Biology and clinical relevance of T-cell allo-HLA reactivity

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INTRODUCTION

ALLOGENEIC STEM CELL TRANSPLANTATION

Allogeneic Stem Cell Transplantation and Donor Lymphocyte Infusion

Allogeneic Stem Cell Transplantation (alloSCT) is applied for the treatment of various hematopoietic malignancies[1;2]. Prior to alloSCT, patients undergo conditioning regimens consisting of high dose chemotherapy, irradiation and immune suppression to eradicate malignant cells, to reduce the hematopoietic system of the patient and to prevent graft rejection in order to allow engraftment of the allogeneic stem cells. Traditionally, myeloablative conditioning regimens have been used which induce considerable toxicity, limiting the procedure to young patients. To also provide the curative potential of alloSCT to older patients with hematological malignancies, reduced intensity conditioning regimens have been developed[3]. Subsequently, patients are transplanted with hematopoietic stem cells from a donor. The donor-derived stem cells have the ability to proliferate and differentiate into mature blood cells and thereby replace the patient hematopoietic system. In addition, based on the observation that the risk of leukemic relapse after autologous or genetically identical SCT, using an identical twin as donor, is significantly higher than after allogeneic SCT[4], it is known that immune cells of the donor can mediate graft versus leukemia (GVL) response. The drawback of an allogeneic SCT is that the immune cells of the donor can also cause graft versus host disease (GVHD), often with detrimental consequences. Depletion of T-cells from the stem cell graft before transplantation results in a substantial decrease in the incidence and severity of GVHD[5-7]. However, T-cell depletion from the graft also increases the incidence of leukemic relapse[8;9]. To restore the GVL response, T-cell depleted SCT can be followed by the postponed administration of donor lymphocyte infusion (DLI)[10;11]. DLI can also lead to GVHD, however, the incidence and severity of GVHD after DLI are decreased as compared to early after non T-cell depleted SCT. It is hypothesized that chemotherapy and irradiation applied before the SCT cause tissue damage, which, by presentation of cellular debris by antigen presenting cells in the context of danger signals provided by pathogens, may lead to the initiation of a cytokine storm and thereby increases the risk and severity of GVHD[12]. If DLI is applied several months after the SCT, cytokine storm is circumvented, and thereby the risk and severity of GVHD is reduced.

GVHD and GVL

GVHD is a severe complication of allogeneic SCT and DLI and is caused by donor T-cell reactivity against tissue cells of the patient. The tissues most commonly affected in GVHD are the skin, liver, gut and lungs. GVHD can present in a range of severities, ranging from mild skin lesions to severe involvement of many organs leading to serious illness or death. GVL response refers to donor-derived immunity directed against malignant cells of the patient which can lead to persistent eradication of hematopoietic malignancies. Both GVHD and GVL can result from T-cell recognition of products of genetic differences between patient and donor. After

HLA matched SCT alloreactive T-cells inducing GVHD or GVL recognize peptides presented in HLA molecules, which are polymorphic between individuals based on single nucleotide polymorphisms, termed minor histocompatibility antigens (MiHAs). T-cell recognition of MiHAs derived from proteins with expression restricted to the hematopoietic system are likely to cause selective GVL, whereas GVHD is caused by T-cell recognition of MiHAs with expression in different tissues[13;14]. After HLA mismachted SCT alloreactive T-cells are mostly directed against the allogeneic HLA molecules. Since HLA molecules are expressed on most cells, allo-immune responses after HLA mismatched transplantation usually lead to GVHD.

HLA mismatched SCT

HLA identical SCT is preferable over HLA mismatched SCT, since in HLA mismatched SCT the incidence and severity of graft rejection and GVHD are increased. However, only 25% of a patient's siblings are HLA identical, resulting in limited chances of finding an HLA identical sibling. For patients lacking an HLA matching sibling, an HLA identical donor can be searched for in the international donor data banks. These data banks contain 9.000.000 donors and provide HLA identical donors for about 70% of the Western European or North American Caucasian patients[15]. However, for non-Caucasian patients, the chance of finding a matched donor may decrease to 10-15%[15-17]. Patients for whom no HLA matched donor is found, can be transplanted with the stem cells of an HLA mismatched donor. Mismatches between patient and donor over each of the A, B, C, DRB1 or DQB1 loci have been demonstrated to have negative effects on the outcome of SCT. The importance of HLA-DPB1 donor–patient matching on the outcome of transplantation is still under debate[18;19]. The negative effect on the outcome of the SCT can be ascribed to higher incidence of GVHD[20] and graft rejection[21], but is also the result of a higher incidence of infections or viral reactivations[21-24] following HLA mismatched SCT as compared to HLA matched SCTs. The risk and severity of GVHD as well as the risk of infectious complications rise with increasing HLA disparity between patient and donor.

IMMUNOBIOLOGY

HLA molecules

HLA molecules are membrane proteins expressed by all nucleated cells. The function of HLA molecules is to present peptides at the cell surface that can then be recognized by T-cells. The peptides presented by HLA molecules are derived from intracellular as well as extracellular proteins and from self as well as foreign proteins.

HLA molecules that are involved in conventional immune responses and in allo-HLA immune responses fall into two classes, I and II, which are structurally and functionally different.

The classic HLA class I molecules are termed HLA-A, B, and C, and are composed of a heavy α-chain linked to a light β-chain (beta$_2$-microglobulin). The α-chain has five domains: two peptide-binding domains (α1 and α2), one immunoglobulin-like domain (α3), the transmembrane region, and the cytoplasmic tail. The α1 and α2 domains form two helices which together form the peptide binding groove. These two domains are the most polymorphic region of the HLA molecule and are the sites of TCR contact with the HLA molecule. The α3 domain contains a CD8 binding site. Peptides presented by HLA class I are generally 8 to 11 amino acids in length[25]. The HLA class II molecules are termed HLA-DR, DQ and DP and consist of two transmembrane chains (α and β). Each of the two chains has four domains: the peptide-binding domain (α1 or β1), the immunoglobulin-like domain (α2 or β2), the transmembrane region, and the cytoplasmic tail[26]. Peptides presented in HLA class II are typically 12-25 amino acids long[25].

HLA class I molecules are expressed on all nucleated cells. In contrast, class II molecules are normally expressed by a subgroup of immune cells that includes B cells, macrophages, dendritic cells, and thymic epithelial cells. However, in the presence of cytokines or after activation, other types of cells can express HLA class II molecules[27]. Although most class I and class II molecules form complexes with peptides derived from endogenous and exogenous proteins, respectively, this distinction is by no means absolute[27]. HLA class I molecules containing peptides derived from exogenous proteins and class II molecules loaded with peptides generated from endogenous proteins exist[28].

The genes coding for the HLA class I A, B and C molecules and HLA class II DR, DQ and DP molecules are among the highest polymorphic gene system in the body. Based on this extensive polymorphism of HLA genes, it very unlikely that two randomly selected individuals will express identical sets of HLA molecules.

T-cells

The main effectors of GVHD and GVL are believed to be T-cells. T-cells are part of the adaptive immune system and play a key role in immune responses against different pathogens, like viruses and parasites. T-cells express T-cell receptors (TCRs) through which they recognize antigens in the form of specific peptides presented in the context of HLA (peptide-HLA complexes, pHLA). The TCR of an individual T-cell is specific for a particular antigenic pHLA. However, it has been argued that TCRs need to be able to react with structurally distinct pHLA ligands[29]. This property T-cells is thought to permit the recognition of a universe of potential antigenic peptides which is estimated to be much larger than the number of T-cell clones present in an individual at a given moment[30].

A typical T-cell receptor is formed by a heterodimer of an α and a β chain which are both, in combination with CD3, embedded in the cell membrane. TCRs formed by a heterodimer of a γ and a δ chain also exist (γδTCRs), but little is known about the specificity of these TCRs and it is unknown whether γδT-cells play a role in allo-HLA directed immune responses.

Each TCRα or -β chain consists of a unique combination of a variable (V), diversity (D) (only in TCRβ chains), joining (J), and constant (C) region, which are formed by complex process of gene rearrangements. The antigen binding surface of a TCR chain is formed by three complementarity-determining regions (CDR1-3). CDR1 and CDR2 are formed by the variable region and are well conserved throughout the different TCR-Vαs and TCR-Vβs[31]. The CDR3 region is encoded by recombination with insertion en deletion of nucleotides in the junctions between de V(D)J rejoins and is therefore highly variable.

The T-cell compartment contains CD8 and CD4 T-cells, which recognize peptides in the context of HLA class I or HLA class II molecules, respectively. CD8 and CD4 molecules, which are termed coreceptors, function as stabilizers of the TCR-HLA interaction and contribute to the intracellular signal transduction after antigen binding to the TCR. Most CD8 T-cells function as cytotoxic T-cells, indicating that they are able to kill cells expressing the recognized antigen on their cell surface. In addition, most CD8 T-cells produce cytokines upon activation by antigen expressing cells. The majority of the CD4 T-cells function as helper T-cells in that they offer help to CD8 T-cells and other cells involved in the immune response, by production of cytokines such as IFNγ, IL2, IL4 and TNFα.

Both CD8 and CD4 T-cells can belong to the naïve, effector or memory subset, which can be distinguished by expression of several cell surface molecules and functional properties[32;33]. Naïve T-cells have not yet been activated by antigen encounter. After activation, T-cells become effector T-cells, enabling them to carry out specialized T-cell function such as cytotoxic activity and cytokine production. Memory T-cells have undergone antigen encounter, but have subsequently returned to a resting state. Upon second encounter with the same antigen, memory T-cells are easily activated and can rapidly expand and be cytotoxic or produce cytokines.

The molecular basis of TCR-HLA interaction

The pHLA-binding site that interacts with the TCR is formed by the α1 and α2 helices, of which the sequence is mostly conserved between different HLA molecules, in combination with a presented peptide, with a high diversity in sequence between the different peptides. The pHLA binding site of the TCR is composed of the conserved CDR1 and CDR2 regions and the highly variable CDR3 region. TCR-pHLA recognition 'pairs' invariant and variant structural components of the TCR and pHLA, in that the most variable regions of the TCR (CDR3) are positioned in the center of the binding interface where they contact the peptide, whereas the more conserved elements of the TCR (CDR1 and CDR2) and the tops of the HLA helices engage in contacts that surround the central CDR3-peptide region like a gasket[34]. It is believed that most of the binding interface (75–80%) involves contact between the germline-encoded CDR1 and CDR2 TCR regions and the HLA helices and that only specific peptides stabilize the half-life of the TCR-pMHC complex sufficiently for signaling to occur[35]. The strength by which a TCR interacts with a pHLA complex is termed TCR-pHLA affinity. The affinity between the

TCRs of a T-cell and the recognized pHLA complexes on a target cell in combinations with additional interactions between the two cells via adhesion and costimulatory molecules, determines the strength by which a T-cell binds to a target cell, termed T-cell avidity.
αβ TCRs are specific for HLA, as they only interact with HLA molecules and not with other molecules, a phenomenon termed HLA bias. On the other hand, TCRs have to be able to interact with multiple different pHLA complexes, in order to cover all possible antigens with the relatively limited TCR repertoire in an individual[30]. How TCRs can be specific for HLA but also crossreactive with many HLA molecules is not completely understood, since the 24 TCR-pHLA crystal structures solved until now do not demonstrate obvious conserved contacts between TCR V regions and HLA helices[36]. However, all solved TCR-pHLA crystal structures were found to share a roughly diagonal docking mode (±75°) with a uniformly stereotyped binding polarity, in which the Vα domain lies mainly over the amino-terminal end of the peptide and the α2 helix (HLA class I) or β1 helix (HLA class II), and the Vβ domain lies mainly over the carboxy-terminal region of the peptide and the respective α1 HLA helices. This conservation of the docking polarity advocates the existence of germline specificity between the Vα and Vβ domains of the TCR and the helices of the HLA.

Thymic selection and self-tolerance

Thymocytes leaving the bone marrow first undergo thymic selection before going to the periphery[37;38]. In the thymus the thymocytes first undergo TCR gene rearrangement and are subsequently selected based on TCR affinity for self pHLA. In the thymic cortex, thymocytes with low avidity for pHLA complexes are positively selected, whereas thymocytes not able to recognize self pHLA die by neglect. This selection imparts restriction to HLA, ensuring that the selected T-cells are able to recognize peptides presented in HLA molecules. In the cortex, thymocytes also undergo CD4 or CD8 lineage commitment. After positive selection and lineage commitment, the thymocytes relocate to the medulla, where they undergo negative selection, implying that cells recognizing self pHLA with high avidity are eliminated in order to prevent autoreactivity. Reactivity against self pHLA can be the result of recognition of a single self peptide presented in self HLA, but can also be due to recognition of multiple different peptides presented in self HLA[39;40]. Thymic selection is therefore thought to be responsible for the removal of polyspecific T-cells, recognizing multiple different peptides in the context of self HLA.

T-cell response

To be able to proliferate and differentiate into effector T-cells in order to mediate immune responses, naïve CD8 T-cells need to encounter their specific antigen presented on activated antigen presenting cells (APCs). APCs are cells specialized in the presentation of antigen in both HLA class I and HLA class II. In addition, activated APCs express the appropriate costimulatory molecules necessary to effectively prime naïve T-cells[41]. APCs can be activated

by inflammatory signals such as ligands for toll like receptors expressed by many microbial pathogens[42;43]. Alternatively, in the absence of inflammatory signals, CD4 T helper cells can activate APCs by CD40-CD40L interaction and cytokine production[44-46]. T-cell responses to non-inflammatory immunogens therefore require dual recognition of antigen or antigen expressing cells, by CD8 as well as the CD4 T-cells, which is thought to serve as a safeguard against autoimmunity[47]. After initiation, T-cells rapidly expand and migrate to infected tissue, where they kill infected cells or produce cytokines upon antigen recognition. After removal of antigen, the contraction phase starts in which most of the proliferated T-cells die and a small part of the T-cells differentiate into memory T-cells. Whereas the naïve T-cell repertoire directed against microbial antigens contains a very broad range of avidities, the memory T-cell repertoire specific for the same antigens exists of T-cells with high avidity for the specific antigens[48]. This indicates that during immune responses high avidity T-cells selectively expand and / or selectively survive the contraction phase[49]. In contrast to naïve T-cells, memory T-cells do not require co-stimulation and therefore do not need to encounter antigen presented on activated APCs in order to become activated, expand and be effective[50;51].

T-CELL ALLO-HLA REACTIVITY

Allo-HLA reactive T-cells

The frequency of T-cells reactive in HLA mismatched mixed lymphocyte reactions (MLR) was demonstrated to be a 1000 fold higher than the frequency of T-cells reactive in HLA identical MLRs[52;53]. By testing alloreactive T-cells against panels of third party target cells expressing different HLA molecules[52;54-56] and against target cells blocked with different HLA antibodies[57-59], it was determined that the recognition exhibited by alloreactive T-cells is directed against non-self HLA (allo-HLA) molecules, and that the frequency of allo-HLA reactive T-cells ranged between 1-10%. This percentage of T-cells suggests that not all T-cells are able to react against foreign HLA molecules, but that only certain T-cells have this ability. On the other hand, based on the demonstration that alloreactivity is presented in the naïve and memory T-cell populations[60], it is not expected that only a specific subgroup of T-cells is able to react against allo-HLA molecules. The hypothesis that TCRs have to be able to interact with multiple different pHLA complexes, in order to cover all possible antigens within the relatively limited TCR repertoire in an individual[30], suggests that most or all TCRs could be allo-HLA reactive based on their ability to react with different pHLAs.

The role of virus specific T-cells in alloreactivity

Although alloreactivity is presented in the naïve and memory T-cell populations[60], the ability of T-cells to cross-react against allo-HLA could especially have serious consequences when exerted by memory T-cells. Since memory T-cells lack the requirement for co-stimulation[50;51],

allo-HLA reactivity of memory T-cells can be efficiently triggered by non-professional antigen presenting cells after HLA mismatched SCT or solid organ transplantation. Based on the restricted TCR repertoire of virus specific memory T-cells[61-64] the number of different virus specific T-cells will be limited, but the total number of virus specific T-cells with an identical TCR will be much higher in the memory pool as compared to the naïve compartment. T-cells directed against latent viruses, like EBV and CMV, are present at high frequencies in blood of healthy individuals and patients[65-68]. Therefore, if certain virus specific T-cells within the memory pool react against the mismatched HLA molecules, they may induce severe GVHD or graft rejection.

Studies of Burrows and colleagues have illustrated that virus specific T-cells exert allo-HLA reactivity by demonstrating that EBV-EBNA3A specific HLA-B8 restricted T-cells cross-react with HLA-B44[69;70]. T-cell specific for HSV-VP13/14 presented in HLA-A2 were also found to cross-react with HLA-B44[71], and CD4 T-cells specific for tetanus toxoid presented in HLA-DR3 were found to be cross-reactive against HLA-DR4[72]. In addition, the association between reactivation of viral infections during organ transplantation and increased graft rejection[73] supports the hypothesis that virus specific T-cells exhibit allo-HLA reactive potential.

Peptide specificity of allo-HLA reactive T-cells

Since T-cells never encounter allo-HLA molecules during thymic development, and therefore no selection based on tolerance for allo-HLA molecules occurs, T-cell allo-HLA reactivity is assumed to be less peptide specific than conventional T-cell reactivity. The degree of peptide specificity of alloreactive T-cells has been studied extensively. Most investigators used cells defective in antigen processing, like Transporter Associated with antigen Processing (TAP) deficient human T2 cells or murine RMA-S cells to address the role of peptide in allorecognition[73-81]. In these studies, groups of alloreactive T-cell clones or lines were tested against these antigen processing deficient cells, unloaded or loaded with peptides. Alloreactive T-cells tested in this manner demonstrated a variation in types of reactivity. Some T-cells only recognized a single peptide and were therefore categorized as peptide specific. T-cells recognizing more than one peptide, without sequence homology, were considered polyspecific. In addition, some alloreactive T-cells were reactive against antigen processing deficient cells in the absence of exogenously loaded peptide, which was initially interpreted as peptide independent recognition, because it was assumed that these cells expressed empty MHC molecules on the cell surface. However, since it was demonstrated that these cells do express a limited number of peptides, which are independent of TAP to be expressed in MHC molecules on the cell membrane[82], reactivity against antigen processing deficient cells may also be based on peptide specific recognition. Reactivity against allo-HLA molecules irrespective of the sequence of the peptide presented has been termed peptide degenerate allorecognition, although it is unclear whether this type of recognition occurs. Based on all these studies

it is believed that alloreactivity is a combination of different reactivities, ranging from peptide specific to peptide degenerate alloreactivity.

Molecular mechanisms of TCR cross reactivity

Previous studies investigating the molecular mechanisms underlying the ability of TCRs to recognize different pMHCs have together identified five distinct mechanisms to explain TCR cross-reactivity[83] . These mechanisms can be summarized as follows. 1) Induced fit, indicating conformational flexibility of the pHLA binding site of the TCR which enables the TCR to accommodate different pHLA ligands while maintaining the same overall docking orientation. 2) Differential TCR docking, referring to the ability of one TCR to bind different pHLA ligands using different docking orientations. 3) Molecular mimicry, means that different pHLA ligands can share key structural and chemical features and thereby form very similar interfaces with the crossreactive TCR. 4) Antigen-dependent tuning of peptide-MHC flexibility refers to conformational flexibility in pHLA which allows recognition of different pHLA ligands by the same TCR, due to structural reorganization upon TCR binding. 5) Structural degeneracy, indicates that absence of specific interactions between TCR and pHLA can lead to TCR cross reactivity against different pHLAs.

Allo-HLA derived peptides

Allo-HLA can be recognized by T-cells as intact HLA molecules on the surface of allogeneic APCs. Alternatively, allo-HLA molecules can be processed into peptides and subsequently presented at the cell surface in the context of other HLA molecules. It has been demonstrated that HLA derived peptides are frequently presented self peptides in the context of HLA class I and HLA class II molecules[84-86], indicating that allogeneic APCs can present the allo-HLA molecules, besides directly, also in the form of peptides presented in shared HLA molecules. Additionally, self APCs can initiate allo-immune responses by cross presenting the allo-HLA antigens as peptides in the context of shared HLA molecules, after the uptake and processing allogeneic cells[87-89]. T-cell reactivity against allo-HLA derived peptides has been extensively described in graft rejections after solid organ transplantations and is believed to be an important cause of chronic solid organ rejection[90-96]. Since in organ transplantion graft rejections can still occur after the disappearance of donor-derived APCs, it is presumed that recipient APCs cross present allo-HLA derived peptides[87;88]. T-cell reactivity against HLA derived peptides has therefore been interpreted as the result of indirect presentation of non-self-HLA by self APCs. However, since HLA derived peptides are frequently presented self peptides, the mismatched HLA molecules will be, besides directly presented, also often presented as peptides in the context of shared HLA molecules by allogeneic APCs after HLA mismatched transplantations. When the peptides derived from the mismatched HLA class I are presented in HLA class II, this could result in CD4 help to the CD8 alloresponse, potentially increasing

toxicity. Therefore, besides mediating chronic organ rejection, T-cell recognition of allo-HLA derived peptides could also be important in acute GVHD and hematopoietic graft rejection.

ADOPTIVE T-CELL THERAPY

Adoptive T-cell therapy and TCR genetransfer

The ability of donor lymfocytes infused after SCT to mediate immune response against relapsed leukemia, demonstrated that T-cells can mediate GVL responses. However, DLI can also lead to GVHD, often with detrimental consequences. Using adoptive T-cell therapy, whereby T-cell populations directed against defined antigens are administered, GVL in the absence of GVHD may be achieved. Antigens which could be targeted in adoptive T-cell therapy directed against leukemia after allogeneic SCT are minor histocompatibility antigens (MiHA) with an expression restricted to hematopoietic cells[97-100]. Infused T-cells may also mediate immune responses against solid tumors, as indicated by the ability of lymphocytes extracted from freshly resected melanomas, termed tumor infiltrating lymphocytes (TILs), to mediated specific lysis of autologos tumor cells and by the observation that re-infusion of in vitro expanded TILs was an effective treatment for patients with metastatic melanoma[101-103]. The antigens targeted in adoptive T-cell therapy directed against different solid tumors could be antigens with expression restricted to tumors, termed tumor associated antigens (TAAs). Broad application of adoptive T-cell therapy could be hampered by the inability to isolate and expand large numbers of antigen specific T-cells[104]. As an alternative approach, genes of TCRs specific for hematopoietic MiHAs or TAAs may be transferred into appropriate T-cell populations which are not expected to cause GVHD. In this strategy, donor or patient derived T-cell populations are equipped with a TCR of defined specificity using short-term in vitro procedures, and the redirected cells are infused to provide T-cell reactivity against defined antigens.

Tumor associated antigens (TAAs)

TAAs are proteins and their derivative peptides which are highly expressed in tumors and are absent or expressed at low levels in healthy tissues. Based on this expression pattern, TAAs may be suitable for adoptive T-cell therapy. TAAs can be divided into three categories. The first category contains tumor associated viral antigens. Epstein-Barr virus (EBV), Hepatitis B and C virus (HBV, HCV) and human papilloma virus (HPV) are involved in the formation of different types of malignancies[105;106]. The viral antigens expressed by these cancers constitute ideal targets for adoptive T-cell therapy, since these antigens are non-self and are only expressed by the tumor cells. Allogeneic EBV-specific T-cells have indeed shown clinical efficacy in immuno-compromised patients at risk of developing EBV associated lympho-proliferative

disease[107;108]. However, only a limited number of cancers are initiated by viruses and express viral antigens.

The second category is comprised of antigens derived from mutated oncogenes or tumor suppressor genes. Since these mutations only occur in the tumors, the potential derived antigens are tumor specific. However, in order to result in antigens useful for adoptive T-cell therapy the altered gene sequence has to be appropriately processed by the proteosome and presented by HLA molecules at the cell surface[109]. To be able to treat large groups of patients with T-cell directed against these antigens, the peptides derived from the mutated proteins have to efficiently bind in frequently expressed HLA molecules, like HLA-A2 or HLA-B7, which is usually not the case[109]. In addition, most of mutations are unique to individual tumors[110], making them incompatible with TCR gene therapy. Few mutations, such as in ras and bcr-abl, are highly conserved in cancers and could serve as broadly applicable targets. However, although HLA-binding peptides derived from these mutated genes have been identified and in vitro T-cell responses against these peptides have been described[109;111;112], there is no definite proof that these epitopes are naturally presented on tumor cells and/or professional antigen presenting cells, and thereby could induce an anti-tumor directed T-cell response.

The third category contains most identified TAAs and represents all tumor associated self-antigens, such as differentiation antigens, aberrantly expressed antigens and cancer testis antigens. Differentiation antigens are not tumor specific, but are specific for the cell-type from which the tumor is derived. Examples are the melanocyte differentiation antigens Melan-A, Tyrosinase, and Gp100 and the B cell lineage specific antigens CD19 and CD20. The aberrantly expressed antigens are antigens which are overexpressed in certain tumors, such as Wilms' tumor 1[113;114], proteinase 3[115] and myeloperoxidase (MPO) which are over-expressed in a variety of myeloid leukemias. Cancer testis antigens are non-mutated genes whose expression, with the exception of testis and fetal tissues, seems to be mostly restricted to tumor cells. Examples of cancer-testis antigens include MAGE, GAGE/PAGE, BAGE, LAGE/NY-ESO-1, and preferentially expressed antigen of melanoma (PRAME). Although these TAAs are mostly known for their association with melanoma[116-119], some of the cancer-testis antigens are also highly expressed in many other cancers including non-small cell lung carcinoma, breast carcinoma and renal cell carcinoma[120;121]. Based on their high expression in many different cancers, cancer-testis antigens may be attractive target for adoptive T-cell therapy.

Allo-HLA reactive T-cells useful for adoptive T-cell therapy

Although the expression of TAAs is high in tumor cells and low or absent in non malignant cells, most TAAs are non-mutated non-polymorphic self antigens[122]. Since T-cells that exhibit high avidity for self-antigens presented by self-HLA are deleted during thymic selection, it is difficult to isolate self restricted high avidity T-cells specific for TAAs. However, for the effective eradication of tumors, T-cells need to recognize the tumor cells with high avidity and therefore need to express TCRs with high affinity for the TAAs[122;123]. Self tolerance to TAAs

can be circumvented by the recognition of TAAs in allogeneic HLA. Since T-cells do not encounter foreign HLA molecules during thymic selection, they can recognize TAAs presented in allogeneic HLA molecules with high avidity. The TCRs of allo-HLA reactive TAA specific T-cells could therefore be used for adoptive T-cell therapy using TCR genetransfer. However, there are risks involved in infusing T cells which recognize tumor associated self antigens presented in allogeneic HLA with high avidity. First of all, since allogeneic HLA molecules are not encountered during thymic development, T-cells recognizing multiple different peptides presented in allo-HLA molecules have not been removed, and therefore allo-HLA recognition is expected to be more cross reactive than conventional T-cell reactivity[39;40]. Usage of allo-HLA T-cells in adoptive T-cell therapy could therefore potentially lead to "off target" toxicity against multiple untargeted tissues based on recognition of other peptides than the TAA[124]. Second, since TAA are self antigens, it is possible that certain healthy cells also highly express the TAA, which could lead to "on target" toxicity against these specific cells[125-127].

AIM OF THE THESIS

Allo-HLA reactive T-cells recognize non-self-HLA molecules that were not encountered during thymic development and can lead to severe GVHD after HLA mismatched SCT or DLI. The ability if these T-cells to recognize allogeneic HLA is a property which is despite extensive previous research not completely understood. How are allo-HLA directed immune responses initiated? Which T-cells are able to exert allo-HLA reactivity? is this a property of a few T-cells or of all T-cells? What is the biological relevant degree of peptide specificity and avidity of T-cell allo-HLA reactivity? And can potentially beneficial allo-HLA reactive T-cells be found, and if so, are they safe for use in the clinic? Understanding these aspects of T-cell allo-HLA reactivity might lead to more insight into general T-cell immunity, TCR function and thymic selection. In addition, further understanding T-cell allo-HLA reactivity may offer new insights into how to circumvent GVHD or how to use T-cell alloreactivity for beneficial purposes. The aim of this thesis is to understand these aspects of T-cell allo-HLA reactivity, and to investigate the possibilities that these understandings offer for beneficial application in the clinic.

In **chapter 2** we investigated how an allo-HLA class I directed immune response is initiated in vivo, which T-cells are involved and what these T-cells recognize. For this purpose the immune response in a patient experiencing GVHD after delayed HLA class I mismatched DLI was characterized. CD8 and CD4 donor derived T-cells which were activated during the GVHD in the patient were investigated for clonal diversity, alloreactivity, HLA restriction and specificity. In addition, patient blood and bone marrow collected during the GVHD were investigated for the presence of patient derived HLA class II positive cells able to activate both the alloreactive CD8 and CD4 T-cells, and therefore possibly responsible for initiation of the immune response.

The frequency of allo-HLA reactive T-cells was previously determined by mixed lymphocyte reactions to range between 1-10%, suggesting that only certain, but not all, T-cells have the ability to react against foreign HLA molecules. It is however hypothesized that in order to cover all possible antigens with the relatively limited TCR repertoire in an individual, each T-cell has to be able to react with different pHLA complexes. In **chapter 3** we investigated whether all T-cells are able to exert allo-HLA reactivity by investigating the ability of memory T-cells with a known specificity to exert allo-HLA reactivity. For this purpose the alloreactivity of virus specific T-cells was investigated by screening single viral antigen specific T-cell lines and clones against a panel of EBV transformed B-cells, together expressing almost all common HLA class I and II molecules. Since it is known that a substantial part of the T-cells naturally express two different TCRs at the cell surface, we investigated whether virus specificity and allo-HLA reactivity were conducted by the same or different TCR.

Since T-cells never encounter allo-HLA molecules during thymic development, and therefore no selection based on tolerance for allo-HLA molecules occurs, T-cell allo-HLA reactivity is assumed to be less peptide specific than conventional T-cell reactivity. Allo-HLA reactivity of

T-cells has been extensively studies and different concepts of what these T-cells recognize have been proposed, including single peptide specificity, polyspecificity, and peptide degeneracy. In **chapter 4** we investigated the biologically relevant peptide specificity of allo-HLA reactivity, by analyzing the degree of peptide specificity of 50 different allo-HLA reactive T-cell clones which were activated and expanded *in vivo* during graft versus host disease. Peptides recognized by the allo-HLA reactive T-cell clones were characterized, and identified using multidimensional HPLC fractionation and mass spectrometry and single peptide specificity was confirmed by downregulation of the expression of the recognized antigens using silencing RNA.

The single peptide specificity of in vivo derived allo-HLA reactive T-cells demonstrated in chapter 4 suggested that allo-HLA reactive T-cells specific for TAAs could be used in adoptive T-cell therapy without the risk of inducing off-target toxicity against multiple untargeted tissues based on recognition of other peptides than the TAA. In **chapter 5** we searched for TAAs specific allo-HLA reactive T-cells within an allo-HLA directed immune response which occurred during GVHD in an HLA class I mismatched transplanted patient. This resulted in the isolation of PRAME specific allo-HLA-A2 reactive T-cell clones. The potential benefits and risks of the use of high avidity PRAME specific TCRs in the clinic was investigated by testing the T-cell clones against multiple different tumor cell lines and leukemia cells and against cells derived from various healthy tissues.

In **chapter 6** the results of this thesis are summarized and discussed, conclusions based on the results of this thesis are drawn and new research questions and ideas are proposed.

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