenogeneic GvHD (phase 1). It is generally assumed that activation, proliferation and differentiation of human T cells occurs in response to histoincompatible murine H-2 molecules presented by murine APC (phase 2), followed by target organ damage through T-effector cell mediated direct cytotoxic activity and/or production of soluble inflammatory mediators (phase 3). Engrafted human CD8+ and CD4+ T cells in xenogeneic GvHD models have been shown to uniformly express an activated/memory phenotype\textsuperscript{199} and produce a variety of pro-inflammatory cytokines\textsuperscript{196}. However, whether xeno-reactivity as mediated by human T cells sufficiently resembles HLA restricted allo-reactivity in humans has not been investigated in much detail. It has been postulated that xenogeneic GvHD resulted predominantly from human CD4+ T cell reactivity against H-2-class II molecules, since human CD8+ T cells specific for H-2-class I molecules have not been isolated from mice during xenogeneic GvHD\textsuperscript{200}. However, the delayed onset of xenogeneic GvHD in H-2-class I or H-2-class II deficient NOD/scid-IL-2Rγnull mice indicated involvement of both CD8+ and CD4+ T cells\textsuperscript{194}. Histopathologic and immunohistochemical analyses of murine organs during the effector phase of xenogeneic GvHD have demonstrated similarities as well as differences as compared to human GvHD. Skin, gut and liver are primary target organs of human GvHD. Skin in xenogeneic GvHD models, similar to human skin GvHD, presents with low T cell infiltrate but a significant pathology\textsuperscript{48,193,194,201}. However, generally low or absent T cell infiltration is observed in the gut without signs of damage in xenogeneic GvHD, whereas significant T cell infiltration and organ damage has been observed in the kidneys, liver, lungs and spleen, suggesting that the typical GvHD pathohistology in xenogeneic GvHD and human GvHD may be different\textsuperscript{193,196,201,202}.

**Humanized mouse models of human haematological malignancies**

As immunodeficient mice can be successfully engrafted with primary human leukemic cells, various immuno-deficient mouse models of human malignancies have been established which enable studies of various aspects of leukemia biology as well as evaluation of different therapeutic approaches\textsuperscript{203}.

Studies with primary human AML cells engrafted in C.B.17-scid and NOD/scid mice revealed the existence of human leukemic stem and progenitor cells\textsuperscript{204-206}. Development of robust mouse models of human AML, however, appeared to be difficult as the majority of primary AML samples failed to engraft in C.B.17-scid mice, NOD/scid mice and other more immunodeficient mice\textsuperscript{206-210}. In addition, the level of engraftment of primary AML cells was often low and inconsistent, which does not make this model ideal for evaluation of therapeutic approaches. In contrast, robust immunodeficient models of human ALL have been developed as the majority of primary ALL samples were shown to readily and reproducibly engraft into NOD/scid mice, resulting in progressive disease\textsuperscript{172,211-213}. As in AML, the existence of human leukemic stem and
progenitor cells capable of initiating human ALL in immunodeficient mice has been confirmed in Ph+ ALL\textsuperscript{214}. Therefore, therapeutic approaches targeting leukemic stem and progenitor cells alongside the bulk of the malignancy are essential for complete eradication of the malignancy.

In our laboratory, a NOD/scid mouse model of human B-lineage ALL or CML-BC has been developed which allows evaluation of various therapeutic modalities\textsuperscript{215-217}, including cellular immune therapy\textsuperscript{218-220}. Primary human ALL and CML-BC cells reproducible engraft in NOD/scid mice, and the kinetics of progression of the malignancy can be monitored over time. Established leukemia can be treated with human \textit{in vitro} generated T cells or DLI, and the anti-tumor efficacy of these treatments can be quantitatively followed by \textit{in vivo} monitoring of human leukemic cells and T cells in murine peripheral blood samples. We used the NOD/scid mouse model to evaluate the anti-tumor efficacy of current and novel DLI based strategies for treatment of human B-lineage ALL and CML-BC.
AIM OF THE STUDY

Relapses of acute leukemia are a major cause of treatment failure after alloSCT. Early intervention with unmanipulated DLI after TCD alloSCT may effectively prevent or treat relapses of these malignancies, but can be accompanied with significant treatment-related morbidity and mortality caused by GvHD. Administration of immunosuppressive therapy as GvHD prophylaxis may effectively control development of GvHD after alloSCT, but is also expected to adversely affect GvL reactivity. Alternatively, patients can be treated with a low dose unmanipulated DLI to prevent development of severe GvHD, but this is unlikely to be able to combat relapsed aggressive acute leukemia. The effectiveness of DLI will be particularly low for acute leukemias with poor immunogenicity after fully HLA-matched alloSCT, as T cell responses induced after HLA-matched DLI are derived from the naïve donor repertoire and require priming by professional APC. Therefore, novel T cell based immunotherapeutic strategies with potent efficacy but limited treatment-related toxicity are highly relevant to improve clinical outcome for patients with aggressive malignancies. Most B-lineage and myeloid malignancies constitutively express HLA-class II molecules, whereas non-hematopoietic cells generally express HLA-class II only under inflammatory conditions. 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 immunotherapy following TCD alloSCT. In this thesis, we addressed this concept by investigating and comparing the efficacy of fully HLA-matched and HLA-class II mismatched unmanipulated DLI and CD4+ T cell selected DLI in a preclinical mouse model of established HLA-class II positive B-lineage ALL and CML-BP. In addition, we investigated the efficacy and toxicity of CD4+ T cell selected HLA-class II mismatched DLI in patients.

As described above, the poor immunogenicity of ALL and CML-BC is expected to limit the potential effectiveness of HLA-matched DLI. This may be overcome by treatment with HLA-class II mismatched DLI, since donor T cells targeting allo-HLA molecules can be derived from the memory compartment, and memory T cells have less APC requirements and a lower threshold for activation than naïve T cells. In chapter 2 we investigated and compared the capacity of fully HLA-matched (12/12 match) and HLA-class II mismatched DLI to induce anti-tumor immune responses against established human ALL and CML-BC in NOD/scid mice. Development of in vivo anti-tumor immunity was followed by quantitative monitoring of human leukemic cells and human T cells in weekly obtained murine peripheral blood samples. The specificity of immune responses induced after DLI was determined by clonal isolation of in vivo expanded T cells and analysis of the specificity of these T cell clones in functional assays.

As the donor T cell repertoire has been shaped based on tolerance for self-HLA molecules, allo-HLA reactive T cell responses induced after HLA-mismatched DLI may exert broad cross-reactivity to multiple disparate allo-HLA molecules. This may confer a risk for detrimental off-target reactivity, and seriously hamper clinical application of allo-HLA restricted T cells. 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-BP 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 xeno-reactivity, we also 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. For this purpose, *in vivo* expanded T cells were clonally isolated at the time of GvL reactivity and xeno-reactivity, and the specificity of T cell clones was determined against human and murine target cells in functional assays.

Allo-reactive CD8+ T cells are potent T-effector cells recognizing allo-antigens in the context of HLA-class I molecules. Since HLA-class I molecules are constitutively expressed on all nucleated cells, allo-reactive CD8+ T cells may mediate severe GvHD. In the clinic, depletion of CD8+ T cells from the alloSCT graft or DLI was associated with a reduced incidence of GvHD without compromising GvL reactivity. However, whether the clinical responses induced in these patients were mediated by CD4+ T cells was not clear due to the presence of residual donor-derived CD8+ T cells in the patients and contaminating CD8+ T cells in the DLI. In **chapter 4**, we investigated whether CD4+ T cells, in the absence of any CD8+ T cells, were capable of mediating GvL immunity as T-effector cells. We compared the capacity of unmanipulated DLI and CD4+ T cell selected DLI to mediate GvL responses against established HLA-class II mismatched human ALL and CML-BC in NOD/scid mice, and the *in vivo* anti-tumor immunity and specificity of immune responses induced after CD4+ DLI were analyzed in detail.

To explore the clinical impact of CD4+ DLI in patients, a clinical study was initiated at our department to investigate the efficacy and toxicity of prophylactic CD4+ T cell selected DLI administered 3 months after TCD alloSCT. In **chapter 5**, we investigated and characterized the clinical impact of CD4+ DLI after HLA-class II mismatched TCD alloSCT in two patients. Both patients suffered from AML and converted to full donor chimerism after prophylactic CD4+ DLI administered 3 months after HLA-DPB1 mismatched TCD unrelated alloSCT. This beneficial response was, however, accompanied with severe GvHD in both patients. We analyzed the clinical course and specificity of T cell responses induced after CD4+ DLI in detail to investigate the immune mechanisms underlying the clinical responses and to acquire insight into clinical circumstances that may have triggered development of GvHD in these patients. T cells were clonally isolated from peripheral blood samples at the time of the clinical responses, and expanded T cell clones were functionally analyzed for specificity against patient- and donor-derived target cells.

Results are summarized and discussed in **chapter 6**.