Adoptive immunotherapy after HLA mismatched stem cell transplantation

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Chapter 1:

General introduction

1. ALLOGENEIC STEM CELL TRANSPLANTATION

Stem cell transplantation (SCT) comprises the intravenous infusion of hematopoietic stem cells to re-establish blood cell formation in patients with damaged or defective bone marrow. Hematopoietic stem cells can be collected from donor bone marrow, peripheral blood mobilized by cytokines[1], or umbilical cord blood[2]. The stem cell donor can be the patient him- or herself (autologous), an identical twin (syngeneic), or a sibling or unrelated donor (allogeneic).

Allogeneic SCT in a clinical setting was first described in 1957. Thomas et al.[3] reported six patients with hematological malignancies that were treated with irradiation and intravenous infusion of bone marrow from a healthy individual. Unfortunately, the results of clinical bone marrow transplantation remained disappointing for many years. Mortality was high due to graft failure, recurrence of the original disease, infections and the so-called secondary disease, now known as graft-versus-host disease (GvHD)[4].

In the 1960s and 1970s, human leukocyte antigens (HLA), first described by Dausset[5] and van Rood[6], were discovered to play key roles in the induction of transplantation reactions[7-9]. Generally, the risk of adverse reactions was found to correlate with the number of HLA-mismatches between the bone marrow donor and the host. In 1975, Thomas' Seattle team published an influential paper on a total of 110 transplantations performed with bone marrow obtained from HLA-matched siblings, and importantly, reporting survivors[10,11]. Improvements in transfusion medicine and treatment of infection further advanced the field. Today, allogeneic SCT is a routine treatment for various conditions with more than 30.000 transplants being carried out every year[12].

The most common indications for allogeneic SCT are hematological malignancies such as acute myeloid leukemia (AML; 27%), acute lymphatic leukemia (ALL; 19%), chronic myeloid leukemia (CML; 14%), myelodysplastic syndrome (MDS; 9%) and chronic lymphatic leukemia (CLL; 3%). Allogeneic SCT can also be used to treat lymphoproliferative disorders such as non-Hodgkin's lymphoma (7%), multiple myeloma (MM; 4%), Hodgkin's lymphoma (1%), and even solid tumors (3%)[13]. First, a conditioning regimen consisting of high-dose chemotherapy combined with total body irradiation eradicates the patient's malignant cells as well as any remaining normal hematopoietic cells. Then, through allogeneic SCT, the patient's hematopoietic system is reconstituted. At present, this is the most effective therapy for patients with ALL or AML, and the only proven curative treatment for CML, CLL, MDS, and low grade non-Hodgkin's lymphoma[14].

Success rates vary greatly depending on the nature of the malignancy. Other factors that influence SCT outcome are time interval from diagnosis to transplantation, prior treatment, dose of infused nucleated stem cells, HLA disparity, patient age and physical condition, and the patient's and donor's cytomegalovirus status[14]. Overall, transplant-related mortality has decreased from around 50% in 1974-1979 to

about 20% in 1997-2001 (IBMTR/ABMTR data). Graft failure, GvHD, opportunistic infections, and recurrence of malignancy however remain the major causes of death. These complications are to a considerable extent driven by the immunologic responses induced by allogeneic SCT.

2. CLINICAL IMMUNOLOGY OF ALLOGENEIC SCT

2.1 IMMUNOLOGIC CONSEQUENCES OF ALLOGENEIC SCT

Allogeneic SCT differs fundamentally from the grafting of solid organs. In solid organ transplantation, the primary clinical concern rests with preventing rejection of the graft by the host immune system. In allogeneic SCT, the preparative conditioning regimen eliminates most of the host immune system, after which the host's immune system is reconstituted by immune cells present in the stem cell graft. Thus, after allogeneic SCT the patient's immune system is donor derived. This condition is called chimerism after the Chimera, a creature from Greek mythology that was lion at the front, goat in the middle, and serpent at the rear[15].

The immune system has evolved as a defensive mechanism against harmful agents such as viruses and bacteria. Cellular elements of the immune system respond to antigens. The T cell compartment is designed to distinguish between self- and non-self-peptides bound by HLA molecules in a context of "danger"[16]. Natural killer (NK) cells are triggered by the absence of self-HLA molecules[17]. After allogeneic SCT, both cell types will elicit immune responses if donor and host are disparate for peptides presented by their respective HLA molecules or for the HLA molecules themselves. These immune responses mediate several important clinical events.

2.2 GRAFT REJECTION

Immunologic graft rejection is an important cause of graft failure, next to drug toxicity, viral infections, and infusion of inadequate numbers of hematopoietic stem cells[18]. Graft rejection may occur directly after allogeneic SCT or weeks to months after initial take of the graft. During engraftment residual host T cells and NK cells mount an immune response against the foreign donor graft. Graft-derived donor T and NK cells in turn attack the residual host immune system. If the host response supercedes, the graft may be rejected[19].

Graft rejection manifests itself as severe pancytopenia and the absence of hematopoietic donor cells. Risk factors are T cell depletion of the graft, the number of HLA-mismatched loci between host and donor, and transfusion- or pregnancy-induced sensitization[18]. High numbers of $CD34^+$ stem cells favor engraftment[20]. Graft rejection is an infrequent but life-threatening situation[18]. The use of non-cytotoxic immunosuppressive drugs and the infusion of donor blood stem cells may salvage patients, but results are not encouraging[21].

2.3 GRAFT-VERSUS-HOST DISEASE

GvHD is one of the main causes of mortality and morbidity after allogeneic SCT. GvHD may occur when immune cells are transferred into an antigenically different host[22]. GvHD can manifest as an acute as well as a chronic disorder. While acute GvHD often precedes chronic GvHD, the two syndromes are clinically distinct and their relationship is not entirely clear.

2.3.1 ACUTE GRAFT-VERSUS-HOST DISEASE

Acute GvHD generally develops within the first three months after SCT and is primarily a T cell-mediated event. NK cells do not cause GvHD because they mainly target the hosts' hematopoietic cells.
The organ systems primarily involved in acute GvHD are skin, gut, and liver. Symptoms include maculopapular rash or erythroderma, liver disease, and gastrointestinal symptoms such as diarrhea, bleeding, and ileus[23]. In severe cases, epidermal necroses and permanent scarring of mucosal tissue may occur. Acute GvHD can be staged and graded according to the Glucksberg criteria[24,25]. Grade I implies limited GvHD, which has a favorable prognosis and does not require treatment. Grade II GvHD is a moderately severe disease requiring therapy. Grade III to IV GvHD is a severe multi-organ disease that may be life threatening.
Incidence and severity of acute GvHD vary with the degree of antigenic difference between donor and host. In a recent clinical study, the incidence of grade II to IV acute GvHD was found to be 40% for HLA-identical sibling donors, 46% for HLA-matched unrelated donors, and 61%-75% for HLA partially-matched family donors[26]. Overall, the number of HLA class I- and class II-mismatched loci strongly corresponds with the risk of acute GvHD. Other risk factors include a gender mismatch between donor and patient, donor parity, donor and recipient age, conditioning regimen, and the use of peripheral blood-derived stem cells[27]. The treatment of acute GvHD mainly consists of immunosuppression and supportive care[28].

2.3.2 CHRONIC GRAFT-VERSUS-HOST DISEASE

Chronic GvHD may develop as early as 60 days or as late as 400 days after SCT. Many transplant centers use day 100 after SCT to distinguish between acute and chronic GvHD. Chronic GvHD may follow acute GvHD, but not all cases of acute GvHD evolve into chronic GvHD, and chronic GvHD may also develop de novo. Its pathogenesis has not yet been elucidated. Clinical features resemble those of autoimmune syndromes, such as lichen planus, scleroderma, mucosal lesions, keratoconjunctivitis, liver disease, pulmonary insufficiency, vaginal strictures, and immunodeficiency[29].
Risk factors are the same as for acute GvHD with previous Graft II to IV acute GvHD being the most powerful predictor[30]. Treatment consists of immunosuppressive drugs, with an average treatment duration of 1–2 years[31]. The combination of immunosuppressive medication and GvHD-mediated deficiencies leaves patients highly susceptible to late infection. Such infections are the leading cause of death in patients with chronic GvHD[32].

2.3.3 PREVENTION OF GRAFT-VERSUS-HOST DISEASE

There are several approaches to prevention of GvHD after allogeneic SCT. Most transplant centers administer prophylactic immunosuppression. Alternatively, allogeneic SCT with stem cells derived from umbilical cord blood is associated with a reduced risk of GvHD[33]. While 0-2 HLA allele-mismatched cord blood transplants have been very successful in children[34], HLA disparity remains a poor risk factor for engraftment and survival in adult patients[35,36]. Moreover, cord blood transplantation is not yet generally applicable for adult patients due to the lower number of stem cells present in these grafts. A third approach is the use of stem cell grafts devoid of potentially harmful donor T cells. Mature donor T cells can be depleted from bone marrow or peripheral blood-derived grafts using ex vivo negative selection of T cells or in vivo anti-T cell antibody administration[37]. This approach has been associated with a considerable decrease in grade II-IV acute GvHD[38]. Unfortunately, T cell depletion is also associated with increased risks of graft failure, delayed immune reconstitution and recurrent leukemia. Thus, the effect of T cell depletion of the stem cell graft on overall survival is limited[38,39]. Increasing the specificity of depletion and optimizing stem cell numbers may decrease the risk of treatment failure[40,41].

2.4 GRAFT-VERSUS-LEUKEMIA

GvHD often coincides with beneficial graft-versus-leukemia (GvL) reactivity. It stands to reason that if donor T cells can damage host tissues, they may also affect the host's leukemic cells. Patients who develop GvHD post-SCT have a lower risk of leukemia relapse[42], whereas risk of relapse is increased after T cell-depleted SCT and after syngeneic or autologous SCT[43,44]. These findings indicate that allogeneic donor T cells indeed mediate a curative GvL effect. Well-defined tumor regressions following allogeneic SCT for renal, breast, and ovarian cancer have extended the GvL effect to include a general graft-versus-tumor effect[45].

2.4.1 DONOR LYMPHOCYTE INFUSIONS

The appreciation that donor alloreactivities can help patient recovery has led to the introduction of donor lymphocyte infusions (DLI) from the original stem cell donor as a treatment for relapse of leukemia after allogeneic SCT[46]. DLI can induce complete remissions in up to 80% of patients with relapsed chronic phase CML. Clinical responses have been shown in 13% to 29% of patients with advanced stages of CML[47,48], and 40% of patients with relapsed MM (40%-52%)[49,50]. DLI has also proven effective in a limited number of patients with relapsed low grade lymphoma or CLL[51]. Relapsed ALL, AML and MDS however hardly respond to DLI. The 2-year survival rates are 20% or less[47,48,52]. The fast pace of these diseases, a larger tumor burden, intrinsic resistance to immune attack, and differences in antigen presentation by the tumor cells, have all been implicated as mechanisms contributing to their decreased sensitivity[53].

The beneficial effect of DLI therapy is unfortunately associated with serious side effects. Due to the infusion of large numbers of unselected host-reactive donor T cells, approximately 60% of patients develop acute GvHD[47,48]. Furthermore, DLI may mediate ablation of host hematopoietic cells before donor hematopoiesis and immune function is sufficiently established resulting in pancytopenia and related infectious complications[47,48]. DLI-related mortality after HLA-matched SCT is up to 18%.
Several strategies have been developed to minimize GvHD after DLI, including step-wise cell-dose escalation and $CD8^+$ T cell depletion[54,55]. Another approach consists of transfecting donor lymphocytes with suicide genes that can be activated if high grade GvHD develops[56,57].

2.4.2 TREATMENT OF LEUKEMIA RELAPSE AFTER HLA-MISMATCHED SCT

HLA-identical sibling donors are regarded as the best stem cell donors in terms of engraftment and acceptable rates of GvHD. Unfortunately, only for about 30% of the patients a suitable sibling donor is available[58]. From the Bone Marrow Donor Worldwide Registries an unrelated highly HLA-matched donor can be identified for 38% of patients, but donor searches are less successful for non-caucasian patients[59]. The remaining patients are transplanted with stem cells from partially HLA-mismatched unrelated or family donors.
Because of the strong association between HLA disparity and GvHD after allogeneic SCT, HLA-mismatched grafts are often depleted of T cells. This puts the patient at increased risk of leukemia relapse. Unfortunately, HLA disparity is associated with GvHD after DLI as well[60,61]. In a recent study, administration of DLI in combination with granulocyte-macrophage colony-stimulating factor was associated with reduced relapse rates after HLA-mismatched SCT. Unfortunately, the majority of patients suffered severe GvHD resulting in 40% GvHD-related mortality within the total treated population[62].
One approach to reduce the risk of relapse after HLA-mismatched SCT is to select donors on the basis of potential NK alloreactivity against the host. A stem cell graft depleted of T cells but still containing NK cells will not cause GvHD but will retain GvL capacity. While this strategy has been shown to reduce the risk of relapse[63], it provides no treatment if relapse does occur.
The optimal treatment of patients who relapse after HLA-mismatched SCT is infusion of donor T cells that have been pre-selected for reactivity to leukemic or patient hematopoietic cells, thus separating the GvL effect from GvHD. This can be achieved by ex vivo generation of donor T cells specific for leukemia- or hematopoietic system-restricted antigens. This thesis focuses on the feasibility of generating T cells specific for hematopoietic system-restricted antigens across HLA-mismatches to facilitate immunotherapy after HLA-mismatched SCT. For this purpose, knowledge of the major players in this particular field of immunology is essential.

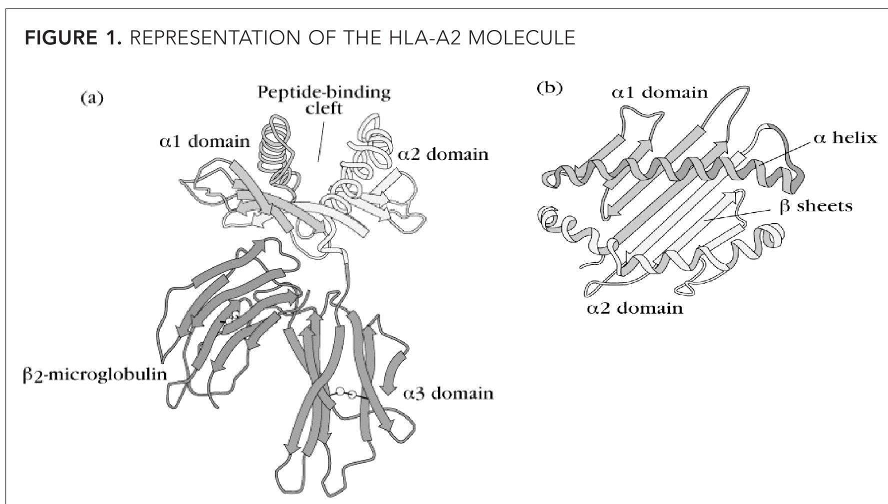
3. BASICS OF THE CELLULAR IMMUNE RESPONSE

3.1 HUMAN LEUKOCYTE ANTIGENS

HLA molecules are cell surface glycoproteins encoded by a cluster of genes in the major histocompatibility complex (MHC) on the short arm of chromosome 6. The unique structure of HLA molecules allows them to bind peptide fragments and to express these fragments on the cell surface for presentation to T cells. Presentation of peptide fragments derived from harmful antigens such as viral proteins, bacterial or parasitical products, or tumor-associated proteins, may be associated with the production of "danger signals" and thus elicit an immune response. Two distinct types of HLA molecules have been described i.e. class I and class II.

HLA class I molecules are encoded by three loci termed HLA-A, HLA-B, and HLA-C. These loci are highly polymorphic. To date 372 HLA-A alleles, 661 HLA-B alleles, and 190 HLA-C alleles have been described[64]. HLA class I molecules consist of a membrane-anchored heavy chain and a non-covalently associated light chain called β2-microglobulin. The extracellular portion of the heavy chain is divided into three domains: α1, α2, and α3. The α1 and α2 domains together form the peptide-binding site, which consists of two α helices supported by an eight-stranded β-pleated sheet (Figure 1). The majority of the polymorphic residues of the HLA class I molecules are located in or near this peptide-binding groove. The conserved residues of the α helices close the ends of the groove, thereby restricting the length of bound peptide to 8-13 amino acids. Binding of peptides to the groove is dictated by the way the side chains of specific amino acids of the peptide, the so-called "anchor residues", fit into "pockets" formed by the polymorphic residues lining the groove. Different HLA

FIGURE 1. REPRESENTATION OF THE HLA-A2 MOLECULE



Ribbon diagram of the extracellular domains of HLA-A2 (A), and top-view of the peptide-binding groove in HLA-A2 as seen by the TCR (B). Adapted from Bjorkman et al.[165]

class I alleles have different binding requirements. In consequence, each HLA class I allele binds its own set of peptides from the available peptide repertoire[65].

HLA class II molecules are encoded by three clusters of loci termed HLA-DR, HLA-DP, and HLA-DQ. HLA class II molecules consist of a polymorphic heavy α chain and light β chain, both containing two extracellular domains (α1, α2, β1, and β2). The α1 and β1 domains form the peptide-binding groove of the HLA class II molecule. Comparable to HLA class I molecules, the peptide-binding groove contains the majority of the polymorphic residues. However, the HLA class II groove is open at both ends, facilitating the binding of longer peptides (13-25 amino acids). Again, each HLA class II allele is associated with its own set of peptides[65].

Because of the highly polymorphic nature of HLA molecules, the probability that two unrelated individuals will have exactly the same HLA alleles is extremely low. The HLA class I and II encoding genes are inherited as haplotypes with low recombination frequencies. In consequence, the probability of two siblings having inherited the same paternal and maternal haplotypes, thus being HLA-identical, is 25%.

All nucleated cells are capable of expressing HLA class I molecules, and are therefore, by definition, antigen-presenting cells (APCs). High levels of HLA class II molecules are expressed by the so-called professional APCs, such as dendritic cells (DCs), macrophages, and activated B cells. On non-professional APCs expression of HLA class II can be induced by pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α)[66]. HLA class II molecules are assembled in the endoplasmatic reticulum (ER) and transported to endosomal compartments where they are loaded with peptides. These peptides are derived mainly from extracellular or membrane-bound proteins that have been endocytosed / phagocytosed and processed by the APC[67]. HLA class II molecules present peptides to $CD4^+$ T cells thereby initiating a T helper (Th) response.

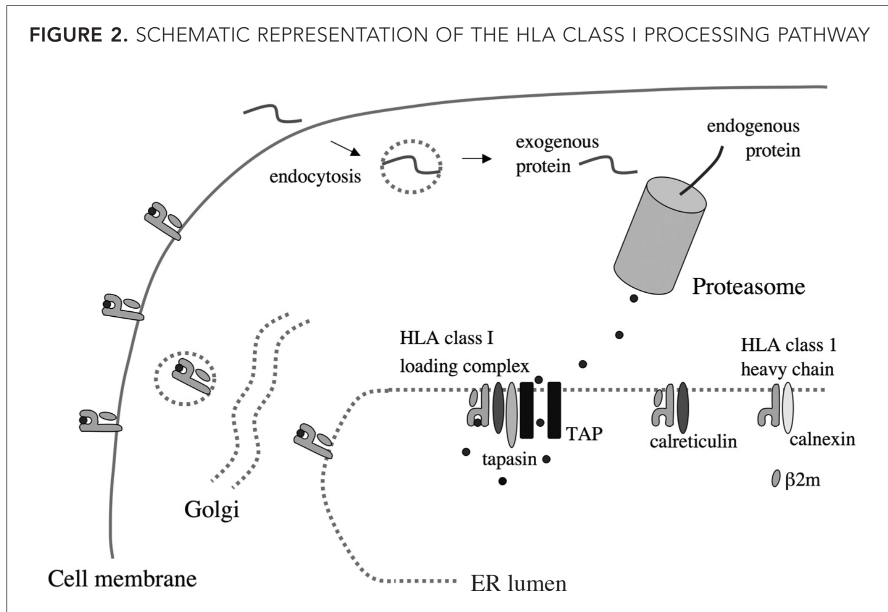
HLA class I molecules are assembled in the ER and there loaded with peptides. These peptides are generally derived from endogenously synthesized proteins, which may be self-proteins, but also viral gene- or oncogene- products. HLA class I molecules present peptides to $CD8^+$ T cells, enabling detection of virus-infected or transformed cells and initiating a cytotoxic response[67]. The total number of HLA class I/peptide complexes at the cell surface amounts to 100.000 to 300.000 per cell[68]. Because of the focus of this thesis, only antigen processing of HLA class I-binding peptides will be examined in detail.

3.2 ANTIGEN PROCESSING OF HLA CLASS I-BINDING PEPTIDES

Most peptides associated with HLA class I molecules are generated in the cytosol by the proteasome, a multicatalytic proteinase complex controlling intracellular protein turnover. The transporter associated with antigen processing (TAP) translocates the peptides into the ER lumen. TAP is a heterodimer, consisting of the subunits TAP1 and TAP2 that are encoded by genes within the MHC. In the ER, newly synthesized HLA class I heavy chains assemble with β2-microglobulin and peptide. This assembly process involves transient interactions with several chaperones including calnexin, calreticulin, and tapasin in the

MHC class I loading complex. The resulting stable HLA class I complex, consisting of peptide, β2-microglobulin, and an HLA class I heavy chain, is then released from the ER and transported to the cell surface via the Golgi apparatus[67] (Figure 2).

FIGURE 2. SCHEMATIC REPRESENTATION OF THE HLA CLASS I PROCESSING PATHWAY



While the above route is the dominant source of HLA class I/peptide complexes at the cell surface, alternative pathways are known to exist. Cells lacking functional TAP still express detectable levels of HLA class I/peptide complexes. These peptides are mostly derived from the signal sequences that guide the translocation of membrane or secretory proteins into the ER or from other protein segments processed not by the proteasome but by proteases situated in the ER[69].

Furthermore, peptides derived from extracellular or membrane-bound proteins can also be presented in HLA class I molecules. Some of the exogenous antigens, that have been internalized by endocytosis / phagocytosis, are released into the cytosol where they enter the conventional HLA class I antigen-processing pathway described above (Figure 2). Alternatively, exogenous antigens may make use of a retrograde pathway leading from endosomal compartments to the Golgi apparatus and from there to the ER. Lastly, some HLA class I complexes may enter the endosomal compartments by internalization or by direct transport from the ER, where they bind or exchange peptides before further transport to the cell surface[67,70]. These pathways function primarily in professional APCs capable of internalizing exogenous antigens.

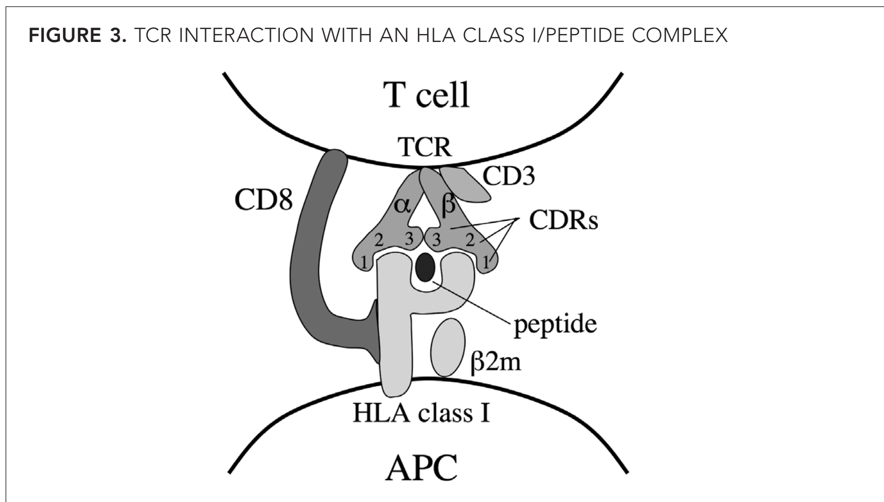
The total peptide repertoire available for binding to HLA class I molecules is determined both by the pathways described above, and by the expression pattern of the peptides'

source proteins. Not all proteins are expressed in every cell type, and thus some peptides will display a tissue-specific distribution. Furthermore, if a protein is generated in a high copy number, its peptides will be present in high copy numbers as well, and vice versa. The binding requirements of the HLA class I alleles then determine which peptides of the total available repertoire will ultimately be bound. In consequence, two individuals who do not express the exact same HLA class I alleles, will not present the same selection of peptides on the cell surface of their APCs.

3.3 GENERATION AND SELECTION OF T CELLS

T cells originate from bone marrow stem cells and migrate to the thymus for further maturation. Within the thymus, developing T cells receive signals that induce them to assemble T cell receptors (TCRs). TCRs consist of highly diverse αβ heterodimers complexed with the invariant accessory protein CD3. While the CD3 chain mediates intracellular signaling after triggering of the TCR, the α and β chains interact with the HLA/peptide complexes. The α and β chain both consist of a constant and a variable domain. The variable domain is generated in the thymus by rearrangement of variable (V), diversity (D), joining (J), and constant (C) gene segments, resulting in great TCR diversity. Furthermore, the V region contains three regions of hypervariable amino acid sequences, called complementarity-determining regions (CDR). Germline V gene segments encode CDR1 and CDR2. CDR1 and CDR2 interact with the α helices of the HLA molecule and establish the configuration of the contact between TCR and HLA/peptide complex. Because CDR1 and CDR2 contain many conserved residues, a measure of HLA reactivity is ensured for each TCR[71]. CDR3 is generated largely by somatic hypermutation during T cell development and is therefore the most variable CDR. Loops of CDR3 engage the peptide, thus determining the specificity of the TCR – HLA/peptide complex interaction (Figure 3). This interaction is so refined that

FIGURE 3. TCR INTERACTION WITH AN HLA CLASS I/PEPTIDE COMPLEX



the TCR can discriminate between peptides that differ in a single amino acid. Whether the mature T cell will ultimately express CD4 or CD8 co-receptors, depends on the preference of the TCR for HLA class I or HLA class II/peptide complexes[72].

During thymic maturation, immature T cells form TCRs that interact with self-HLA/self-peptide complexes expressed in the thymus. Only T cells capable of engaging their TCRs with these HLA/peptide complexes are thought to receive the signals necessary for the next step in the maturation process (positive selection). Positive selection ensures that the T cell repertoire will be fine-tuned to react strongly to potentially harmful peptides bound to the same HLA molecules in the periphery.

Next, T cells that react to the HLA/self-peptide complexes in the thymus with high avidity are deleted (negative selection). Negative selection is required to purge the T cell repertoire of potentially self-reactive T cells that may initiate an autoimmune response in the periphery[72]. Induction of non-responsiveness, ignorance, and generation of regulatory T cells represent additional mechanisms for establishing self-tolerance[73]. Thus, only T cells that display a TCR capable of interacting with self-HLA molecules but with low avidity for self-HLA/self-peptide complexes receive appropriate survival signals and will subsequently enter the periphery as naive T cells.

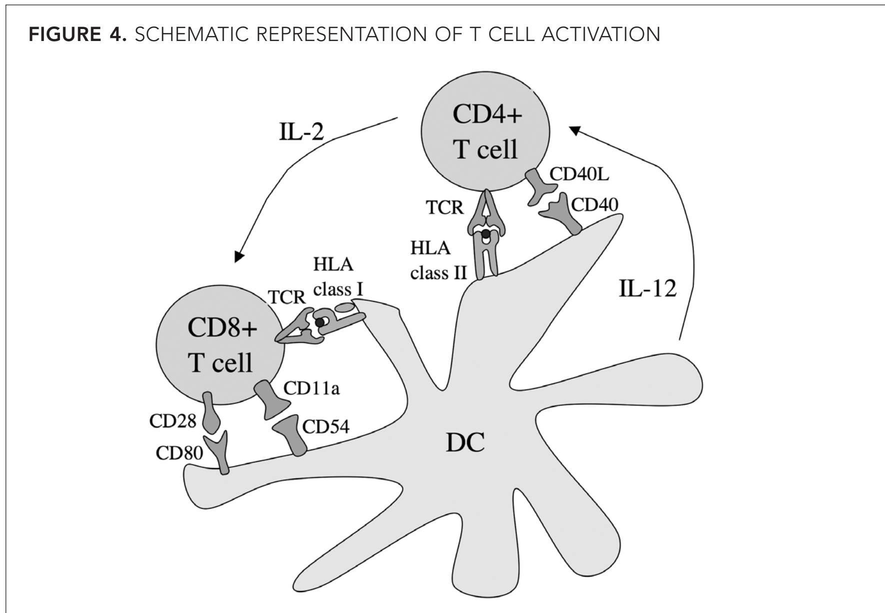
3.3.1 ACTIVATION OF T CELLS

Naive T cells continuously circulate in the periphery through lymphatic vessels and lymph nodes until they become activated. Activation of naive T cells is the prerogative of professional APCs, especially DCs. Immature DCs are located in the peripheral tissues, preferentially at potential pathogen-entry sites such as skin and mucosae, and continuously sample antigens. Encounter of “danger signals” like microbial products, inflammatory cytokines, or tissue damage triggers particular Toll-like receptors thereby initiating DC maturation. Maturation increases processing and presentation of peptides derived from the antigenic sample collected by the immature DC at the time of “danger”, decreases new antigen uptake, and initiates migration to regional draining lymph nodes. These lymph nodes become enlarged, thus increasing the traffic of naive T cells[74].

Upon recognition of their specific HLA/peptide complexes, naive T cells become trapped on the DC cell surface. Accessory molecules expressed by the DC provide additional costimulatory and adhesive signals required for optimal T cell activation (Figure 4). The most important costimulatory molecules are CD80 and CD86 that interact with CD28 on T cells. Adhesion is mediated by the binding of CD54 and CD58 on DCs to CD11a/CD18 and CD2 on T cells. Recently it was shown that members of the TNF-family provide additional signals necessary for prolonged survival and memory formation, mainly via CD27 – CD70 and CD137L – CD137 ligations. Optimal activation often requires three way interactions between DCs, $CD4^+$ T cells, and $CD8^+$ T cells. Activated $CD4^+$ T cells assist in the induction of $CD8^+$ T cells by increasing the DC’s expression of costimulatory molecules via CD40 – CD154 ligation and by secreting cytokines such as interleukin-2 (IL-2). HLA/peptide complex recognition by the TCR in the absence of costimulatory signals may induce naive

T cells to become unresponsive (anergic) or to die. Only under conditions of high antigen concentrations, TCR triggering alone is sufficient to activate naive T cells[75].

FIGURE 4. SCHEMATIC REPRESENTATION OF T CELL ACTIVATION



3.3.2 EFFECTOR FUNCTIONS OF T CELLS

Activated T cells differentiate into effector cells capable of proliferation, cytokine production, and cytotoxic activity. The main effector function of CD8$^+$ T cells is lysis of target cells through release of lytic granules or through the induction of apoptosis by TNF-α or FAS ligand. Effector CD8$^+$ T cells also secrete pro-inflammatory cytokines such as IFN-γ or TNF-α that can be tumoricidal and recruit additional effector cells. CD4$^+$ T cells can differentiate into cytotoxic effectors or evolve into Th1 or Th2 type T cells. Th1 cells produce IL-2 and IFN-γ that activate macrophages and stimulate cell-mediated cytotoxicity. Th2 cells secrete IL-4 and IL-10 thus regulating B cell proliferation and antibody-production. Th cell class is largely determined by the cytokines secreted by DCs during T cell activation. This cytokine profile, in turn, is skewed during DC maturation in the periphery by the triggering of different Toll-like receptors by different pathogenic products and by the production of particular cytokines by damaged tissues. Thus, the immune system actively tailors T cell effector functions to the most efficient elimination of a particular pathogen in a particular tissue[16,76,77].

After activation, effector T cells undergo clonal expansion and migrate into the periphery. Recognition of their specific HLA/peptide complex on the surface of (infected) cells results in the destruction of these cells, but not of innocent bystander cells. Expansion is followed by contraction once the target antigen is cleared. A small proportion of effector T cells

will remain to form a memory T cell population. Memory T cells are characterized by high TCR affinity, longevity, limited dependence on costimulation, rapid turnover, and easy access to the periphery. Memory T cells, in short, are poised to respond quickly after a repeated encounter with antigen when antigen levels and the availability of costimulation may still be low[78].

3.4 IMMUNE RECONSTITUTION AFTER ALLOGENEIC SCT

After allogeneic SCT, stem cells and mature immune cells in the donor graft reconstitute the immune system of the patient who has been left immunodeficient by the conditioning regimen. The time for restoration varies for the different components and functions of the immune system. Newly developing DCs and NK cells integrate quickly, but the development of a new T cell repertoire may take up to three years.

T cell reconstitution early after SCT occurs primarily through thymus-independent expansion of mature T cells transferred with the donor graft. $CD8^+$ T cell numbers normalize more rapidly than $CD4^+$ T cell numbers[79]. During the first year post-SCT, the T cell repertoire is limited and unstable. Massive clonal T cell expansions are frequent, driven by reactivity to pathogens or to the host. Because these T cells originally matured in the thymus of the donor, they are rendered tolerant to the donor, but not necessarily to the host. HLA/peptide complexes presented by host APCs but foreign to the donor, can serve as targets for immune responses by donor-derived T cells[80]. Newly generated, donor stem cell-derived T cells that mature in the host thymus are rendered tolerant to the host and will not cause GvHD[81]. However, the timeframe of immune reconstitution by host-maturated T cells depends on residual thymic function, which declines with age and is additionally damaged by the conditioning regimen and GvHD.

3.5 CELLULAR IMMUNE RESPONSES AFTER ALLOGENEIC SCT

The development of immune responses against the host can be considered a three step process. First, the pretransplant conditioning regimen meant to eradicate the malignancy leads to tissue damage throughout the body and the production of the pro-inflammatory cytokines IL-1 and TNF-α. These "danger signals" cause DCs in the host to more efficiently present host-specific HLA/peptide complexes to donor-derived T cells. Once activated, donor T cells secrete IL-2 and IFN-γ, inducing inflammatory responses and cytotoxic reactivity by T cells and NK cells. This mechanism results in the amplification of tissue damage, further promotion of a pro-inflammatory response, and enhanced T cell reactivity against other potential antigens. GvHD, GvL, or both, ensue depending on the tissue-distribution of the targeted HLA/peptide complexes[82,83].

Factors influencing the strength of the immune response are the type of conditioning regimen, cytokine gene polymorphisms that affect cytokine production[84], concomitant infections, and the survival of host-derived DCs. Early after allogeneic SCT, residual host-derived DCs and new donor-derived DCs co-exist in the periphery. Host DCs directly present disparate HLA/peptide complexes to donor T cells, donor DCs present disparate

peptides indirectly after internalization and degradation of host antigens. Overall, direct presentation seems to be dominant. Persistence of host DCs after allogeneic SCT correlates with development of more severe GvHD, but also with stronger GvL effects after DLI[85,86]. The presence of active infections during DLI increases the incidence and severity of DLI-induced GvHD. In consequence, timing of immunotherapeutic interventions is important.

4. TARGETS OF THE CELLULAR IMMUNE RESPONSE AFTER ALLOGENEIC SCT

4.1 MINOR HISTOCOMPATIBILITY ANTIGENS

After HLA-matched SCT, donor T cell immune responses are directed against disparate peptides presented by HLA molecules that are identical between donor and host. Theoretically, APCs of HLA-identical individuals express the same HLA/peptide complexes, as their HLA alleles bind the same peptide selection. However, some peptides are differentially expressed between HLA-matched individuals.

Such peptides most often arise from polymorphic self-proteins. The human genome contains a great number of single nucleotide polymorphisms. Single nucleotide polymorphisms may cause amino acid substitutions in a protein. These changes do not usually affect the biological activity of the protein, but may influence the processing or presentation of peptides derived from that protein. A single amino acid difference can alter TCR recognition[87], affect intracellular processing of the peptide[88], influence TAP transport efficiency[89], or change affinity for binding by certain HLA molecules. Also, peptide expression can vary with deletion or absence of the gene encoding the source protein[90]. Any of these factors may result in differential peptide expression between HLA-matched individuals. If these differentially expressed peptides are immunogenic, they are called minor histocompatibility antigens (mHags). Table I contains a list of mHags identified to date.

Depending on its HLA-binding properties, a particular mHag will be presented on the cell surface in the context of a particular HLA class I or class II molecule. mHag presentation is therefore HLA allele-restricted. Furthermore, mHag presentation can be ubiquitous or tissue restricted. If the source protein of an mHag is expressed in a limited number of tissues, only those tissues will present that mHag. Tissue distribution of some mHags can be affected by inflammatory conditions as inflammatory cytokines alter protein expression patterns and upregulate HLA class II expression[91].

mHags are inherited as Mendelian traits, independent of HLA alleles. Therefore, HLA-identical siblings, unlike identical twins, may be disparate for mHags. Because the general population has a greater diversity of mHag alleles than single families, the probability of mHag disparity is higher between HLA-matched unrelated individuals than between HLA-identical siblings. These mHag differences contribute to the higher GvH reactivities observed after SCT from HLA-matched unrelated donors.

TABLE I. HUMAN MHAGS CHARACTERIZED TO DATE

mHag	Restriction molecule	Gene	Tissue distribution	Phenotype frequency*	References
HY-A1	HLA-A1	DFFRY	Ubiquitous	Male	155,156
HY-A2	HLA-A2	SMCY	Ubiquitous	Male	157
HY-B7	HLA-B7	SMCY	Ubiquitous	Male	158
HY-B8	HLA-B8	UTY	Ubiquitous	Male	159
HY-B60	HLA-B60	UTY	Ubiquitous	Male	160
HY-DQ5	HLA-DQ5	DBY	Ubiquitous	Male	161
HY-DR52	HLA-DR52	RPS4Y	Unknown	Male	162
HA-1-A2	HLA-A2	KIAA0023	Hematopoietic	69%	87
HA-1-B60	HLA-B60	KIAA0023	Hematopoietic	69%	163
HA-2	HLA-A2	Myosin I G	Hematopoietic	95%	91
HA-3	HLA-A1	AKAP-13	Ubiquitous	88%	88
HA-8	HLA-A2	KIAA0020	Ubiquitous	65%	89
HA-9	HLA-A3	SP110	Hematopoietic	54%	164
HB-1	HLA-B44	Unknown	Hematopoietic	79%	102
ACC-1	HLA-A24	BCL2A1	Hematopoietic	79%	103
BLCL	HLA-B44	BCL2A1	Hematopoietic	50%	103
Unknown	HLA-A29	UGT2B17	Liver/Colon	90%	90

**Phenotype frequency in the caucasian population.*

4.1.1 MHAG-SPECIFIC T CELLS, GVHD AND GVL

Human mHags were originally detected owing to their role as histocompatibility barriers in graft rejection[92]. Since then, mHag-specific T cells have been isolated from patients after allogeneic HLA-matched SCT[93]. The role of mHag-specific T cells in the induction of GvHD and GvL is indicated by the correlation with mHag disparity[94,95]. Both CD8$^+$ and CD4$^+$ T cells appear to contribute to these responses[93,96].

Not much is known about the diversity of mHag-specific T cell responses and the relative contribution of each separate response to the overall donor-derived immune response. Evidence suggests however that the majority of the response is directed against a limited number of immunodominant mHags[94,97,98]. Responses against mHags with a broad tissue distribution are thought to mediate both GvHD and GvL. For instance, male patients receiving a stem cell graft from a female donor have a higher risk of developing GvHD and a lower risk of leukemia relapse[99]. High frequencies of circulating male-associated mHag HY-specific T cells are detectable in the peripheral blood of such patients[100] and in an in vitro skin explant model HY-specific T cells could infiltrate male skin and induce GvHD reactions[101]. T cell responses directed against mHags expressed by only hematopoietic cells or leukemic cells are likely to mediate mainly GvL. They eliminate the patient's residual

hematopoietic cells and leukemic cells while sparing the donor-derived hematopoietic cells and the patient's non-hematopoietic tissues. Hematopoietic system-restricted mHags are therefore excellent targets for adoptive immunotherapy.

4.1.2 mHAGs AS TARGETS FOR ADOPTIVE IMMUNOTHERAPY

Several mHags have been described that display a hematopoietic system-restricted tissue distribution[87,91,102,103]. T cells specific for the hematopoietic system-restricted mHags HA-1, HA-2, HB-1, and BCL2A1 have been isolated from patients after allogeneic SCT and were shown to lyse leukemic cells and their progenitors in vitro [96,102-105]. In addition, the emergence of HA-1- and HA-2-specific T cells coincides with the remission of CML and MM after DLI[95]. T cell responses against HA-1 and HA-2 do not induce clinically relevant GvHD in the ex vivo skin explant model described before, although some grade I skin GvHD may be observed due to T cell responses against residual host-derived hematopoietic cells[101]. Generation, selection, and infusion of HA-1- and HA-2-specific T cells for the adoptive therapy of relapsed leukemia after allogeneic HLA-matched SCT as well as HA-1 and HA-2 peptide vaccination strategies are currently being investigated[106,107]. Furthermore, most of the autosomally encoded mHags identified so far appear to be derived from genes involved in tumorigenesis[108]. HA-1 in fact shows aberrant expression in certain solid tumors[109]. Malignancy-associated mHags could therefore serve as targets for adoptive immunotherapy of solid tumors. A limitation of adoptive immunotherapy with mHag-specific T cells is the small number of suitable mHags identified so far, and the need to find specific mHag disparities between stem cell donor and patient.

4.2 TARGETS OF THE CELLULAR IMMUNE RESPONSE AFTER ALLOGENEIC HLA-MISMATCHED SCT

After HLA-mismatched SCT, donor T cell immune responses can be directed against both disparate peptides presented by non-self (allo) HLA molecules and against the disparate HLA molecules themselves. T cells have not been negatively selected in the thymus against recognition of alloHLA molecules. As a result, 1 to 10% of the total T cell repertoire displays alloHLA-reactivity[110]. Two models have been proposed to account for this high frequency. The high determinant density model proposes that alloHLA-reactive T cells recognize alloHLA molecules independent of bound peptide. In this case, all the HLA molecules of a given allele serve as ligands for the alloHLA-reactive T cell, creating a high ligand density and a high precursor frequency[111]. Peptide-independent alloHLA-recognition may result from high-affinity interactions between polymorphic residues of the alloHLA α helices and the relatively conserved CDR2 and CDR3 loops of the TCR. Negative selection in the thymus supposedly deletes T cells that might be activated by peptide-independent interactions with self-HLA molecules, but not T cells that display reactivity to alloHLA molecules.

The second model, the multiple binary complex hypothesis, proposes that alloHLA-reactive T cells respond to both alloHLA-molecule and peptide[112]. Because peptide-binding requirements differ between HLA alleles, an alloHLA allele is associated with

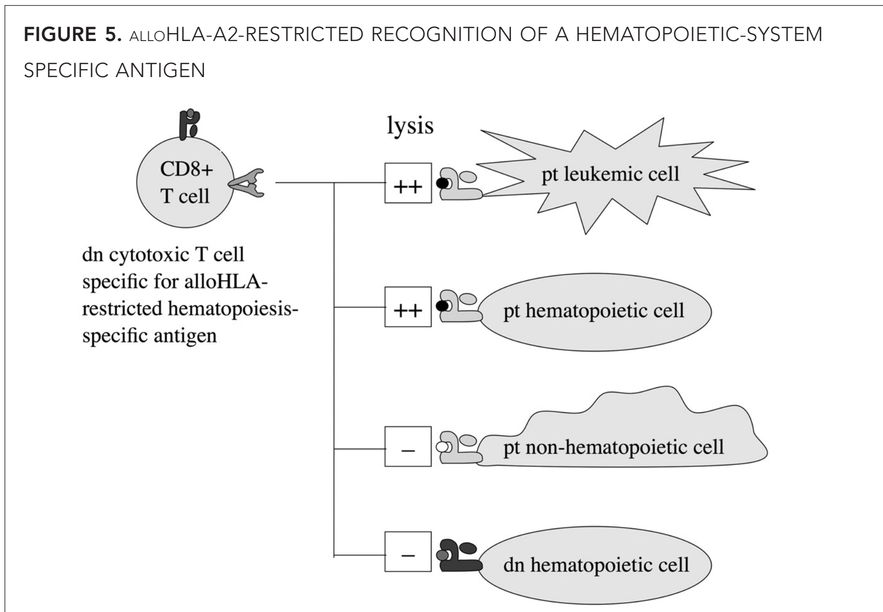
a whole new peptide repertoire. Each of the resulting alloHLA/peptide complexes can elicit alloHLA-restricted T cell responses, comparable to responses induced by self-HLA molecules presenting foreign peptides. The cumulative effect of many different T cells recognizing alloHLA-restricted peptides could account for the strength of the alloHLA-response. Peptide-independent and peptide-specific alloHLA-recognition have both been reported[41,113-117], indicating that both models may contribute to alloHLA-reactivity.
Crystallography of a murine TCR – alloMHC class I/peptide structure revealed that the orientation of TCRs bound to alloMHC is very similar to the one used in contacting self-MHC. Furthermore, peptide residues constitute an integral part of the surface of the alloMHC/peptide complex, contacted by the TCR[118]. In a murine system, a $CD8^+$ T cell alloresponse against an alloMHC molecule closely related to self-MHC, as defined by amino acid differences in the peptide-binding groove only, contained more peptide-specific T cells than a response against an alloMHC molecule with both groove and α helical replacements[119]. Thus, thymic selection for self-MHC reactivity influences the alloMHC-reactive T cell repertoire. However, for humans no association between peptide-specific alloHLA-recognition and HLA similitude could be detected[120]. Probably, the alloHLA-reactive T cell repertoires differ between humans that have similar HLA alleles due to environmental influences such as viral infections[121], that are absent in laboratory animals kept under strict hygienic conditions.
The high frequency of T cells that can respond to alloHLA molecules explains the high incidence and the severity of GvHD after HLA-mismatched SCT. The relative contribution of the two models described above to the total alloHLA-response remains a subject of debate and many questions are yet unanswered. However, the feasibility of generating alloHLA-restricted peptide-specific T cells may provide us with an excellent tool for adoptive immunotherapy.

5. ADOPTIVE IMMUNOTHERAPY WITH ALLOHLA-RESTRICTED T CELLS

5.1 ADOPTIVE IMMUNOTHERAPY USING ALLOHLA-RESTRICTED T CELLS

The ideal scenario for immunotherapy without GvHD would be to target donor T cells specifically to the patient's leukemic or hematopoietic cells in general. Elimination of all residual hematopoietic cells of the patient will also eradicate the patient's hematological malignancy, but not the newly grafted donor hematopoietic cells.
Suitable target antigens can be derived from leukemia-specific fusion proteins, from self-proteins that are over-expressed in leukemic cells, or from peptides expressed only by the hematopoietic cells of the patient, e.g. hematopoietic system-restricted mHags. After HLA-matched SCT, donor T cells recognize these antigens in the context of self-HLA class I. After HLA-mismatched SCT, donor T cells can recognize these antigens in two different modes. If the HLA allele presenting the antigen is shared between donor and patient, recognition is similar to that after HLA-matched SCT. If the antigen-presenting HLA allele is disparate between donor and patient, donor T cells recognize the antigen in the context of alloHLA (Figure 5).

FIGURE 5. ALLOHLA-A2-RESTRICTED RECOGNITION OF A HEMATOPOIETIC-SYSTEM SPECIFIC ANTIGEN



dn = donor, pt = patient

Using alloHLA-restricted T cells for adoptive immunotherapy has several hypothetical advantages. First of all, using the alloHLA-restricted T cell repertoire circumvents tolerance to antigens derived from self-proteins over-expressed in leukemic cells. T cell responses generated against these antigens in an HLA-matched setting are often of low avidity, due to previous deletion or tolerization of high avidity donor-derived T cells. If the donor is mismatched for the antigen-restricting HLA allele, tolerization is not an issue and generation of high avidity alloHLA-restricted antigen-specific T cells becomes feasible.
In addition, the alloHLA-restricted T cell repertoire provides antigen specificities that cannot be found in the self-HLA-restricted repertoire. After HLA-matched SCT, peptides derived from "normal" non-polymorphic hematopoietic system-restricted proteins cannot serve as antigens for immunotherapy. Patient and donor APCs present these peptides in equal numbers so that potentially reactive donor T cells will have been deleted in the donor thymus. Moreover, due to the limited number of hematopoietic system-restricted mHags identified so far, immunotherapy is applicable for a few donor – patient combinations only. HLA-mismatched donor APCs however present a different selection of peptides from the same proteins, so that donor T cells specific for the hematopoietic system-restricted peptides of the patient are preserved. Thus, the use of alloHLA-restricted T cells eliminates the need to find specific mHag disparities between stem cell donor and patient. In consequence, the use of the alloHLA-restricted T cell repertoire greatly expands the number of patients eligible for immunotherapy of leukemia relapse post-SCT. This concept was first proposed by H.J. Stauss et al. who showed the generation of alloHLA-restricted human cyclin-D1- and WT1 tumor antigen-specific cytotoxic T cells[122,123].

5.2 TARGETS OF ADOPTIVE IMMUNOTHERAPY BY ALLOHLA-RESTRICTED T CELLS

Hematopoietic system-restricted, leukemia-specific or leukemia-associated antigens suitable for immunotherapy should fulfill the following criteria: (1) sufficient expression on the patient's malignant and/or hematopoietic cells; (2) no expression on non-hematopoietic cells of the patient; (3) no expression on hematopoietic cells of the donor; (4) elicitation of cytotoxic T cell responses of sufficient avidity. Potential targets are discussed in more detail below.

5.2.1 LEUKEMIA-SPECIFIC ANTIGENS

Leukemia-specific fusion proteins such as BCR-ABL in CML, PML-RAR in acute promyelocytic leukemia, and DEK-CAN and ETV6-AML in AML, have been extensively studied as possible sources of leukemia-specific antigens in vivo. Unfortunately, few peptides have been identified that are (a) derived from the fusion sites, (b) are naturally processed, and (c) are sufficiently immunogenic to reproducibly elicit T cell responses. So far, only the BCR-ABL b3a2 fusion point appears to be a suitable target for adoptive immunotherapy. B3a2 fusion point-derived peptides are presented by leukemic cells and can induce HLA-A3-, HLA-A11-, and HLA-B8-restricted cytotoxic T cell responses[124]. B3a2-specific, HLA-A3- and HLA-B8-restricted T cells were detected in patients after allogeneic SCT[125]. However, a direct link between the presence of b3a2-specific T cells and remission of CML has not yet been established.

5.2.2 LEUKEMIA-ASSOCIATED ANTIGENS

Antigens derived from over-expressed self-proteins are attractive targets for adoptive immunotherapy only if they are expressed at levels substantially higher than normal (approximately 10-fold or more). Otherwise, a T cell response directed against these antigens would induce GvHD. The antigens are preferably present in all hematological malignancies of a particular type and associated with the malignant phenotype, so that downregulation affects tumor cell survival. Self-proteins that meet these criteria are Wilms' tumor antigen (WT1), proteinase-3 (PR-3), survivin, and telomerase. T cells directed against these antigens can be generated from healthy individuals and selectively lyse leukemic cells and their progenitors in vitro[123,126-136]. Furthermore, WT1-, PR-3-, and survivin-specific T cells can be detected in the peripheral blood of patients with hematological malignancies[137-139]. PR-3-specific T cells have also been detected in the peripheral blood of CML patients after allogeneic HLA-matched SCT and correlated with a good prognosis[137]. So far, successful in vitro generated alloHLA-restricted T cell responses have only been elicited against WT1[123,126].

5.2.3 HEMATOPOIETIC SYSTEM-RESTRICTED NON-POLYMORPHIC ANTIGENS

An individual's hematopoietic cells, including the hematological malignancies, express certain non-polymorphic hematopoietic system-restricted proteins, such as CD20 (B lymphocytes), CD68 (mononuclear phagocytes), and CD45 (all hematological cells). After HLA-mismatched SCT, these proteins can serve as targets for immunotherapy with

alloHLA-restricted T cells. Already it has been shown that alloHLA-A2-restricted T cells can be generated against CD68- and CD45-derived peptides[115,117].

5.2.4 MINOR HISTOCOMPATIBILITY ANTIGENS

As described before, adoptive immunotherapy of relapsed leukemia after allogeneic HLA-matched SCT using HA-1- and HA-2-specific T cells is currently being investigated. HA-1 and HA-2 are also suitable targets for adoptive immunotherapy after HLA-mismatched SCT. The immunogenic HA-2 allele is expressed by 95% of HLA-A2^{pos} individuals[97]. The immunogenic HA-1 allele is present in 69% of HLA-A2^{pos} individuals. Indeed, the feasibility of generating nonself-HLA-A2-restricted HA-1-specific T cells has recently been demonstrated[140].

5.2.4.1 GENERATION OF mHAG-SPECIFIC alloHLA-RESTRICTED T CELLS

mHag-specific T cells can be generated in vitro by co-culture of lymphocytes with DCs that express the relevant peptide. In this cell culture system, the cytokines IL-2, IL-12, and IL-7[141-143] as well as activated CD4$^+$ T cells are added to enhance expansion, cytotoxic activity, and memory formation. However, the generation of large numbers of mHag-specific T cells ex vivo, while preserving their ability to proliferate and execute their function in vivo, remains a major challenge.

Self-HLA-restricted mHag-specific T cells are usually generated using autologous DCs as stimulators that have been pulsed or retrovirally transduced with mHag peptide[144,145]. Because the new peptide is the only disparate antigen presented, T cells will mount alloresponses against that peptide only. Generation of alloHLA-restricted T cells however requires DCs that express the alloHLA molecule in combination with the mHag peptide. Pulsing or transducing an APC with peptide enforces its preferential presentation on the cell surface. Yet, presentation of endogenous peptide is not completely abrogated. An APC expressing an alloHLA molecule associated with a completely new peptide repertoire will not only induce T cells with the desired mHag peptide specificity, but also T cells with broad and unpredictable alloHLA-reactivities. These T cells could be very harmful to the patient.

5.3 STRATEGIES TO REDUCE UNDESIRED alloHLA-REACTIVITIES

Several strategies have been designed to reduce the co-generation of undesired alloHLA-reactivities. Most studies describing the generation of alloHLA-restricted peptide-specific T cells have used TAP-deficient T2 cells pulsed with peptide as stimulators[116,117,146,147]. TAP-deficient cell lines present only a limited set of endogenous peptides, so that HLA molecules can be uniformly loaded with exogenous peptides on the cell surface[148]. In a recent study HLA-A2^{neg} lymphocytes were stimulated with HLA-A2^{pos} T2 cells that had been loaded with a synthetic HLA-A2 peptide library. Peptide-specific T cells constituted only half of the resulting alloHLA-A2-reactive T cell repertoire[116]. In several studies, T cells were stimulated alternatively with T2 cells, and with C1R cells or Drosophila-derived cells expressing a single HLA-A2 allele to avoid repeated exposure to undesired endogenous peptides. Despite these precautions considerable levels of undesired alloHLA-reactivity were commonly detected[116,117,149].

An alternative approach was based on the removal of undesired alloHLA-reactive T cells from the donor lymphocyte population prior to stimulation with the target antigen. T cells that upregulate the activation markers CD69 and CD25 after exposure to antigenically disparate cells can be efficiently removed using magnetic beads[150]. In an HLA-mismatched setting, this approach indeed reduced alloHLA-reactivity of CD69/CD25-depleted T cell populations in the initial weeks of culture. However, strong alloHLA-responses developed upon restimulation with alloHLA-expressing target cells after several weeks[140].
As yet, the only secure method of obtaining peptide-specific alloHLA-restricted T cells consists of limiting dilution and cloning. This method is laborious and time-consuming. Furthermore, many rounds of cell division are required to obtain sufficient numbers of clonal T cells of a desired specificity for adoptive immunotherapy. Because in vitro induced T cells have a limited lifespan, their activity and persistence in the patient will be impaired[151]. The clinical applicability of immunotherapy with alloHLA-restricted T cells therefore requires the development of protocols for the generation and propagation of peptide-specific T cells without co-induction of undesired alloHLA-reactivity.

6. AIM OF THIS THESIS

In this thesis we investigated several strategies to generate peptide-specific alloHLA-restricted T cells with minimal co-induction of potentially harmful alloHLA-reactivities. The HLA-A2-associated mHags, HA-1 and HA-2, and the $CD8^+$ T cell responses directed against these mHags, have been extensively studied. HA-1 and HA-2 are promising reagents for adoptive immunotherapy of leukemia in the HLA-matched as well as the HLA-mismatched setting as addressed in paragraph 5.2.4. We therefore used HA-1 and HA-2 as model antigens in our studies.
In chapter 2, we explored the feasibility of using artificial antigen-presenting constructs (aAPCs) to stimulate T cells. aAPCs that express a single type of HLA/peptide complex, will stimulate only alloHLA-restricted T cells specific for that particular complex. To generate aAPCs, we coated latex beads with HLA-A2/mHag monomers and recombinant human CD80-Fc and CD54-Fc. Optimal ligand density was determined by flowcytometric analysis and functional testing. The resulting aAPCs were used to stimulate mHag-specific T cell clones and polyclonal mHag-specific T cell lines. We analyzed ligand-specific expansion, cytokine production, and cytotoxic activity of the T cells before and after stimulation with aAPCs.
In chapter 3, we examined the effects of TAP inhibition on the ability of APCs to stimulate alloHLA-reactive T cells. An APC expressing a TAP inhibitor and exogenously pulsed with peptides could serve as an antigen-specific stimulator for HLA-mismatched T cells. To test this, we transduced Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCLs) with viral TAP inhibitors. EBV-LCLs have previously been shown to efficiently present antigens to both self- and alloHLA-reactive T cells[117]. For transduction we used retroviral vectors encoding US6, ICP47, or UL49.5, derived from human cytomegalovirus,

herpes simplexvirus type 1, and bovine herpes virus type 1, respectively. These proteins inhibit peptide translocation by TAP in human cells, thus affecting endogenous peptide presentation. We compared their effects on the EBV-LCL's HLA class I expression, and recognition by mHag-specific and alloHLA-reactive T cell clones.
In chapter 4, we investigated the alloHLA-A2-specific response in more detail. We aimed to determine whether the majority of alloHLA-restricted T cells generated after repeated stimulation with alloHLA-expressing APCs are peptide-specific or peptide-independent. To this end, HLA-A2^{neg} lymphocytes were stimulated with HLA-A2^{pos} EBV-LCLs or with HLA-A2^{pos} T2 cells. We determined peptide-specific alloHLA-recognition using a panel of HLA-A2 tetramers representing five known antigens derived from ubiquitously expressed self-proteins. Tetramer-binding profiles of the alloHLA-reactive T cells were analyzed. We investigated peptide-independent alloHLA-recognition using aAPCs expressing single type HLA-A2/peptide complexes. IFN-γ production of alloHLA-reactive T cells after stimulation with aAPCs was assessed and compared to IFN-γ production after stimulation with HLA-A2^{pos} EBV-LCLs.
In chapter 5, we combined the results of two different approaches. In the first part of our study, we explored the possibility of coating DCs with a single type of alloHLA/peptide complex. This strategy may allow us to generate peptide-specific alloHLA-restricted T cells using autologous DCs as stimulators. The stimulatory capacity of these complex-coated DCs was tested using ligand-specific proliferation, cytokine production, and cytotoxic activity of mHag-specific T cells as a read-out system. In addition, we used HLA-A2/HA-1 coated DCs as stimulators for the generation of alloHLA-A2-restricted HA-1-specific T cells from HLA-A2^{neg} lymphocytes. In the second part of this study, we examined the feasibility of directly isolating alloHLA-restricted peptide-specific T cells from peripheral blood of HLA-A2^{neg} donors. The use of HLA/peptide tetramers enables the detection and isolation of tetramer-binding T cells present at very low frequencies[152], including non-self-HLA-restricted CTLs[153]. In vivo primed alloHLA-restricted peptide-specific T cells may thus be obtained without a stimulatory in vitro procedure that could increase the risk of co-inducing undesired alloHLA-reactivities. To optimize our chances of success, we selected female donors who had delivered at least one HLA-A2^{pos} child, as pregnancy has been shown to induce mHag-specific T cells[154].
In chapter 6, the overall results are discussed and summarized.

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