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

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Citation

Amir, A. (2012, June 28). *Biology and clinical relevance of T-cell allo-HLA reactivity*. Retrieved from https://hdl.handle.net/1887/19160

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Author: Amir, Avital
Title: Biology and clinical relevance of T-cell allo-HLA reactivity
Date: 2012-06-28

BIOLOGY AND CLINICAL RELEVANCE OF T-CELL ALLO-HLA REACTIVITY



AVITAL AMIR

BIOLOGY AND CLINICAL RELEVANCE OF T-CELL ALLO-HLA REACTIVITY

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FINANCIAL SUPPORT

The work described in this thesis was financially supported by the Landsteiner Foundation for Blood Transfusion and the Dutch Cancer Society (KWF). Printing of this thesis was financially supported by the Dutch Cancer Society and the J.E. Jurriaanse Stichting.

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Cover Design by Joshua Verheijen, De Huurlingen
Thesis Layout and Print by Optima Grafische Communicatie, Rotterdam



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

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 28 juni 2012
klokke 13.45 uur
door

Avital Amir

geboren te Hadera (Israël)
in 1979

PROMOTIECOMMISSIE

Promotor	Prof. Dr. J.H.F. Falkenburg
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	Prof. Dr. F.H.J. Claas
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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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IDENTIFICATION OF A COORDINATED CD8 AND CD4 T-CELL RESPONSE DIRECTED AGAINST MISMATCHED HLA CLASS I CAUSING SEVERE ACUTE GVHD

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Biol Blood Marrow Transplant. 2012 Feb;18(2):210-9



ABSTRACT

After HLA class I mismatched stem cell transplantation allo-HLA directed CD8 T-cell responses can be activated without the help of CD4 T-cells if memory CD8 T-cells crossreactive against the allo-HLA class I are present, or if naïve CD8 T-cells are administered during inflammatory conditions. However, in the absence of inflammatory conditions, cooperation between CD4 and CD8 T-cells is likely to be required for an effective primary CD8 T-cell response directed against allo-HLA class I. In this study we investigated whether a coordinated response of CD8 and CD4 T-cells could be demonstrated in an HLA class I directed immune response in a patient who developed severe graft versus host disease (GVHD) after the administration HLA-A2 mismatched donor lymphocyte infusion (DLI) in the absence of inflammatory conditions. A previously administered DLI from the same donor did not lead to an immune response, excluding the presence of a substantial pool of CD8 T-cells crossreactive against HLA-A2 within the memory T-cell compartment of the donor. Analysis of isolated donor CD8 and CD4 T-cell clones which were activated during the GVHD in the patient illustrated a polyclonal CD8 T-cell response directed against the mismatched HLA-A2 and a polyclonal CD4 T-cell response recognizing HLA-A2 derived peptides presented in HLA class II. In addition, we demonstrated that patient leukemic blasts present at the time of the emergence of GVHD expressed HLA-A2 and HLA class II and could activate both the CD4 and CD8 alloreactive T-cells. The results demonstrate that the GVHD was mediated by a cooperative CD4 and CD8 response directed against the mismatched HLA-A2 and suggest that leukemic blasts possibly activated both the CD8 and CD4 T-cell response.

INTRODUCTION

In vivo, naive CD8 T-cells require priming by activated antigen presenting cells (APCs) to be able to proliferate and differentiate to effector T-cells. APCs can be activated by the inflammatory signals from pathogens[1;2]. Alternatively, in the absence of inflammatory signals, CD4 T-cells can activate APCs by CD40-CD40L interaction and cytokine production[3-5]. 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[6]. In contrast to naïve CD8 T-cells, memory CD8 T-cells do not need priming by activated APCs[7;8], indicating that CD4 T-cell help is not required for the effective activation of memory CD8 T-cell responses.

Allo-HLA reactive T-cells involved in the generation of graft versus host disease (GVHD) and graft rejection after HLA mismatched stem cell transplantation (SCT) can be derived from the naïve as well as the memory T-cell pool[9]. As we have previously shown, allo-HLA reactivity exerted by virus specific T-cells is common, suggesting that many memory T-cells are able to exert allo-HLA reactivity[10]. Based on the lower activation threshold of memory CD8 T-cells, an allo-HLA class I directed immune response can easily be activated without the presence of activated APCs and CD4 T-cell help, if T-cells cross reactive against the mismatched HLA class I molecule are present within the memory CD8 T-cell compartment. If these CD8 T-cells are not present in the memory T-cell pool, allo-HLA directed immune response has to develop from the naïve CD8 T-cell compartment, probably requiring activated APCs. Prior to SCT patients are treated with conditioning regimens including irradiation and/or chemotherapy which cause tissue damage leading to production of pro-inflammatory cytokines and thereby activation of APCs[11]. In addition, SCT is often complicated by infections which can lead to systemic inflammatory signals[12]. In the presence of these inflammatory conditions, cooperation between CD8 and CD4 T-cells may not be required for effective activation of primary allo-immune responses, as was demonstrated by Korngold et al. who showed that in heavily irradiated MHC class I mismatched mice purified CD8 T-cells were able to initiate GVHD without the help of CD4 T-cells[13;14].

However, in the absence of tissue damage or infection, allogeneic cells are non-inflammatory immunogens. Under these circumstances cooperation between CD4 and CD8 T-cells is likely to be required for an effective primary CD8 T-cell response. Chakraverty et al. demonstrated that in delayed MHC class I mismatched donor lymphocyte infusion (DLI), not preceded by irradiation, CD4 T-cells were necessary for the expansion of GVHD inducing CD8 T-cells[15]. Since for a coordinated response CD4 and CD8 T-cells need to be activated by the same antigen expressing cells, allogeneic cells expressing both HLA class I as well as HLA class II would be required.

In this study we investigated whether a coordinated response of CD8 and CD4 T-cells could be demonstrated in a HLA class I directed immune response in a patient with acute myeloid

leukemia (AML), who received a T-cell depleted SCT and two DLIs from the same HLA-A2 mismatched donor. The first DLI did not lead to an immune response, indicating that there was not a substantial pool of CD8 T-cells crossreactive against HLA-A2 within the memory T-cell compartment of the donor. The second DLI administered in the presence of HLA class II positive leukemic cells, led to a coordinated response of CD8 and CD4 T-cells resulting in a severe acute GVHD. Characterization of the allo-immune response leading to the GVHD showed that the CD8 T-cell response was directed against the mismatched HLA-A2 molecule and that the CD4 T-cell response recognized HLA-A2 derived peptides presented in the context of the HLA-DR1 molecule shared between patient and donor. Patient leukemic cells present at the time of the emergence of GVHD expressed HLA-A2 and HLA-DR, and were able to activate the CD8 as well as the CD4 alloreactive T-cells, suggesting that the leukemic blasts may have activated both the CD8 and the CD4 T-cell response.

DESIGN AND METHODS

Patient

The patient was a 55 year old male with acute myeloid leukemia (AML) secondary to myelodysplastic syndrome in complete remission. After a non-myeloablative conditioning regimen consisting of ATG, Fludarabine and Busulfan[16], he received a T-cell depleted SCT from a sibling donor. No immune suppression was administered post transplantation. Six months after the SCT, a DLI of $2.5x10^6$ T-cells/kg was given for mixed chimerism and 12 months after the SCT, AML for which a second DLI containing 7.5*10e6 T-cells/kg was given. Five weeks after the second DLI the patient died of grade IV GVHD.

HLA typing. Patient: *A0201*, A0301, B0702, B3501, C0401, C0701, DRB1-0101, DRB1-1501, DQB1-0501, DQB1-0602, DPB1-0402. Donor: *A2601*, A0301, B0702, B3501, C0401, C0701, DRB1-0101, DRB1-1501, DQB1-0501, DQB1-0602, DPB1-0402

Cell collection and preparation

After informed consent, peripheral blood and bone marrow samples were obtained from the patient and donor as well as from other patients and healthy donors. Mononuclear cells were isolated by Ficoll-Isopaque separation and cryopreserved. Stable Epstein–Barr virus (EBV)-transformed B-cell lines (EBV-LCLs) were generated using standard procedures. HLA-A2+ donor EBV-LCLs were generated by transduction of donor derived EBV-LCLs with a retroviral vector encoding for HLA-A*0201[17]. Fibroblasts were cultured from skin biopsies in Dulbecco's modified Eagle's medium with 1g/l glucose (BioWhittaker, Verviers, Belgium) and 10% fetal bovine serum (FBS, BioWhittaker). Keratinocytes were cultured from skin biopsies in keratinocyte serum free medium supplemented with 30 µg/ml bovine pituitary extract and 2 ng/ml epithelial growth factor (Invitrogen, Carlsbad, CA, USA).

Flow cytometry

The monoclonal antibodies (mAbs) anti-HLA-A2-FITC, anti-HLA-DR-APC, anti-CD40-PE and anti-CD86-PE, were obtained from Pharmingen (San Jose, CA, USA). Anti-CD45-PerCp, anti-CD3-APC, anti-CD33-APC, anti-CD80-PE, anti-CD54-PE and anti-CD11c-PE were derived from Becton Dickinson (BD, San Jose, CA, USA). Anti-CD4-PE, anti-CD8-PE, anti-CD19-PE and anti-CD14-PE were purchased from Caltag (Carlsbad, CA, USA). Anti-BDCA1-PE and anti-BDCA2-FITC antibodies were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). For determination of TCR Vβ usage of the CD4 T-cell clones, the TCR Vβ kit (Beckman Coulter, Fullerton, CA) was used. Flow cytometric analysis was performed on a BD flow cytometer.

Generation of alloreactive T-cell clones

Patient peripheral blood mononuclear cells (PBMCs) were collected during the start of the GVHD, three weeks after the second DLI, in the absence of administration of immune suppressive drugs. The PBMCs were stained with anti-HLA-A2, anti-HLA-DR, anti-CD4 and anti-CD8 mAbs at 4°C for 30 min and washed once. HLA-DR positive (activated), HLA-A2 negative (donor) CD4+ or CD8+ T-cells were sorted single cell per well into U-bottom microtiter plates containing 100 µl of feeder mixture consisting of Iscove's Modified Dulbecco's Medium (IMDM, Cambrex, Rutherford, NJ, USA), 5% FBS, 5% human serum (HS), IL-2 (120 IU/ml, Chiron, Novartis, Emeryville, CA, USA), phytohemagglutinin (PHA, 0.8 µg/ml, Murex Biotec Limited, Dartford, UK), and 50 Gy irradiated allogeneic third-party PBMCs ($0.5x10^6$ /ml). Proliferating T-cell clones were selected and further expanded using nonspecific stimulation and third-party feeder cells.

Characterization of T-cell clones

To analyze the alloreactivity of the expanded CD8 T-cell clones, cytotoxicity assays and cytokine production assays were performed. In the cytotoxicity assays the CD8 T-cells clones were tested in a standard 4 hour ^{51}Cr-release assay against patient EBV-LCLs, donor EBV-LCLs, and third party HLA-A2+ and HLA-A2- EBV-LCLs in an effector to target ratio of 10:1. In the cytokine production assays IFNγ production of the CD8 T-cells clones in response to patient EBV-LCLs, donor EBV-LCLs and third party HLA-A2+ and HLA-A2- EBV-LCLs was tested. To determine IFNγ production, 5,000 T-cells were cultured with 20,000 stimulator cells in a final volume of 150µl IMDM culture medium supplemented with 60 IU/ml IL-2. After 18 hours of incubation, supernatants were harvested, and IFNγ production was measured by standard ELISA (CLB, Amsterdam, the Netherlands). To determine the HLA-restriction of the CD8 T-cell clones, blocking studies were performed using BB7.2 (anti-HLA-A2), W6.32 (anti-HLA class I) or B1.23.2 (anti-HLA-B and C) mAbs. Patient EBV-LCLs, donor EBV-LCLs, donor EBV-LCLs transduced with HLA-A2 and HLA-A2+ EBV-LCLs were preincubated with saturating concentrations of mAbs for 1 hour at 20°C before addition of T-cells.

To analyze the alloreactivity of the expanded CD4 T-cell clones, IFNγ production in response to patient EBV-LCLs and donor EBV-LCLs was tested. To determine the HLA-restriction of the alloreactive CD4 T-cell clones, blocking studies were performed using W6.32 (anti-HLA class I), PdV5.2 (anti-HLA class II), B8.11.2 (anti-HLA-DR), SPV-L3 (anti-HLA-DQ) or B7.21 (anti-HLA-DP) mAbs. Patient EBV-LCLs, donor EBV-LCLs and donor EBV-LCLs transduced with HLA-A2 were preincubated with saturating concentrations of mAbs for 1 hour at RT before addition of T-cells. In addition, the IFNγ production in response to a panel of HLA-DR*0101+ HLA-A*0201+, HLA-DR*0101+ HLA- A*0201-, HLA-DR*1501+ HLA- A*0201+ and HLA-DR*1501+ HLA- A*0201- EBV-LCLs was tested. To identify the recognized HLA-A2 derived peptides and the minimal recognized epitope of the HLA-A2 derived peptides, IFNγ production of the CD4 T-cells clones in response to donor EBV-LCLs loaded overnight with the different HLA-A2 derived peptides was measured after 18 hours of co-culture.

HLA-A2 derived peptides

To investigate epitopes of HLA-A*0201 which could be recognized in the context of HLA class II by the CD4 T-cell clones, synthetic 20-mer peptides covering the whole sequence of the HLA-A*0201 molecule, with an overlap of 7 amino acids between each two subsequent peptides, were made by solid phase peptide synthesis. To further analyze the minimal recognized epitope, truncated peptides of the recognized region of amino acids 99-122 of the HLA-A2 molecule were generated. All peptides were checked for purity by analytic reversed phase HPLC and amino acid analysis.

Stimulatory capacity of patient hematopoietic cells and non-hematopoietic cells

Bone marrow cells (BMCs) and PBMCs collected from the patient prior to the first and second DLI were stained with anti-HLA-A2 mAb, and the HLA-A2+ cells from the two time points were selected by FACS sorting. In addition, BMCs collected from the patient prior to the second DLI were stained with HLA-A2, CD33 and CD3 mAbs, and patient T-cells (HLA-A2+, CD3+) and leukemic cells (HLA-A2+, CD33+) were selected by FACS sort. IFNγ production of the CD4 and CD8 clones in response to the different T-cell subsets was measured by ELISA. Recognition of non-hematopoietic cells was analyzed using fibroblasts and keratinocytes as stimulator cells. Following cell culture of 3 days in the presence or absence of 200 U/ml IFNγ (Immukine, Boehringer Ingelheim, Alkmaar, the Netherlands), cells were thoroughly washed and 10,000 stimulator cells were co-cultured with 5,000 T-cells. IFNγ production was measured by ELISA after overnight incubation.

RESULTS

CD8 and CD4 T-cells activated during GVHD after single HLA class I mismatched DLI

In this study we characterized the allo-immune response in a patient experiencing GVHD after single HLA class I mismatched DLI. Based on a cross-over, patient and donor had a disparity in HLA-A2, whereas the other HLA class I and II molecules were completely matched. The patient received a T-cell depleted SCT and was treated 6 months after SCT with DLI for mixed chimerism. No change in chimerism was observed and in addition no GVHD developed. 12 months after the SCT, AML relapse occurred with 9% blasts in bone marrow, and 0.1% malignant cells in peripheral blood for which a second DLI was given. In contrast, the second DLI induced severe acute GVHD, and chimerism analyses on bone marrow derived mononuclear cells demonstrated a rapid change in patient chimerism from 41% before second DLI to 2% after the second DLI (data not shown).

To investigate whether the occurrence of GVHD correlated with activation of donor T-cells, the expression of the T-cell activation marker HLA-DR on donor T-cells before and after the

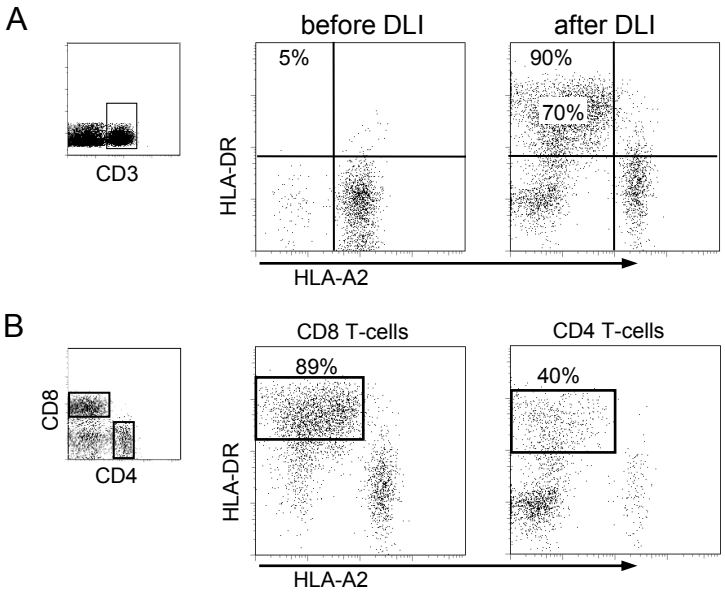


Figure 1. Correlation of GVHD and donor CD4 and CD8 T-cell activation.
Patient (HLA-A2+) and donor (HLA-A2-) cells could be discriminated by the expression of HLA-A2. HLA-DR expression marks the activated T-cells. **(A)** PBMCs collected three weeks before and after the DLI leading to GVHD were stained with mAbs against CD3, HLA-A2 and HLA-DR. The gated CD3+ cells are shown. After the DLI, the percentage of donor T-cells increased from 5% to 90% and 70% of donor T-cells were activated **(B)** PBMCs collected during the GVHD, were stained with anti-HLA-A2 and anti-HLA-DR mAbs in combination with anti-CD4 and anti-CD8. The gated CD4 and CD8 T-cells are shown. 89% of the CD8 donor T-cells and 40% of the CD4 donor T-cells were activated during the GVHD.

second DLI was determined by flow cytometric analysis. After the second DLI, the percentage of donor T-cells increased from 5% to 90%, indicating strong T-cell expansion. In addition, 70% of donor T-cells was activated, as shown by high HLA-DR expression (figure 1A). To investigate whether donor CD8 as well as donor CD4 T-cells were activated, the expression of HLA-DR on the CD8 and CD4 T-cells derived from the GVHD was determined by flow cytometry. As shown in figure 1B, 89% of donor CD8 T-cells and 40% of donor CD4 T-cells were activated during the GVHD.

Isolation of alloreactive CD8 and CD4 T-cells

To characterize the CD8 and CD4 allo-immune responses, activated donor CD8 and CD4 T-cells were isolated from patient PBMCs collected during the GVHD by single cell sort based on the expression of HLA-DR and the absence of HLA-A2. Isolation and expansion of these T-cells resulted in 56 CD8 T-cell clones and 88 CD4 T-cell clones for further analysis

To investigate whether the CD8 T-cell clones were alloreactive, the clones were tested for reactivity against patient and donor EBV-LCLs. 50 of 56 isolated CD8 clones, were shown to be alloreactive, since these T-cells were cytotoxic against patient EBV-LCLs, but not against donor EBV-LCLs (data not shown). In addition to cytotoxicity, these 50 CD8 T-cell clones also produced IFNγ against patient EBV-LCLs, but not against donor EBV-LCLs (data not shown). To determine the alloreactivity of the CD4 T-cell clones, the 88 clones were tested against patient and donor EBV-LCLs in cytokine production assays. 21 of the 88 CD4 clones were alloreactive as shown by IFNγ production upon stimulation with patient EBV-LCLs but not with donor EBV-LCLs (data not shown).

Polyclonal CD8 response directed against allo-HLA-A2

To determine the diversity of the CD8 alloresponse, the TCR Vβ usage of the 50 alloreactive CD8 T-cell clones was analyzed by flow cytometric analysis with Vβ mAbs and sequencing of the CDR3 region of the TCR Vβ chains. The CD8 clones showed usage of at least 13 different TCR Vβs, and all TCR Vβ chains had a different CDR3 sequence (data not shown), demonstrating that all 50 CD8 T-cell clones were of different clonal origin, and that the CD8 response in the patient was polyclonal.

To define the HLA restriction, the CD8 T-cell clones were tested against patient EBV-LCLs, donor EBV-LCLs, donor EBV-LCLs transduced with HLA-A*0201 and a panel of 8 HLA-A*0201+ EBV-LCLs in combination with HLA blocking mAbs. In figure 2A recognition of one representative clone is shown. Donor EBV-LCLs transduced with HLA-A*0201 and the panel of HLA-A*0201+ EBV-LCLs, of which one is shown, were recognized by all CD8 T-cell clones. Recognition of patient EBV-LCLs, donor EBV-LCLs transduced with HLA-A*0201 and third party HLA-A*0201+ EBV LCLs was blocked by HLA-A2 and HLA class I mAbs, but not by HLA-B/C mAb. These results demonstrate that all 50 alloreactive CD8 clones were restricted to HLA-A*0201, the only mismatched HLA allele between patient and donor.

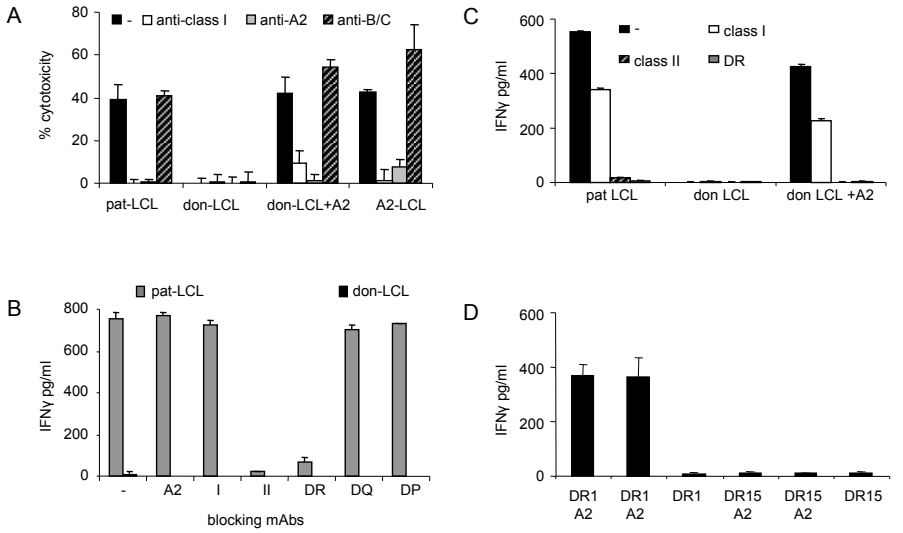


Figure 2. Characterization of the HLA restriction and specificity of donor CD8 and CD4 T-cell clones isolated from the GVHD.

(A) CD8 T-cell clones were tested for cytotoxicity against patient EBV-LCLs (pat-LCL), donor EBV-LCLs (don-LCL), donor EBV-LCLs transduced with HLA-A2 (don-LCL+A2) and a panel of 8 HLA-A2+ EBV-LCLs of which one is shown (A2-LCL). During the 4 hour incubation either no (-), anti-HLA class I (anti-class I), anti-HLA-A2 (anti-A2) or anti-HLA B/C (anti-B/C) mAbs were present. All clones showed HLA-A2 restricted reactivity. Cytotoxicity of one representative clone (8.26) is shown. **(B)** CD4 T-cell clones were stimulated with patient EBV-LCLs (pat-LCL) and donor EBV-LCLs (don-LCL) in the presence of either no (-), anti-HLA- anti-A2 (A2), anti-HLA class I (I), anti-HLA-class II (II), anti-HLA-DR (DR), anti-HLA-DQ (DQ) or anti-HLA-DP (DP) mAbs, and IFNγ production was measured by ELISA. 14 of the 21 CD4 T-cell clones were HLA-DR restricted. IFNγ production of one representative HLA-DR restricted clone (4.12) is shown. **(C)** The HLA-DR restricted alloreactive CD4 T-cell clones were tested against patient EBV-LCLs (pat-LCL), donor EBV-LCLs (don-LCL) or donor EBV-LCLs transduced with HLA-A2 (don-LCL+A2) in the presence of either no (-), anti-HLA class I (class I), anti-HLA-class II (class II) or anti-HLA-DR (DR) mAbs. 13 of the 14 HLA-DR restricted CD4 T-cell clones showed recognition of patient EBV-LCLs as well as of donor EBV-LCLs transduced with HLA-A2 and recognition could be blocked by anti-HLA-class II and anti-HLA-DR mAbs. IFNγ production of one representative clone (4.12) is shown. **(D)** CD4 T-cell clones were stimulated with a panel of HLA-DR1+HLA-A2+ (DR1 A2), HLA-DR1+HLA-A2- (DR1), HLA-DR15+HLA-A2+ (DR15 A2) and HLA-DR15+HLA-A2- (DR15) EBV-LCLs. IFNγ production of one representative clone (4.12) is shown. These results indicate that 13 of the 21 alloreactive CD4 clones recognized an HLA-A2 derived peptide presented in the context of HLA-DR1.

Polyclonal CD4 response directed against HLA-A2 derived peptide presented in HLA class II

To determine the diversity of the CD4 response, the TCR Vβ usage of the alloreactive CD4 T-cell clones was analyzed by flow cytometric analysis. This showed that the 21 alloreactive CD4 T-cell expressed at least 10 different TCR Vβ chains (data not shown), indicating that the CD4 response was also polyclonal. To define the HLA-restriction, the alloreactive CD4 T-cell clones were tested against patient EBV-LCLs blocked with different HLA mAbs. IFNγ production of

14 of the 21 clones could be blocked by HLA class II and HLA-DR mAbs, indicative for HLA-DR restriction. In figure 2B the recognition of patient EBV-LCLs by one representative CD4 T-cell clone is shown.

To analyze whether the CD4 T-cells recognized an HLA-A2 derived peptide presented in HLA class II, the CD4 clones were tested against donor EBV-LCLs transduced with HLA-A*0201. 13 of the 14 HLA-DR restricted alloreactive CD4 clones, of which one is shown in figure 2C, recognized donor EBV-LCLs transduced with HLA-A*0201, and this recognition could be blocked by HLA-class II and HLA-DR mAbs but not by HLA class I mAb. To further analyze the HLA-DR restriction, the CD4 T-cell clones were tested against a panel of HLA-DR*0101+ HLA-A*0201+, HLA-DR*0101+ HLA- A*0201-, HLA-DR*1501+ HLA- A*0201+ and HLA-DR*1501+ HLA- A*0201- EBV-LCLs. All 13 CD4 T-cell clones recognizing donor EBV transduced with HLA-A*0201 recognized the HLA-DR*0101+ HLA-A*0201+ EBV-LCLs. The recognition of one representative clone is shown in figure 2D. These results demonstrate that 13 of the 21 isolated CD4 clones recognized an HLA-A2 derived peptide presented in HLA-DR1.

Eight of the 21 alloreactive CD4 T-cell clones did not recognize donor EBV-LCLs transduced with HLA-A*0201 (data not shown). Since these clones produced only low amounts of IFNγ, further characterization of their specificity was not pursued.

To investigate which epitopes of HLA-A*0201 were recognized in the context of HLA-DR1, the CD4 T-cell clones were tested against donor EBV-LCLs loaded with overlapping 20 mer peptides covering the whole sequence of the HLA-A*0201 molecule. All 13 CD4 T-cell clones recognizing donor EBV transduced with HLA-A*0201, showed recognition of sequence 101-122 derived from a hypervariable region of the HLA-A*0201 molecule. Three representative clones are shown in figure 3A. To further analyze the minimal recognized epitope, the CD4 T-cell clones were tested against truncated peptides of the recognized region. Based on the recognition pattern, the CD4 clones could be subdivided into three groups (figure 3B). The first group represented by clone 4.12, recognized the 15-mer epitope of aa 106-120 and the 17-mer epitope of aa 103-119. This group covered 9 of the 13 HLA-DR1 restricted HLA-A2 specific CD4 clones, which showed usage of at least 5 different TCR Vβ chains. The second group, representing 3 clones with different TCR Vβ chains including clone 4.79, showed recognition of aa 105-117. Clone 4.44, recognized the 14-mer epitope 101-114. These results show that, although the majority of the CD4 T-cell clones all recognize a peptide derived from the same region of the HLA-A2 molecule, there was diversity between the clones in minimal epitope recognition.

The results of the characterization of the allo-immune response demonstrated a coordinated allo-immune response consisting of a polyclonal CD8 response directed against the mismatched HLA-A2, and an also polyclonal CD4 response directed against peptides derived from the mismatched HLA-A2 molecule presented in HLA class II.

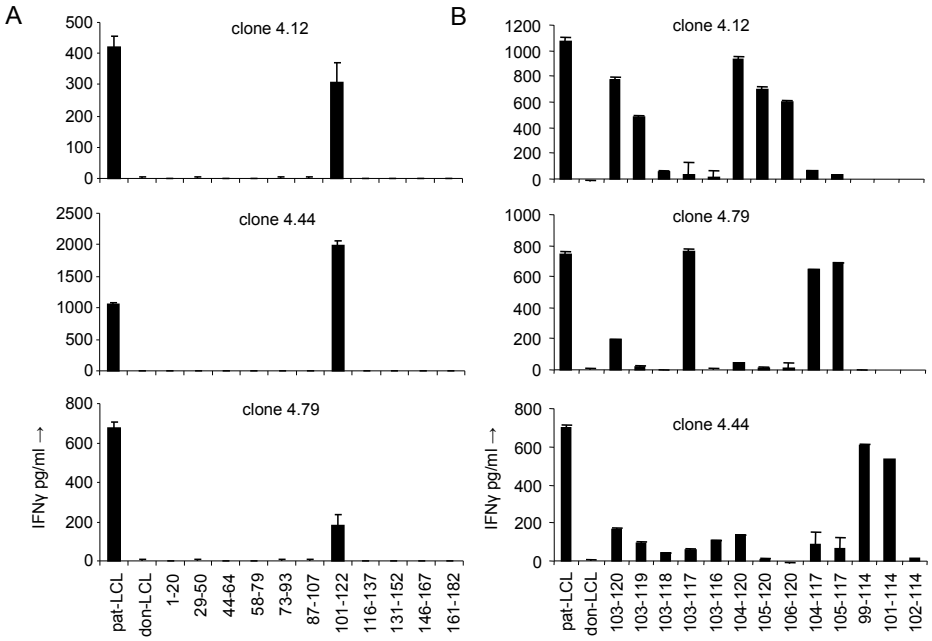


Figure 3. Identification of the HLA-A2 derived peptide and of the minimal epitopes recognized by the CD4 T-cell clones.

(A) The alloreactive CD4 T-cell clones which showed recognition of HLA-A2+ donor EBV-LCLs, were stimulated with donor EBV-LCLs loaded with different peptides covering the whole HLA-A2 sequence. All tested clones, of which one representative clone (4.12) is shown, recognized aa 101-122 of the HLA-A2 molecule. **(B)** To analyze the minimal recognized epitope, the CD4 T-cell clones were stimulated with donor EBV-LCLs loaded with truncated peptides of the recognized region. The clones can be divided into three groups based on their minimal epitope recognition. The first group, represented by clone 4.12, showed recognition of the 15-mer epitope of aa 106-120 and the 17-mer epitope of aa 103-119. The second group, represented by clone 4.79, showed recognition of aa 103-117. Clone 4.44 recognized the 14-mer epitope of aa 101-114.

Patient leukemic blasts possibly mediated the crosstalk between the CD4 and CD8 response

Since severe acute GVHD developed after administration of the second DLI, whereas no clinical signs of GVHD were observed after first DLI, we investigated whether a difference in the composition of the hematopoietic compartment in the patient at the time of the first and second DLI could explain the difference in clinical outcome. Patient and donor chimerism in different cell subsets was measured at the two time points by flow cytometric analysis using lineage specific mAbs in combination with HLA-A2 mAb. The only significant difference in the composition of the hematopoietic compartment in the patient at the time of the DLIs was the absence of leukemic blasts at the time of the first DLI and the presence of leukemic blasts at the time of the second DLI. In the bone marrow high numbers (figure 4A) and in the

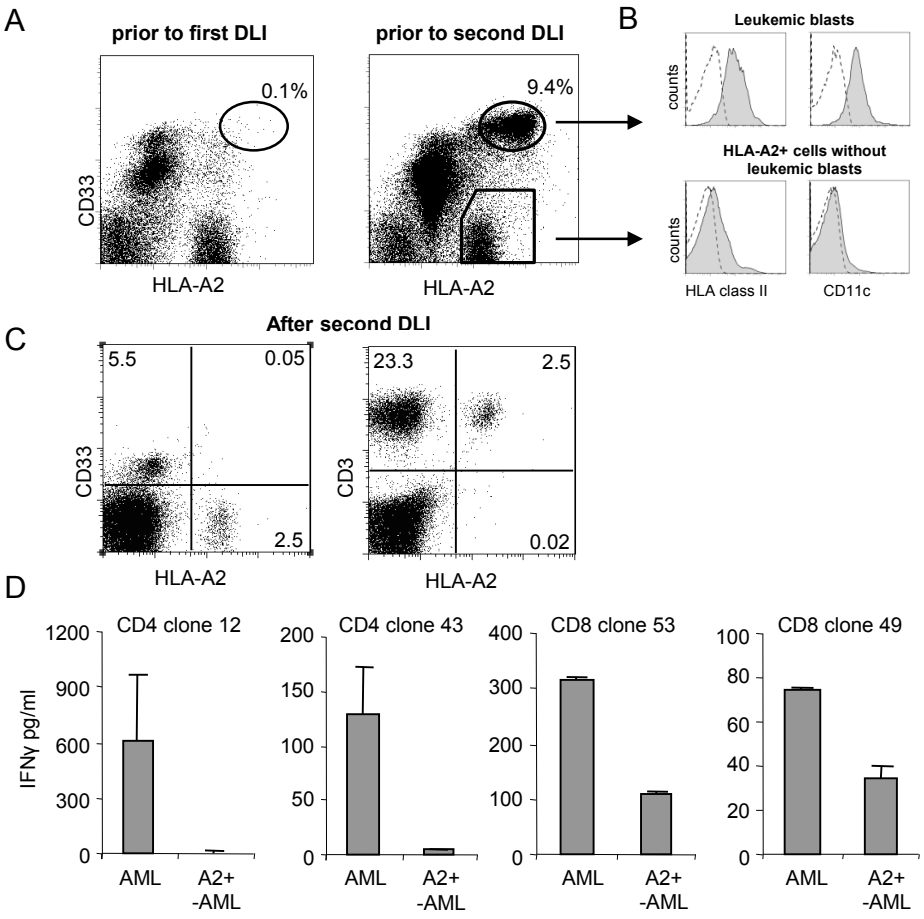


Figure 4. The presence, HLA class II and CD11c expression and stimulatory capacity of patient leukemic blasts.

(A) To visualize leukemic blasts, BMCs were stained with mAbs against HLA-A2 and CD33. At the time of the first DLI no leukemic blasts could be detected, whereas at the time of the second DLI 9% leukemic blasts were present in bone marrow. This percentage of leukemic blasts was confirmed by bone marrow morphology (data not shown). **(B)** Patient BMCs collected prior to the second DLI were stained with anti-HLA-A2, anti-CD33 mAbs, and either anti-HLA class II or anti-CD11c mAbs. Cells gated on HLA-A2 and CD33 positivity represent the leukemic blasts and cells gated on HLA-A2 positivity and absence of CD33 expression represent the HLA-A2+ cells without leukemic blasts. The dashed lines are the isotype controls and the grey lines represent the expression of HLA class II and CD11c on the gated cells. The leukemic blasts showed a high expression of HLA class II and CD11c. The remaining HLA-A2+ cells showed a marginal expression of HLA class II and no expression of CD11c. **(C)** To investigate whether at the time of GVHD also a GVL effect was seen, patient BMCs collected at 4 weeks after second DLI were stained with mAbs against HLA-A2, CD33, and CD3. **(D)** Two CD8 clones (8.53 and 8.49) and two CD4 clones (4.12 and 4.43) were stimulated with AML blasts (AML) or with HLA-A2+ PBMCs deprived of the leukemic blasts (A2+ -AML) from the time of the second DLI. The CD4 T-cell clones only recognized the leukemic blasts and the CD8 T-cell clones recognized all HLA-A2+ cells.

peripheral blood low numbers of leukemic blasts (data not shown) were present at the time of second DLI.

To investigate whether the leukemic blasts could have activated both the CD8 and CD4 alloimmune responses, we determined the expression of HLA class II on the patient leukemic blasts by flow cytometric analysis. In addition, we determined the expression of the costimulatory molecules CD80, CD86 and CD40 and the adhesion molecules CD54 and CD11c, also relevant for immune response initiation. No expression of CD40, CD80, CD86 or CD54 was found on leukemic cells (data not shown). However, a clear expression of HLA class II and CD11c was observed (figure 4B). The remaining HLA-A2+ cells present in patients blood and bone marrow at the time of the second DLI, consisting mostly of patient T-cells (data not shown), showed a marginal expression of HLA class II and no expression of CD11c (figure 4B). Since GVHD and graft versus leukemia (GVL) are often associated, we investigated if a decrease in the numbers of leukemic blasts could be measured at the time of severe GVHD. Results in Figure 4C demonstrate that the leukemic blasts completely disappeared from the bone marrow after the second DLI at the time of GVHD, leaving only some residual patient T-cells present in bone marrow, indicative for a GVL effect.

To investigate which cells were able to activate the donor T-cells, different CD8 and CD4 T-cell clones were tested against the leukemic blasts and against the HLA-A2+ PBMCs deprived of the leukemic blasts. The results show that the leukemic blasts were the only cells in the patient hematopoietic compartment able to activate both the CD8 and CD4 T-cell clones (figure 4D).

CD4 T-cells can recognize non-hematopoietic cells during inflammatory conditions, possibly enhancing the GVHD

Since at the time of administration of the second DLI the patient did not suffer from infections or GVHD, HLA class II expression of non-hematopoietic cells was unlikely. However, to investigate whether during the cytokine storm after development of GVHD, tissues could be targets for both alloreactive CD8 and CD4 T-cells, these T-cell clones were tested for recognition of fibroblasts and keratinocytes derived from HLA-A*0201+ HLA-DR*0101+ individuals. A three days pre-incubation with IFNγ of the fibroblasts and keratinocytes was used to mimic inflammatory conditions. As shown in figure 5A, after pre-treatment with IFNγ the fibroblasts and keratinocytes expressed HLA class II. In agreement with this, the CD4 clones recognized the fibroblasts and keratinocytes only after upregulation of HLA class II with IFNγ. Some of these CD4 clones clearly recognized the fibroblasts and keratinocytes after treatment with IFNγ, whereas other CD4 clones only showed minimal recognition of HLA class II expressing fibroblasts and keratinocytes (figure 5B). The CD8 clones, of which one representative clone is shown in figure 5B, recognized all HLA-A2+ target cells. These results indicate that once the GVHD was initiated, the non-hematopoietic cells were able to serve as target cells for the CD4 alloreactive T-cells, thereby possibly amplifying the GVH response.

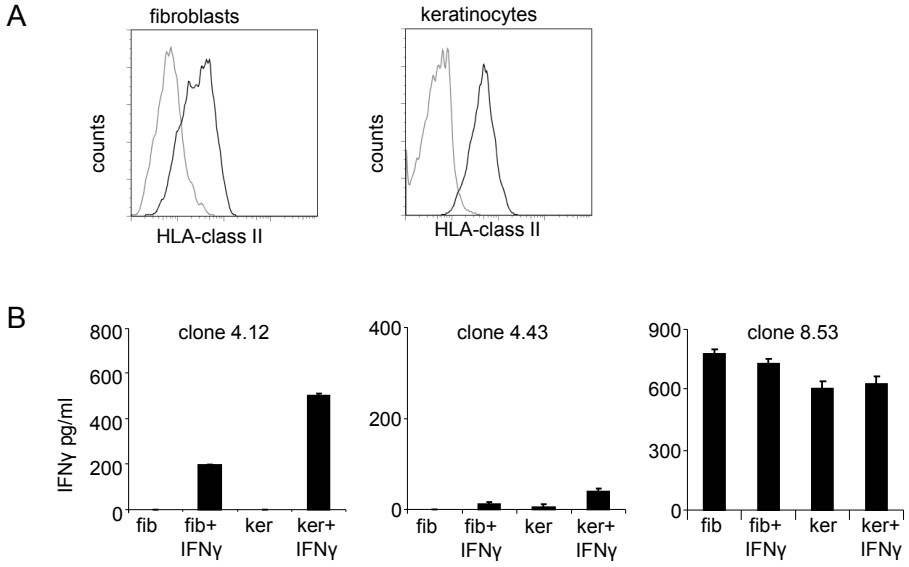


Figure 5. The stimulatory capacity of cells derived from the non-hematopoietic compartment on donor CD4 and CD8 T-cell clones.

(A) Fibroblasts and keratinocytes incubated for three days with or without IFNγ were stained with HLA-class II mAb. The black and grey lines represent the keratinocytes and fibroblasts with or without IFNγ pre-treatment, respectively. Only after the pre-treatment with IFNγ the fibroblast and keratinocytes expressed HLA class II. **(B)** CD4 and CD8 clones were stimulated with fibroblasts and keratinocytes derived from HLA-A2+ HLA-DR1+ individuals, which were pre-incubated for three days with or without IFNγ. The CD8 clones, as represented by clone 8.53, recognized all HLA-A2+ target cells. The CD4 clones only recognized fibroblasts and keratinocytes after upregulation of HLA class II with IFNγ. Some CD4 clones, as represented by clone 4.12, showed a strong recognition, whereas other CD4 clones, as represented by clone 4.43, showed a low recognition of the fibroblasts and keratinocytes which were pre-incubated with IFNγ.

DISCUSSION

In this study the allo-immune response in a patient suffering from GVHD after an HLA-A2 mismatched DLI was characterized. A polyclonal CD8 response, directed against the allo-HLA-A2 molecule was found in conjunction with a polyclonal CD4 response, of which a substantial part was directed against HLA-A2 derived peptides presented in HLA-DR1.

The first DLI that the patient received did not lead to an immune response, whereas the second DLI with a 3-fold higher dose led to severe GVHD. A higher dose of administered lymphocytes could explain the difference in emergence of GVHD, since the occurrence of GVHD has been previously correlated with the dosage of lymphocytes in DLI. Testing donor PBMCs against patient PBMCs in mixed lymphocyte reaction (MLR) demonstrated a very low frequency of alloreactivity (<1:100.000). We therefore assume that the difference in DLI dose was too small to explain the difference between complete absence of GVHD with the first DLI

dose and development of lethal grade 4 GVHD with the second DLI dose. We can, however, not completely rule out that higher T-cell dose contributed to the emergence of GVHD after the second DLI.

Based on the absence of an immune response after the first DLI and the low frequency of alloreactive donor cells measured by MLR, we additionally presume that there was not a substantial pool of CD8 T-cells crossreactive against HLA-A2 present within the memory repertoire of the donor. The DLIs were administered a long time after the conditioning regimen and in the absence of active infections, indicating that no significant inflammatory conditions were apparent around the time of initiation of the GVHD. We hypothesize that due to the absence of allo-HLA-A2 reactive memory CD8 T-cells and inflammatory conditions, a coordinated response of CD8 and CD4 T-cells directed against the allogeneic HLA class I was required for the development of GVHD.

In the immune response analyzed in this study, all alloreactive CD8 T-cells were directed against the mismatched HLA-A2, and the majority of the CD4 T-cells recognized HLA-A2 derived peptides presented in HLA class II, indicating that these HLA-A2 derived peptides were abundantly expressed by the antigen presenting cells responsible for the initiation of the response. The other CD4 T-cell clones most likely recognized peptides polymorphic between the patient and the donor based on single nucleotide polymorphisms, termed minor histocompatibility antigens (MiHAs), presented in HLA class II. HLA derived peptides are frequently presented in both HLA class I and HLA class II[18;19], indicating that after HLA mismatched transplantation, the mismatched HLA molecules will also be presented as peptides in the context of self-HLA. Therefore, the common direction of the CD8 and CD4 T-cell responses against allo-HLA-A2 suggests initiation of the two responses by the same HLA-A2 and HLA class II expressing cells.

The leukemic blasts were the only cells in patient blood and bone marrow which highly expressed HLA class II and HLA-A2 and were able to activate the alloreactive CD8 as well as the CD4 T-cells, suggesting a role for the leukemic blasts in the initiation of the allo-immune response. Conversely, no expression of costimulatory molecules, previously shown to be relevant for initiation of a primary immune response, could be detected on the AML blasts. However, upon activation AML cells express costimulatory molecules[20;21], and these activated AML-DCs can induce autologous anti-leukemic reactive T-cells. In addition, it was recently shown that cross-talk between CD4+ T-cells and leukemic cells in vivo can change leukemic cells into an APC phenotype[22]. We therefore postulate that a small part of the allo-HLA reactive T-cells exhibiting high avidity allo-HLA reactivity can become activated in the absence of costimulatory molecules, and subsequently induce expression of costimulatory molecule on the AML cells, which on their turn more broadly activate the allo-HLA reactive T-cells.

It cannot be excluded that patient dendritic cells (DCs) were still present in other tissues during the second DLI. Merad et al.[23] demonstrated that host Langerhans cells can self renew and thereby remain present in the skin for long periods of time after allogeneic SCT. It is possible

that Langerhans cells and other tissue-resident DCs of the patient remained in the patient post-transplant and thus represented a major priming population for the immune response leading to the GVHD. However, this would indicate that patient DCs were also present at the time of the first DLI, which did not lead to GVHD. Alternatively, donor DCs may have cross presented HLA-A2 peptides. Lechler and colleagues described cross presentation of HLA class I derived peptide in HLA class II by host APCs which migrated to the donor kidney, thereby causing chronic graft rejection[24;25]. However, since donor DCs were HLA-A2 negative, the CD8 alloresponse could not have been initiated by these cells. Although it has been demonstrated in vitro that DCs are able to cross present intact HLA molecules, a phenomenon termed semi-direct presentation[26], this phenomenon has not been demonstrated in vivo and is far less likely to occur than direct presentation by patient APCs.

GVHD and GVL effects are often closely associated. In this study we demonstrate a complete elimination of the AML blasts, indicative for a GVL effect at the time of GVHD. The elimination of the leukemia can be mediated by allo-HLA reactive T-cells that recognize both the malignant as well as healthy tissue of the patient and thereby mediate both GVL and GVHD effects. T-cells selectively directed against the malignancy may also have contributed to the GVL effect. Separation of the GVHD/GVL and solely GVL inducing T-cells may lead to the identification of T-cells directed against tumor specific antigens, useful for adoptive T-cell therapy. Although non-hematopoietic cells, which under normal conditions do not express HLA class II, were unlikely to have initiated the coordinated CD4 and CD8 T-cell response, upregulation of HLA class II molecules under inflammatory conditions during GVHD may have amplified the effector phase of the immune response. As shown in figure 5, some of the alloreactive CD4 T-cells could be activated by non-hematopoietic cells forced to express HLA class II by culturing under conditions mimicking inflammation. We hypothesize that, once GVHD was initiated, activated T-cells may have generated a cytokine storm leading to upregulation of HLA class II on non-hematopoietic cells, and thereby increasing the destruction of these cells. Based on the results in this study we conclude that the GVHD following a delayed HLA-A2 mismatched DLI was mediated by a cooperative CD4 and CD8 response directed against the mismatched HLA-A2. We postulate that the CD8 T-cells directed against the allo-HLA-A2 molecule acted as the primary effector cells and thereby caused the majority of tissue damage. We hypothesize that the CD4 T-cell response, mostly directed against HLA-A2 derived peptide presented in HLA class II, was essential for the initiation and possibly also the amplification of the response, and we speculate that the leukemic blasts expressing HLA-A2 and HLA class II may have activated both the CD8 and CD4 responses.

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3

ALLO-HLA REACTIVITY OF VIRUS-SPECIFIC MEMORY T-CELLS IS COMMON

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Blood. 2010 Dec 2;116(23):5079



ABSTRACT

Graft versus host disease and graft rejection are major complications of allogeneic HLA mismatched stem cell transplantations or organ transplantation that are caused by alloreactive T-cells. Since a range of acute viral infections have been linked to initiating these complications, we hypothesized that the cross-reactive potential of virus specific memory T-cells to allogeneic (allo) HLA molecules may be able to mediate these complications. To analyze the allo-HLA reactivity, T-cells specific for Epstein Barr virus, cytomegalovirus, varicella zoster virus and influenza virus were tested against a panel of HLA typed target cells, and target cells transduced with single HLA molecules. 80% of T-cell lines and 45% of virus specific T-cell clones were shown to cross-react against allo-HLA molecules. The cross-reactivity of the CD8 and CD4 T-cell clones was primarily directed against HLA class I and II, respectively. However, a restricted number of CD8 T-cells exhibited cross-reactivity to HLA class II. T-cell receptor (TCR) gene transfer confirmed that allo-HLA reactivity and virus specificity were mediated via the same TCR. These results demonstrate that a substantial proportion of virus specific T-cells exert allo-HLA reactivity, which may have important clinical implications in transplantation settings as well as adoptive transfer of third party virus specific T-cells.

INTRODUCTION

HLA disparity between donor and recipient increases the risk and the severity of graft versus host disease (GVHD) after stem cell transplantation (SCT). The risk of graft rejection is also significantly increased in HLA mismatched as compared to HLA matched SCTs, and solid organ transplantations. The negative effect of HLA disparity on the clinical outcome of transplantations is the result of high frequencies of alloreactive T-cells. In HLA mismatched mixed lymphocyte reactions (MLR) the frequency of reactive T-cells was demonstrated to be a 1000 fold higher than the frequency of T-cells reactive in HLA identical MLRs.[1;2] By testing alloreactive T-cells against panels of third party target cells expressing different HLA molecules[1;3-5] and against target cells blocked with different HLA antibodies,[6-8] 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%.

During thymic development T-cells undergo an instruction process of positive and negative selection which results in the composition of a mature T-cell repertoire that is selected on the basis of tolerance for self-HLA molecules presenting self peptides.[9;10] However, during thymic development T-cells never encounter allo-HLA molecules, and therefore no selection based on tolerance for allo-HLA molecules occurs. We therefore hypothesize that every antigen specific self-HLA restricted T-cell could potentially cross-react with non-self HLA molecules and exert allo-HLA reactivity.

Although it was shown that alloreactivity is equally presented in the naïve and memory T-cell populations,[11] the ability of T-cells to exhibit allo-HLA reactivity could especially have serious consequences when exerted by memory T-cells. Memory T-cells lack the requirement for co-stimulation,[12;13] and therefore 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[14-17] 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.[18-21] Therefore, if certain virus specific T-cells with cross-reactive potential against the mismatched allo-HLA molecule are triggered by viral activation and expanded in the memory pool, these T-cells will react against the mismatched HLA molecule, and may induce severe GVHD or graft rejection.

Studies of Burrows and colleagues have first 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.[22;23] T-cell specific for HSV-VP13/14 presented in HLA-A2 were also found to cross-react with HLA-B44,[24] and CD4 T-cells specific for tetanus toxoid presented in HLA-DR3 were found to be cross-reactive against HLA-DR4.[25] In addition, the association between

reactivation of viral infections during organ transplantation and increased graft rejection[26] supports the hypothesis that virus specific T-cells exhibit allo-HLA reactive potential.
In this study we investigate the ability of a large panel of virus specific T-cells to exert allo-HLA reactivity. We determined the cross-reactive potential of virus specific T-cells to allo-HLA molecules by screening single viral antigen specific T-cell lines and clones against a panel of EBV transformed B-cells (EBV-LCLs), together covering almost all prevalent HLA class I and II molecules, as well as single HLA transduced target cells. The tested CD8 and CD4 virus specific memory T-cells were specific for Epstein Barr virus (EBV), cytomegalovirus (CMV), varicella zoster virus (VZV) and influenza virus (Flu). Most virus specific T-cell lines and 45% of the virus specific T-cell clones were demonstrated to be cross-reactive against allo-HLA molecules. TCR gene transfer demonstrated that the virus specificity as well as the cross-reactivity to allo-HLA molecules was mediated by the same TCR. These results demonstrate that T-cells specific for different viruses exert cross-reactivity to allo-HLA molecules, and illustrate the high frequency of T-cells able to exert allo-HLA reactivity.

MATERIAL AND METHODS

Cell collection and preparation

After informed consent, peripheral blood (PB) was obtained from different individuals. Mononuclear cells (MNC) were isolated by Ficoll-Isopaque separation and cryopreserved. Stable Epstein–Barr virus (EBV)-transformed B-cell lines (EBV-LCLs) were generated using standard procedures. EBV-LCLs and K562 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Lonza, Basel, Switzerland) and 10% fetal bovine serum (FBS, Lonza). PHA blasts were generated by stimulation of PB-MNCs with phytohemagglutinin (PHA, 0.8 ug/ml, Murex Biotec Limited, Dartford, UK) in IMDM, 5% FBS, 5% human serum (HS) and IL-2 (120 IU/ml). K562 expressing single allo-HLA molecules and HLA transduced EBV-LCLs were generated by transduction with retroviral vectors encoding for the allo-HLA molecules or by transfection of allo-HLA molecules.[27;28] For the isolation of T-cells, B-cells and monocytes PBMC of healthy donors were stained with either anti-CD3, anti-CD19 or anti-CD14 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) respectively and isolated according to manufacturer's instructions. CD40L activated B-cells were generated by culturing the CD19+ fraction for 3 days on CD40L transduced murine fibroblasts[29] in medium containing CpG (10 µg/ml) and IL-4 (500 IU/ml) (Schering-Plough, Innishammon, Cork, Ireland). Monocyte derived DCs were generated by culturing the CD14+ fraction in medium containing activating cytokines as described previously.[30] Fibroblasts were cultured from skin biopsies in Dulbecco's modified Eagle medium (DMEM, Lonza) with 1g/l glucose (BioWhittaker, Verviers, Belgium) and 10% FBS.

Generation of virus specific T-cell lines and clones

PB-MNCs from healthy individuals were stained with tetramer and anti-CD8 mAb for 1 h at 4°C and washed once. The tetramers used were constructed as described previously[31] and are shown in table 1. Tetramer positive, CD8 positive T-cells were sorted 50 cells per well for the generation of lines or single cell per well for the generation of clones into U-bottom microtiter plates containing 100 µl of feeder mixture. Sorting was performed at 4°C using the FACS Vantage (BD). The feeder mixture consisted of IMDM, 5% FBS, 5% HS, IL-2 (120 IU/ml), PHA and 30 Gy irradiated allogeneic third-party PB-MNCs ($0.5x10^6$/ml). Proliferating T-cell clones were selected and further expanded by nonspecific stimulation every 14 days using the previously mentioned feeder mixture. The viral specificity of the expanded lines and clones was confirmed by tetramer staining, cytotoxicity and cytokine production assays. Polyclonality or monoclonality of the T-cell lines and clones was analyzed by TCR Vβ analysis using the TCR Vβ kit (Beckman Coulter, Fullerton, CA, USA).

Table 1. Tetramers used for the generation of virus specific T-cell lines and clones

virus	viral antigen	HLA	epitope
CMV	pp50	HLA-A*0101	VTEHDTLLY
CMV	pp65	HLA-A*0101	YSEHPTFTSQY
CMV	pp65	HLA-A*0201	NLVPMVATV
CMV	pp65	HLA-B*0702	RPHERNGFTVL
CMV	pp65	HLA-B*03501	IPSINVHHY
CMV	pp65	HLA-DRB1*0101	KYQEFFWDANDIYRI
CMV	IE1	HLA-B*0801	ELRRKMMYM
EBV	EBNA3A	HLA-A*0301	RLRAEAQVK
EBV	EBNA3A	HLA-B*0702	RPPIFIRRL
EBV	EBNA3A	HLA-B*0801	FLRGRAYGL
EBV	BMLF1	HLA-A*0201	GLCTLVAML
EBV	BRLF1	HLA-A*0301	RVRAYTYSK
EBV	BZLF1	HLA-B*0801	RAKFKQLL
EBV	LMP2	HLA-A*0201	CLGGLLTMV
FLU	IMP	HLA-A*0201	GILGFVFTL
FLU	HA	HLA-DRB1*0401	PKYVKQNTLKLAT
VZV	IE62	HLA-A*0201	ALWALPHAA

Allo-HLA reactivity of the virus specific T-cell lines and clones

In the IFNγ production assays 5000 T-cells were co-cultured with 20.000 stimulator cells in a final volume of 150µl IMDM culture medium supplemented with 100 IU/ml IL-2. After 18 h of incubation, supernatants were harvested and IFNγ production was measured by standard ELISA (ELISA; CLB, Amsterdam, the Netherlands). In the cytotoxicity assays the virus specific T-cells clones were tested in a standard 6 hour ^{51}Cr-release assay[32] against EBV-LCLs in an effector to target ratio of 10:1.

TCR gene transfer

The TCRAV and TCRBV gene usage of the BRLF1/HLA-A3 clone 19 the and the VZV-IE62/HLA-A2 specific T-cell clone 7 was determined using reverse transcriptase (RT)–PCR and sequencing.[27] Retroviral vectors were constructed that encoded the TCRα chain in combination with GFP and the TCRβ chain in combination with the marker gene ΔNGF-R.[27] CMV-IE1/HLA-A1 specific T-cells derived from an HLA-A*0301 negative healthy individual were transduced with the TCR α and β of the BRLF1/HLA-A3 clone 19. CMV-pp50/HLA-A1 specific T-cells derived from peripheral blood of a healthy individual negative for HLA-A*0201 and HLA-A*0205 were transduced with the retroviral vectors encoding for the TCR α and β chain of the VZV specific T-cell clone.[27] The TCR transduced T-cells were sorted on basis of double positivity for GFP and ΔNGF-R, and after expansion the T-cells were tested for viral specificity and allo-HLA reactivity in stimulation assays.

RESULTS

Alloreactivity of virus specific T-cell lines

To investigate the ability of virus specific T-cells to exert alloreactivity, virus specific T-cell lines were tested against a panel of EBV-LCLs, together covering almost all frequently occurring HLA class I and II molecules. The HLA typing of the EBV-LCLs used in the panel is listed in table 2. The virus specific T-cell lines were generated by isolation of tetramer positive CD8 positive T-cells by FACS sort and subsequent expansion. Tetramer and CD8 staining confirmed the purity of the virus specific lines and showed that all T-cell lines were more than 98% tetramer positive (figure 1A-G). In total eleven virus specific lines were tested, derived from 9 different donors, specific for 7 different antigens of CMV, EBV and VZV and restricted to 3 different HLA molecules. In the figures in which EBV specific T-cell lines were tested, the EBV-LCLs expressing the HLA molecules to which the T-cell lines were restricted are not shown in the figures, to present only the alloreactivity of the T-cells and not the virus specificity. All LCLs were tested for IFNγ production in the absence of T-cells and did not show production of IFNγ (data not shown). In figure 1(A-G) seven representative lines are shown. Nine of the eleven tested virus specific T-cell lines were shown to be alloreactive, since these lines produced IFNγ upon stimulation with at least one of the EBV-LCLs of the panel. Two of the eleven tested lines exerted no alloreactivity against the EBV-LCLs tested in our panel (including figure 1C). The recognition pattern of the virus specific lines that demonstrated alloreactivity ranged from recognition of almost all EBV-LCLs, as shown in figure 1A and 1B by the CMV-pp50/HLA-A1 specific lines of individuals MBX and UKL, to recognition of a limited number of EBV-LCLs, as shown by the IE1/HLA-B8, the BMLF1/HLA-A2, the VZV-IE62/HLA-A2 and the EBNA3A/HLA-B8 specific lines (figure 1D, 1E, 1F and 1G respectively). The pattern of allo-recognition of some of the T-cell lines suggested that the alloreactivity was directed against one or several

Table 2. HLA expression of the EBV-LCL panel

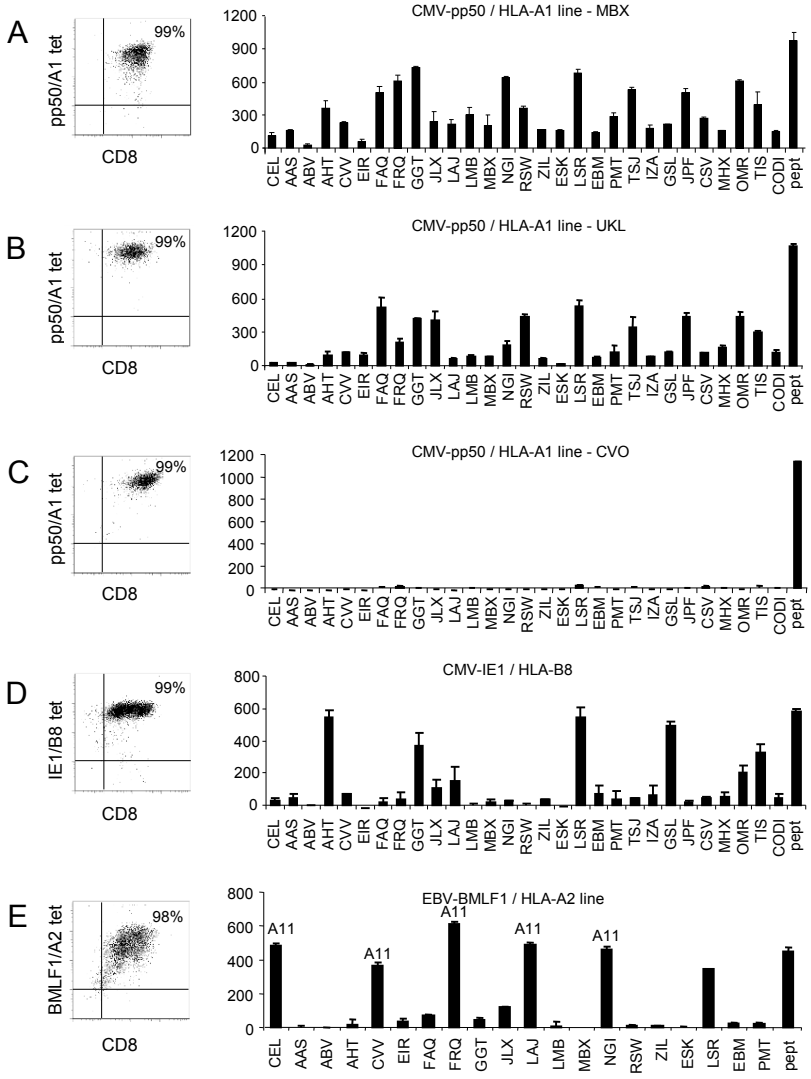
ID	HLA class I			HLA class II		
	A	B	C	DR	DQ	DP
CEL	1101	1502, 4001	0401, 0801	0901	0202, 0303	0501
AAS	3101, 6601	2705, 4102	0202, 1703	1101, 1303	0301	0401, 0402
ABV	0301, 2902	0702, 4403	0702, 1601	0701, 1454	0502, 0202	0401, 1101
AHT	2402, 2501	5501, 1501	0303	0806, 1501	0501, 0602	0401
CVV	11, 31*	57*, 1501	3*, 0602	0401, 7*	0301, 0303	0201, 0401
EIR	0101, 2402	3502, 3701	0401, 0602	0404, 1104	0301, 0402	0301, 0401
FAQ	2301, 6802	1402, 3801	0802, 1203	1301, 1303	0301, 0603	0201
FRQ	0101, 1101	0801, 4402	0501, 0701	0301, 0401	0201, 0301	0101, 0401
GGT	2601, 3101	1401, 4901	0701, 0802	0101, 0701	0202, 0504	0402, 1101
JLX	0101, 6802	0801, 5301	0401, 0701	0301, 1302	0201, 0604	0101, 0401
LAJ	1101, 2402	1302, 5501	0303, 0602	0701, 1601	0202, 0502	0401, 1701
LMB	2902	4402 , 5101	1402, 1601	0701, 0801	0202, 0402	0401, 1101
MBX	0101	0801, 1517	0701	1202, 0301	201,0301	0101, 0401
NGI	1101, 2402	0801, 3906	0701	0301, 0801	0201, 0402	0101, 1401
RSW	3001, 6802	4201	1701	0302	0402	0101, 0402
ZIL	2402, 2601	5601, 5801	0101, 0701	0101, 0804	0402, 0501	0201, 0301
ESK	23, 66*	51, 72*	2, 18*	12, 13*	1, 3*	ND
LSR	3201, 6801	3503, 5201	1202, 1203	1502, 1602	0502, 0601	0401, 1401
EBM	2301	1401	0802	0401	0302	0201
PMT	0301, 3301	1402	0802	0102	0501	0301, '0401
TSJ	2, 24	1501, 75	4	12, 15	6, 0301	ND
IZA	0201, 2402	0801, 4001	0304, 0701	0301, 1301	0603	0401, 1401
GSL	2,3*	47, 1501*	3, 6*	11, 13*	1*, 0301	ND
JPF	0201, 0205	4002, 1501	0202, 0304	0701, 1104	0301, 0303	0402, 1401
CSV	0201, 3101	5101, 5501	0303, 1402	0404, 1001	0501, 0301	0401, 0402
MHX	0101, 0205	1801, 5001	0602, 0701	0701, 0901	0201, 0303	0201, 0301
OMR	201	4501	1601	1301	0603	0101
TIS	0206, 0207	4601	0102, 0801	0901	0303	1301
CODI	2*, 8001	58, 70*	2, 6*	17, 11*	2, 7*	1, 4*
AKB	0101, 0201	3701, 3901	0602, 0702	0101, 1001	501	0201, 0401
PSJ	0201, 3001	1302, 4402	0501, 0602	0401, 0701	0202, 0301	0402, 1401
MWX	0101, 3401	1521, 3503	0403, 1203	0101, 1502	0501, 0601	0601, 1301
NGZ	1101, 2401	5201, 4002	0202, 1202	0101, 1101	0501, 0301	0201, 0301
RSB	0201, 0301	5701, 4402	0602, 0704	0701, 1101	0301, 0303	0201, 0401
WOH	1, 28	8, 27	2, 7	ND	ND	ND
FBV	0201, 1101	0702, 5501	0303, 0702	1454, 1501	0503, 0602	0401
RTN	0101, 1101	0801, 5701	0602, 0701	0301, 0701	0201, 0301	0401, 1401

The panel of EBV-LCLs was composed of HLA-typed EBV-LCLs which together covered almost all frequently occurring HLA molecules. The HLA typing was mainly determined molecularly; however some EBV-LCLs were only serologically typed.

*) indicates that the HLA expression was determined by serological typing

ND) indicates that the HLA expression was *not determined*

of the allo-HLA molecules presented by the EBV-LCL panel. The BMLF1/HLA-A2 specific line showed high reactivity against 6 EBV-LCLs, of which 5 EBV-LCLs expressed HLA-A*1101. All HLA-A*1101 expressing EBV-LCLs within this panel were highly recognized by this T-cell line. The EBV-LCLs recognized by the EBNA3A/HLA-B8 specific line expressed either HLA-B*4402 or HLA-B*5501. TCR Vβ analysis of the lines demonstrated that the complexity of the TCR composition was correlated with the broadness of the alloreactivity. T-cell lines that did not show alloreactivity expressed maximally two different TCR Vβ chains. T-cell lines with limited alloreactivity expressed one to four different TCR Vβs and lines that recognized almost all



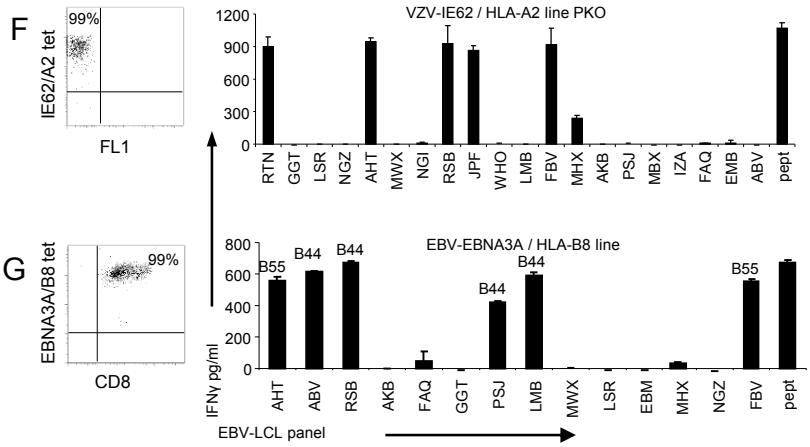


Figure 1. Alloreactivity of virus specific T-cell lines.
Eleven virus specific T-cell lines, of which seven are shown in this figure, were stimulated with a panel of EBV-LCLs for 18h and IFNγ production was measured by ELISA. In experiments in which EBV specific T-cell lines were tested, we excluded the EBV-LCLs expressing the HLA molecules to which the T-cell lines were restricted. The purity of the virus specific lines was analyzed by tetramers and CD8 staining, and all T-cell lines proved to be more than 98% pure. As a positive control, the lines were tested against EBV-LCLs expressing the HLA restricting molecule of the viral epitope, loaded with the viral peptide recognized by the T-cell lines (pept). **(A)** The CMV-pp50/HLA-A1 specific lines of individual MBX recognized almost all EBV-LCLs. **(B)** The CMV-pp50/HLA-A1 specific line of individual UKL showed broad alloreactivity. **(C)** Two of the ten tested T-cell lines exerted no alloreactivity against the tested EBV-LCLs of which one, the pp50/HLA-A1 specific line, is shown. **(D)** The CMV-IE1/HLA-B8 recognized a limited number of EBV-LCLs. **(E)** The BMLF1/HLA-A2 specific line showed high reactivity against all HLA-A11 positive EBV-LCLs and one HLA-A11 negative EBV-LCL. **(F)** The VZV-IE62/HLA-A2 specific line of individual PKO recognized a limited number of EBV-LCLs. **(G)** EBNA3A/HLA-B8 specific line recognized EBV-LCLs expressing either HLA-B44 or HLA-B55.

EBV-LCLs expressed at least 8 different TCR Vβs. This relation between the clonal composition and the recognition of the lines suggests that the alloreactivity of the lines is the sum of the alloreactivity of the various clonal populations present within the lines.
The results demonstrate that 80% of the tested virus specific T-cell lines were able to exert alloreactivity. Some virus specific lines showed a pattern of alloreactivity suggestive for allo-HLA reactivity. However, for most of the virus specific cell lines allo-HLA reactivity could not be determined since the exerted alloreactivity was very broad.

Allo-HLA reactivity of virus specific T-cell clones

Since we were unable to determine allo-HLA reactivity with the oligoclonal virus specific T-cell lines, further characterization of the allo-HLA reactivity of virus specific T-cells was performed with T-cell clones. For this purpose tetramer positive T-cells were sorted single cell per well and expanded. The specificity of the T-cell clones was confirmed by tetramer staining and the

TCR Vβ usage of the clones was analyzed using the TCR Vβ kit. The T-cell clones as shown in table 3 were different based either on their different origin, TCR Vβ usage or their recognition pattern. In total, 41 virus specific CD8 and CD4 T-cell clones were tested against the EBV-LCL panel. These virus specific T-cell clones were derived from 16 individuals, specific for 13 different CMV, EBV, VZV and Flu antigens and restricted to 8 different HLA molecules. The results demonstrated that 18 of the 41 virus specific CD8 and CD4 T-cell clones were alloreactive, as shown by recognition of at least one of the EBV-LCLs from the panel. Most alloreactive T-cell clones exhibited cross-reactivity against EBV-LCLs that shared an HLA molecule, suggesting allo-HLA reactivity. To confirm that the reactivity of a T-cell clone was directed against a specific allo-HLA molecule, the virus specific T-cell clones were tested against HLA negative K562 cell line or EBV-LCLs negative for the recognized allo-HLA molecules, which were transduced with the particular allo-HLA molecule. The allo-HLA reactivity of seven representative T-cell clones is shown in figure 2(A-G). The EBV-EBNA3A/HLA-A3 specific CD8 T-cell clone exhibited alloreactivity against all HLA-A*3101 expressing EBV-LCLs within the panel. The reactivity directed against allo-HLA-A*3101 was confirmed by transfection of K562 with HLA-A*3101 and subsequent recognition by this T-cell clone (figure 2A). The EBV-EBNA3A/HLA-A3 clone also recognized the HLA-A*3101 negative EBV-LCL RSW, which however expressed HLA-A*3001. HLA-A*3101 and HLA-A*3001 are very similar in sequence and therefore we hypothesize that the molecules exhibit strong similarity in structure and peptide presentation, explaining recognition by this T-cell clone. To analyze whether the EBV-EBNA3A/HLA-A3 clone recognized HLA-A*3001, the clone was tested against three HLA-A*3001+ EBV-LCLs, of which one is shown in figure 2A, and one HLA-A*3101+ EBV-LCL with or without blocking mAbs directed against HLA class I, HLA-A30/A31 and HLA-A2. All HLA-A*3001+ EBV-LCLs and the HLA-A*3101+ EBV-LCL were recognized and this recognition was blocked by anti-HLA class I and anti-HLA-A30/A31 mAbs and not by anti-HLA-A2 mAb, indicating that the clone indeed also recognized HLA-A*3001. The CMV-pp50/HLA-A1 specific T-cell clone exhibited alloreactivity against all HLA-A*1101 expressing EBV-LCLs (figure 2B). The allo-HLA-A*1101 reactivity could be confirmed by specific recognition of K562 transduced with HLA-A*1101 by this T-cell clone. In addition to reactivity against HLA-A*1101, the T-cell clone also exhibited low IFNγ production upon stimulation with a few EBV-LCLs negative for HLA-A*1101. Since these lower recognized EBV-LCLs did not share one HLA molecule, we did not determine whether this alloreactivity was also based on allo-HLA cross-reactivity. The EBV-BRLF1 specific HLA-A3 restricted clone, shown in figure 2C, exerted alloreactivity against all HLA-A*0201+ EBV-LCLs. The CD8 T-cell clone did not recognize K562 transduced with HLA-A*0201, however showed recognition of EBV-LCLs transduced with HLA-A*0201, suggesting that the peptide recognized by the clone in the context of HLA-A*0201 is not presented by K562 cells. The clone also showed recognition of HLA-A*0201+ PHA stimulated T-cell blasts (figure 2C, right figure), excluding the possibility that the clone recognized an EBV derived peptide presented in HLA-A*0201. Next to allo-HLA-A*0201 reactivity, this T-cell clone also

Table 3. Allo-HLA reactivity of virus specific T-cell clones

specificity	donor	TCR Vβ	EBV panel	HLA trans/block	figure
pp50/A1	MBX	#	UD		
pp50/A1	MBX	1			
pp50/A1	MBX	5.1			
pp50/A1	MBX	3	A*1101	A*1101	2B
pp65/A2	MRJ	2			
pp65/A2	MRJ	13			
pp65/A2	HRN	8	UD		2G
pp65/A2	HRN	2			
pp65/A2	AMJ	3			
BMLF1/A2	GFS	#			
LMP2/A2	JVW	#			
FLU/A2	FKR	17	B*6401		
FLU/A2	FKR	17			
VZV/A2	PKN	14	B*5501	B*5501	3A
VZV/A2	PKN	#	B*5701	B*5701	3B
VZV/A2	PKN	21,3	A*0205	A*0205	3C
EBNA3A/A3	HRN	#	A*3101	A*3101	2A
BRLF1/A3	AKO	7.1			
BRLF1/A3	AKO	14			
BRLF1/A3	AKO	17			
BRLF1/A3	AKO	1			
BRLF1/A3	DVO	7.1	UD		
BRLF1/A3	DVO	8			
BRLF1/A3	DVO	14	UD		
BRLF1/A3	DVO	17	UD		
BRLF1/A3	DVO	#			
BRLF1/A3	DVO	7.2	A*0201	A*0201	2C
pp65/B7	BDV	7.2			
pp65/B7	BDV	7.2	DRB1*0801	DRB1*0801	2D
EBNA3A/B8	LDO	#	B*4402, B*5501	B*4402, B*5501	2E
BZLF/B8	AVK	7.1			
BZLF/B8	AVK	#			
BZLF/B8	AVK	5.1			
pp65/B35	MED	3			
pp65/B35	MED	5.1	DRB1*0401	DRB1*0401	
pp65/B35	MED	#			
pp65/DR1	CBH	2			
pp65/DR1	CBH	#			
pp65/DR1	MSV	8	DRB1*0901		
pp65/DR1	MSV	#	DRB3*0101	DRB3*0101	
FLU/DR4	VKY	3	DRB1*1301	DRB1*1301	2F

#) indicates that the TCR Vβ of the clone could not be determined with the TCR Vβ kit
UD) *undetermined*, indicates allo-HLA reactivity, however allo-HLA reactivity could not be characterized since the recognized EBV-LCLs did not share one particular allo-HLA molecule.

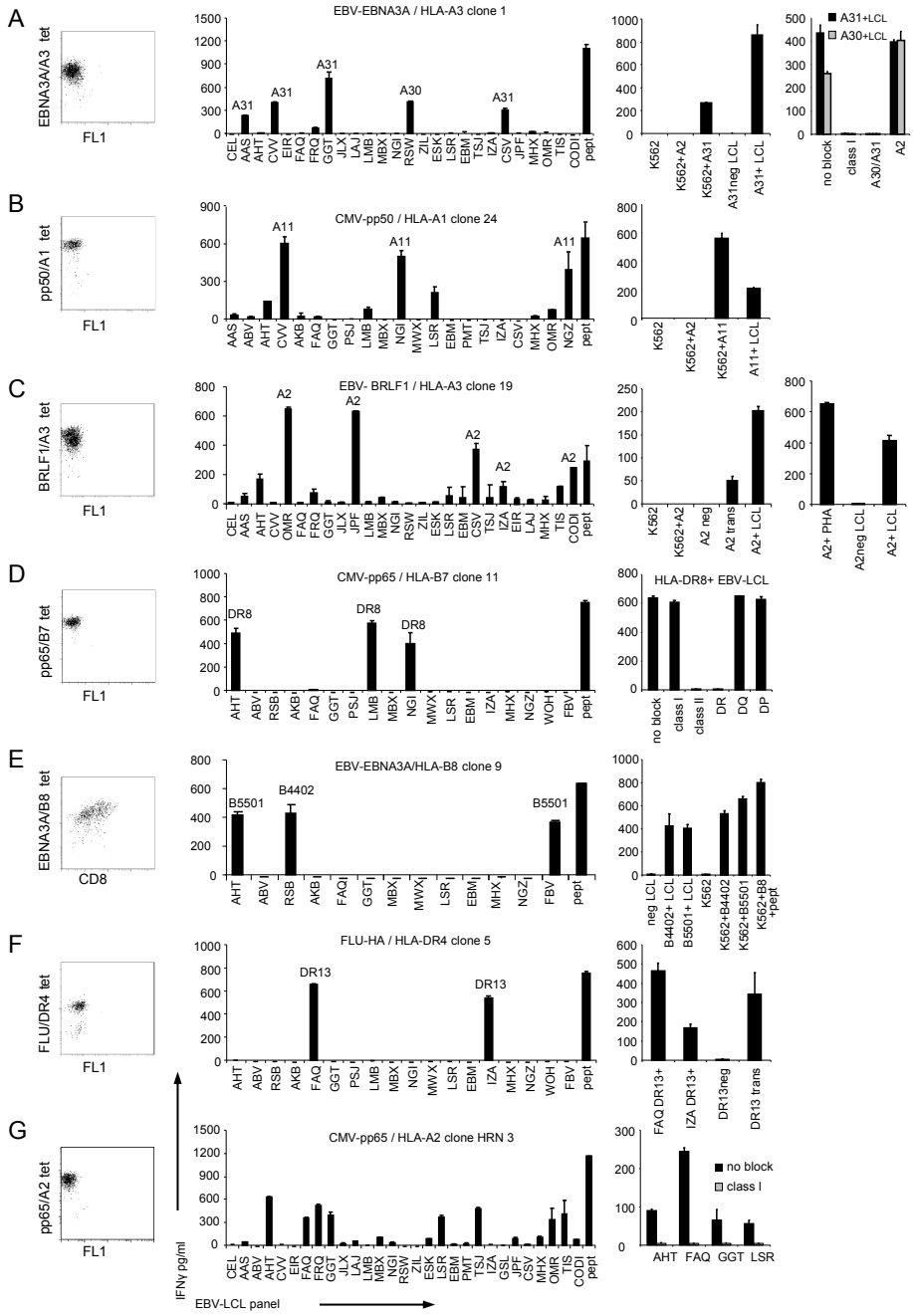

A
EBNA3A/A3 tet
FL1
EBV-EBNA3A / HLA-A3 clone 1
A31
A31
A31
A30
A31
CEL AAS AHT CVV EIR FAQ FRQ GGT JLX LAJ LMB MBX NGI RSW ZIL ESK LSR EBM TSJ IZA CSV JPF MHX OMR TIS CODI pept
K562
K562+A2
K562+A31
A31neg LCL
A31+ LCL
A31+LCL
A30+LCL
no block
class I
A30/A31
A2
B
pp50/A1 tet
FL1
CMV-pp50 / HLA-A1 clone 24
A11
A11
A11
AAS ABV AHT CVV AKB FAQ GGT PSJ LMB MBX NGI MWX LSR EBM PMT TSJ IZA CSV MHX OMR NGZ pept
K562
K562+A2
K562+A11
A11+ LCL
C
BRLF1/A3 tet
FL1
EBV- BRLF1 / HLA-A3 clone 19
A2
A2
A2
A2
A2
CEL AAS AHT CVV OMR FAQ FRQ GGT JLX JPF LMB MBX NGI RSW ZIL ESK LSR EBM CSV TSJ IZA EIR LAJ MHX TIS CODI pept
K562
K562+A2
A2 neg
A2 trans
A2+ LCL
A2+ PHA
A2neg LCL
A2+ LCL
D
pp65/B7 tet
FL1
CMV-pp65 / HLA-B7 clone 11
DR8
DR8
DR8
AHT ABV RSB AKB FAQ GGT PSJ LMB MBX NGI MWX LSR EBM IZA MHX NGZ WOH FBV pept
HLA-DR8+ EBV-LCL
no block
class I
class II
DR
DQ
DP
E
EBNA3A/B8 tet
CD8
EBV-EBNA3A/HLA-B8 clone 9
B5501
B4402
B5501
AHT ABV RSB AKB FAQ GGT MBX MWX LSR EBM MHX NGZ FBV pept
neg LCL
B4402+ LCL
B5501+ LCL
K562
K562+B4402
K562+B5501
K562+B8 pept
F
FLU/DR4 tet
FL1
FLU-HA / HLA-DR4 clone 5
DR13
DR13
AHT ABV RSB AKB FAQ GGT PSJ LMB MBX NGI MWX LSR EBM IZA MHX NGZ WOH FBV pept
FAQ DR13+
IZA DR13+
DR13neg
DR13 trans
G
pp65/A2 tet
FL1
CMV-pp65 / HLA-A2 clone HRN 3
IFNγ pg/ml
EBV-LCL panel
CEL AAS ABV AHT CVV EIR FAQ FRQ GGT JLX LAJ LMB MBX NGI RSW ZIL ESK LSR EBM PMT TSJ IZA GSL JPF CSV MHX OMR TIS CODI pept
no block
class I
AHT FAQ GGT LSR

Figure 2. Allo-HLA reactivity of virus specific T-cell clones.
41 virus specific T-cell clones, of which seven are shown in this figure, were stimulated with a panel of EBV-LCLs for 18h and IFNγ production was measured by ELISA. **(A)** The EBV-EBNA3A/HLA-A3 specific T-cell clone 1 exhibited alloreactivity against all EBV-LCLs expressing HLA-A*3101 and one EBV-LCL expressing HLA-A*3001. To confirm allo-HLA-A*3101 reactivity, clone 1 was tested against K562 cells (K562), K562 cells transduced with HLA-A*0201 (K562+A2), K562 cells transfected with HLA-A*3101 (K562+A31), HLA-A*3101 negative (A31neg LCL) and HLA-A*3101 positive EBV-LCLs (A31+ LCL). To confirm the reactivity against HLA-A*3001 the clone was tested against three HLA-A*3001+ EBV-LCLs, of which one is shown, and one HLA-A*3101+ EBV-LCLs with or without blocking mAbs directed against HLA class I, HLA-A30/A31 and HLA-A2. **(B)** The CMV-pp50/HLA-A1 specific T-cell clone 24 exhibited alloreactivity against all HLA-A*1101 expressing EBV-LCLs. To confirm allo-HLA-A*1101 reactivity, clone 24 was tested against K562 cells (K562), K562 cells transduced with HLA-A*0201 (K562+A2), K562 cells transduced with HLA-A*1101 (K562+A11) and HLA-A*1101 positive EBV-LCLs (A11+ LCL). **(C)** The EBV-BRLF1/ HLA-A3 specific clone 19 exerted alloreactivity against all HLA-A0201+ EBV-LCLs. This T-cell clone did not recognize K562 transduced with HLA-A*0201 (K562+A2). To confirm allo-HLA- A*0201 reactivity clone 19 was tested against untransduced HLA-A*0201 negative EBV-LCLs (HLA-A2neg LCL) or transduced with HLA-A*0201 (A2 trans), and HLA-A*0201 positive EBV-LCLs (A2+ LCL). To investigate whether this clone recognized an EBV derived peptide in the context of HLA-A*0201, the clone was tested against HLA-A*0201+ PHA blasts and HLA-A*0201 positive and negative EBV-LCLs as controls. **(D)** The CMV-pp65/HLA-B7 specific T-cell clone 11 exhibited reactivity against all three HLA-DRB1*0801+ EBV-LCLs. To confirm allo-HLA-DRB1*0801 reactivity, clone 11 was tested against the three HLA-DRB1*0801+ EBV-LCLs, of which one is shown, in the presence of either no (no block), anti-HLA class I (class I), anti-HLA-class II (class II), anti-HLA-DR (DR), anti-HLA-DQ (DQ), anti-HLA-DP (DP) blocking mAbs. **(E)** The EBV-EBNA3A/HLA-B8 specific T-cell clone 9 exhibited alloreactivity against all EBV-LCLs expressing either HLA-B*4402 or HLA-B*5501. To confirm HLA-B*4402 and HLA-B*5501 cross-reactivity, the clone was tested against K562 cells (K562), K562 cells transfected with HLA-B*4402 (K562+B4402) or HLA-B*5501 (K562+B5501). As controls, the clone was tested against HLA-B*4402 and HLA-B*5501 negative EBV-LCL (neg LCL), HLA-B*4402+ EBV-LCL (B4402+ LCL), HLA-B*5501+ EBV-LCL (B5501+ LCL) and HLA-B*0801+ K562 loaded with viral peptide (K562+B8+pept). **(F)** The Flu-HA/HLA-DR4 specific clone 5 recognized all HLA-DRB1*1301+ EBV-LCLs. To confirm allo-HLA-DRB1*1301 reactivity, clone 5 was tested against HLA-DRB1*1301+ EBV-LCLs (FAQ DR13+ and IZA DR13+) as well as HLA-DR13 negative EBV-LCLs non-transduced (DR13 neg) or transduced with HLA-DRB1*1301 (DR13 trans). **(G)** The CMV-pp65/HLA-A2 specific clone HRN 3 recognized EBV-LCLs which did not share one particular allo-HLA molecule. To investigate whether this reactivity was based on allo-HLA recognition, the clone was tested against four of the recognized EBV-LCLs with and without blocking mAb directed against HLA class I.

exhibited low cross-reactivity against EBV-LCLs negative for HLA-A*0201. Since these lower recognized EBV-LCLs did not share one HLA molecule, we did not determine the alloreactivity in detail. The CMV-pp65/HLA-B7 specific CD8 T-cell clone exhibited alloreactivity against two HLA-DRB1*0801 and one HLA-DRB1*0806 expressing EBV-LCLs present in the panel. The cross-reactivity exerted by this CD8 T-cell clone could be blocked with antibodies directed against HLA class II and HLA-DR and not by HLA class I, HLA-DQ or HLA-DP antibodies, confirming that the cross-reactivity of this virus specific CD8 T-cell clone was directed against HLA-DR8. The ability of CD8 T-cells to cross-react against allo-HLA class II molecules was also demonstrated by the CMV-pp65 specific HLA-B35 restricted CD8 T-cell clone that was shown to be cross-reactive against HLA-DRB1*0401 (table 3). As previously described[22], we also observed that EBV-EBNA3A/HLA-B8 specific T-cell clone exhibited alloreactivity against

all EBV-LCLs expressing HLA-B*4402. Furthermore, we observed alloreactivity against EBV-LCLs expressing HLA-B*5501 as was recently described by us.[33] Allo-HLA reactivity against HLA-B*4402 and HLA-B*5501 was confirmed by recognition of K562 transfected with either HLA-B*4402 or HLA-B*5501 by this T-cell clone (figure 2E).

In addition to CD8+ T-cell lines and clones we also analyzed the cross-reactive potential of CD4+ T-cell clones to allo-HLA molecules. The alloreactivity of a Flu-HA/HLA-DR4 specific T-cell clone demonstrated to be directed against HLA-DRB1*1301. As shown in figure 2F both HLA-DRB1*1301 positive EBV-LCLs present in the panel were efficiently recognized by this T-cell clone. Allo-HLA-DRB1*1301 reactivity of the T-cell clone could be confirmed since the T-cells recognized EBV-LCLs transduced with HLA-DRB1*1301 whereas non-transduced EBV-LCLs were not recognized. In addition, allo-HLA reactivity was demonstrated for two other CD4+ T-cell clones of the in total 5 CD4+ T-cell clones tested, indicating that virus specific CD4+ T-cells also exert allo-HLA reactivity (table 3).

For 5 of the 18 allo-HLA reactive virus specific T-cell clones the recognized HLA molecules could not be determined since the recognized EBV-LCLs did not share one particular allo-HLA molecule. The alloreactivity of one of these clones, CMV-pp65 / HLA-A2 specific clone HRN 3, is shown in figure 2G. We hypothesize that this recognition is mediated by the recognition of several HLA molecules since the reactivity could be blocked by mAb specific for HLA class I.

The results of the allo-HLA reactivity exerted by the virus specific T-cell clones are summarized in table 3, and demonstrate that approximately 45% of the virus specific memory CD4 and CD8 T-cell clones exhibit cross-reactivity to allo-HLA molecules. The cross-reactivity of the CD8 and CD4 T-cell clones was primarily directed against HLA class I and II, respectively. However, cross-reactivity of CD8 T-cells directed against HLA class II was also observed.

Different allo-HLA recognition by T-cell clones with the same specificity but different TCR usage

Burrows et al. showed that EBV-EBNA3A specific HLA-B8 restricted T-cell clones, derived from different HLA-B44 negative individuals were all alloreactive against HLA-B44.[22;23] It could therefore be suggested that allo-HLA reactivity of virus specific T-cells can be predicted. The EBV-EBNA3A response in HLA-B8+, HLA-B44- individuals is, however, a very homogeneous response in which all T-cells express an almost identical public TCR.[34] This is in contrast with most anti-virus responses, which are usually oligoclonal and different between individuals[16;35] (table 3). To assess whether virus specific T-cells sharing the same antigen specificity but expressing different TCRs exert the same allo-HLA reactivity, we tested the alloreactivity of three T-cell clones derived from the same individual, all specific for a peptide of the IE62 protein of VZV presented in HLA-A*0201 but with different TCR usage. As demonstrated in figure 3 A, B and C all 3 VZV specific T-cell clones recognized different allo-HLA molecules. Clone 5 showed alloreactivity against HLA-B*5501, clone 6 was alloreactive against HLA-B*5701 and clone 7 exhibited allo-HLA-A*0205 and allo-HLA-A*0207 reactivity. The recognition of the

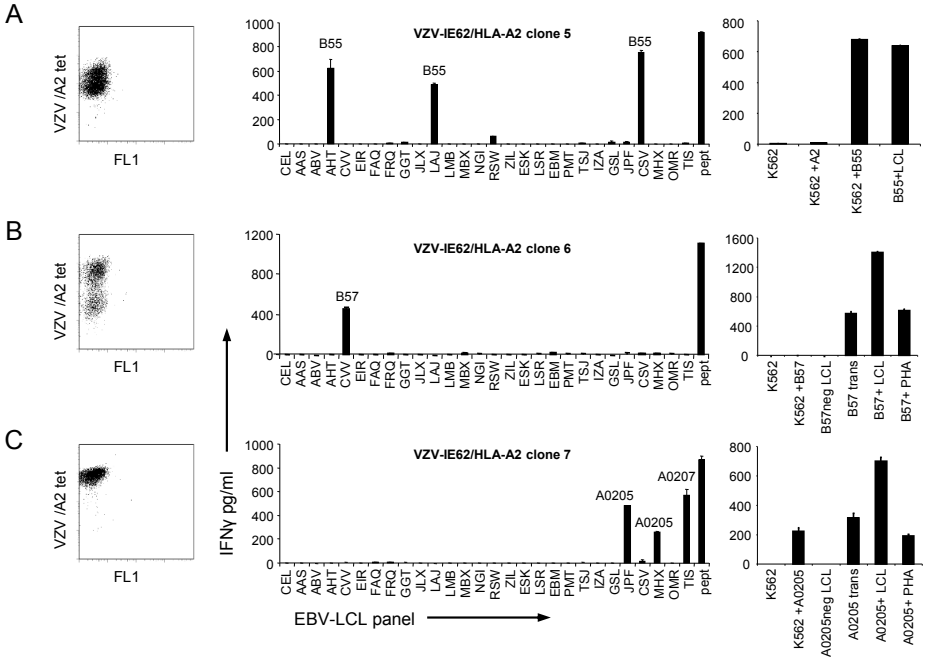


Figure 3. Variable allo-HLA recognition by T-cell clones with the same specificity but different TCR Vβ usage.

To investigate whether virus specific T-cells sharing the same antigen specificity but expressing different TCRs exert the same allo-HLA reactivity, three VZV-IE62/HLA-A2 specific T-cell clones expressing different TCRs were stimulated for 18h with a panel of EBV-LCLs and IFNγ production was measured by ELISA. **(A)** VZV clone 5 showed alloreactivity against all HLA-B*5501+ EBV-LCLs. To confirm allo-HLA-B*5501 reactivity, the clone was tested against K562 cells (K562), K562 cells transduced with HLA-A*0201 (K562+A2), K562 cells transduced with HLA-B*5501 (K562+B55) and HLA-B55+ EBV-LCLs (B55+ LCL). **(B)** VZV clone 6 was alloreactive against the HLA-B*5701+ EBV-LCL. Clone 6 did not show reactivity against K562 cells transduced with HLA-B*5701 (K562+B57). To confirm allo-HLA-B*5701 reactivity clone 6 was tested against HLA-B*5701 negative EBV-LCLs (HLA-B57neg LCL) or transduced with HLA-B*5701 (B57 trans), HLA-B*5701+ EBV-LCLs (B57+ LCL) and HLA-B*5701+ PHA blasts (B57+ PHA). **(C)** VZV clone 7 exhibited cross-reactivity against all HLA-A*0205+ and HLA-A*0207+ EBV-LCLs. Allo-HLA-A*0205 reactivity was confirmed by testing the clone against K562 cells (K562), K562 transduced with HLA-HLA-A*0205 (K562+ HLA-A0205), HLA-A*0205 negative EBV-LCLs (HLA-A0205neg LCL) or these EBV-LCLs transduced with HLA-A*0205 (A0205 trans), HLA-A*0205+ EBV-LCLs (A0205+ LCL) and HLA-A*0205+ PHA blasts (A0205+ PHA). The results demonstrate that virus specific T-cells with the same antigen specificity, but with different TCR usage exert alloreactivity against different HLA molecules.

three clones together was comparable to the recognition exerted by the VZV-IE62 specific line derived from the same individual. This confirms that alloreactivity exerted by the virus specific lines shown in figure 1 is the sum of the alloreactivity of the various clonal populations present within the lines. The allo-HLA reactivities of the T-cell clones were confirmed by transduction of the allo-HLA molecules in K562 or in non-recognized EBV-LCLs. Clone 5

and 7 recognized K562 transduced with HLA-B*5501 and HLA-*0205, respectively. Clone 6 was unable to recognize K562 transduced with HLA-B*5701, whereas EBV-LCLs transduced with HLA-B*5701 were recognized. Since this clone also recognized HLA-B*5701 expressing PHA stimulated T-cell blasts, specificity against EBV derived peptide in the context of allo-HLA-B*5701 is excluded. These results demonstrate that virus specific T-cells with the same antigen specificity, but with different TCR complexes can exert alloreactivity against different HLA molecules. Since T-cell responses against viruses are usually oligoclonal and different between individuals these results indicate that allo-HLA reactivity cannot be predicted.

The cytotoxic potential and affinity of the alloreactivity exerted by virus specific T-cells

Since cytotoxicity might be a relevant measure to predict the potency of the virus-specific T-cells to induce GVHD or graft rejection in vivo, we investigated the allo-HLA reactive cytotoxic capacity of the virus specific T-cells. Six virus specific T-cell clones were tested against a panel of EBV-LCLs positive and negative for the recognized allo-HLA molecules. As shown in figure 4A, all six T-cell clones tested showed cytotoxicity against the allo-HLA expressing EBV-LCLs. To investigate whether the affinity of the allo-HLA reactive response was comparable to the affinity of the virus specific response, the kinetics of recognition and antigen threshold were tested for both specificities. For this purpose the allo-HLA-A*3101/A*3001 reactive EBV-EBNA3A/HLA-A3 specific clone 19 was tested against HLA-A*0301+ EBV-LCL AST transduced with a retrovirus encoding for EBNA3A and against two HLA-A*3101+ EBV-LCLs GGT and DSP at different effector : stimulator ratios. As is shown in figure 4B, the T-cell clone produced comparable amounts of IFNγ against the virus antigen expressing EBV-LCL as against the allo-HLA expressing EBV-LCLs in the different effector : stimulator ratios, indicating that the kinetics of recognition and antigen threshold of the alloreactive response and the virus-specific T-cell response are comparable.

Normal cell subsets are recognized by virus specific T-cells

To extrapolate the results obtained with the EBV-LCLs and K562 cells to the recognition of normal cell subsets in vivo, we tested virus specific T-cell clones against allo-HLA-expressing B cells, CD40L activated B-cells, T-cells, PHA-blasts, monocytes, monocyte derived DCs and fibroblasts with and without IFNγ pre-treatment. The results shown in figure 4C demonstrate the reactivity of three virus specific T-cell clones directed against the different cell subsets. Pp65/HLA-B*0702 specific clone 11 showed high recognition of HLA-DRB1*0801 positive CD40L activated B-cells and low recognition of B-cells and PHA blasts. VZV-IE62/HLA-A*0201 specific clone 5 showed high recognition of HLA-B*5501 positive CD40L activated B-cells, DCs and PHA blast and low recognition of monocytes and T-cells. This clone could unfortunately not be tested against fibroblasts since HLA-B*5501 positive fibroblasts were not available. VZV-IE62/HLA-A*0201 specific clone 6 highly recognized HLA-B*5701 positive DCs and

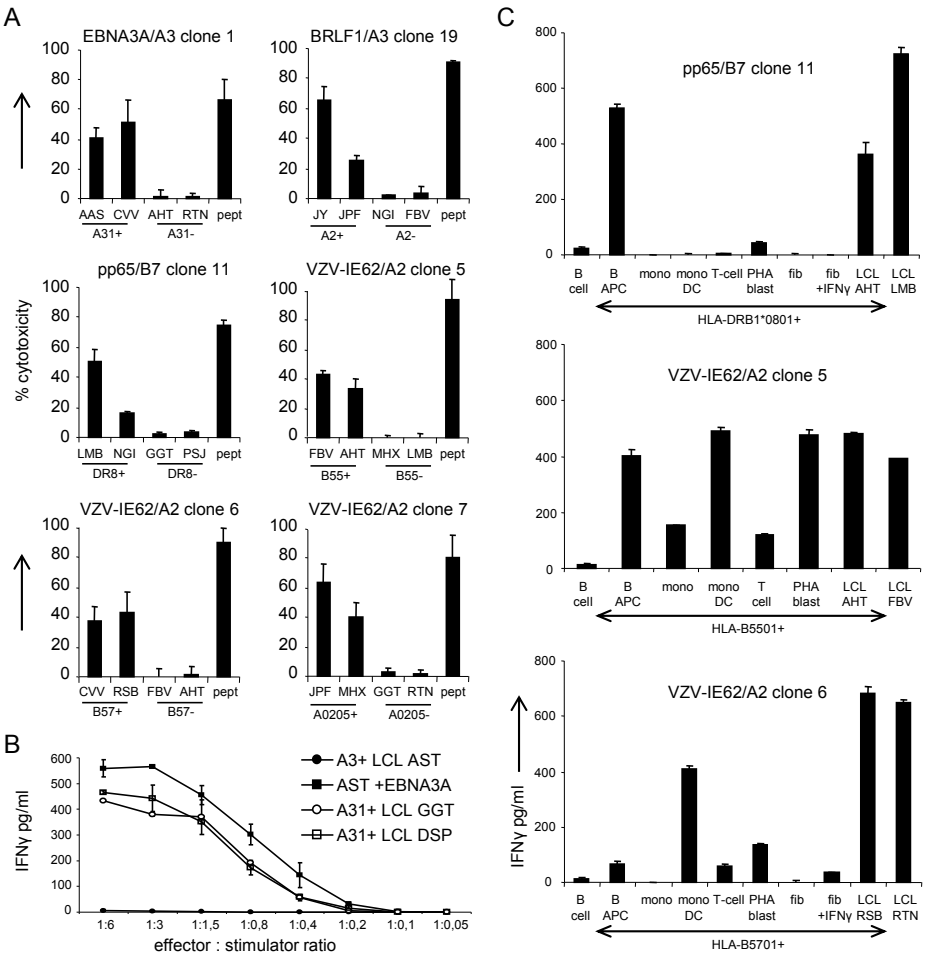


Figure 4. The potency of the alloreactivity exerted by virus specific T-cells.
(A) To investigate the allo-HLA reactive cytotoxic capacity of virus specific T-cells, 6 virus specific cell clones were tested in cytotoxicity assays against 2 EBV-LCLs expressing the recognized allo-HLA molecules and 2 EBV-LCLs negative for the allo-HLA molecules. EBV-LCLs expressing the virus specific restriction molecule were loaded with the viral peptide and used as positive control for cytotoxicity. **(B)** To compare the affinities of the allo-HLA reactive response and the virus specific response, the allo-HLA-A30/A31 reactive EBV-EBNA3A/HLA-A3 specific clone 19 was tested against the HLA-A*0301+ EBV-LCL AST transduced with a retrovirus encoding for EBNA3A and against two HLA-A*3101+ EBV-LCLs GGT and DSP. To compare the kinetics of the two responses, the clone was tested against the EBV-LCLs in different effector : stimulator ratios. **(C)** To extrapolate the results obtained with the EBV-LCLs and K562 cells to the recognition of normal cell subsets in vivo, we tested virus specific T-cell clones against allo-HLA-expressing B cells, CD40 ligand activated B-cells (B APC), monocytes, monocyte derived DCs, T-cells, PHA-blasts and fibroblasts with and without IFNγ pre-treatment.

showed low reactivity against CD40L activated B-cells, T-cells, PHA blasts and IFNγ stimulated fibroblasts. These results indicate that virus specific T-cells can also be reactive against in vivo relevant normal cell subsets.

One TCR complex mediates both virus specificity and allo-HLA reactivity

Since alloreactivity mediated by T-cells may be explained by T-cells expressing 2 TCR complexes at the cell surface,[36;37] we wanted to exclude that the allo-HLA reactivity was mediated via another TCR than the virus specific TCR. For this purpose we determined the TCR usage of two representative allo-HLA reactive virus specific clones, the allo-HLA-A*0201 reactive BRLF1/HLA-A*0301 specific clone 19 (figure 2C) and the allo-HLA-A*0205 reactive VZV-IE62/HLA-A*0201 specific clone 7 (figure 3C). By RT–PCR, we established that the BRLF1/HLA-A*0301 specific clone 19 expressed one TCRβ gene transcript, BV7S2, and two TCRα transcripts, AV12S1 and AV18S1. However, one of the TCRα chains, AV12S1, contained a stopcodon in the CDR3 region, indicating that this TCRα was not expressed. Flow cytometric analysis confirmed that 100% of the T-cells expressed TCR BV7S2 at the cell surface (data not shown). No antibodies were available for analysis of the specific TCRα chain expression at the cell surface. To investigate whether the BV7S2 and the AV18S1 mediated the dual recognition, IE1/HLA-A1 specific T-cells were transduced with retroviral vectors encoding for these TCR α

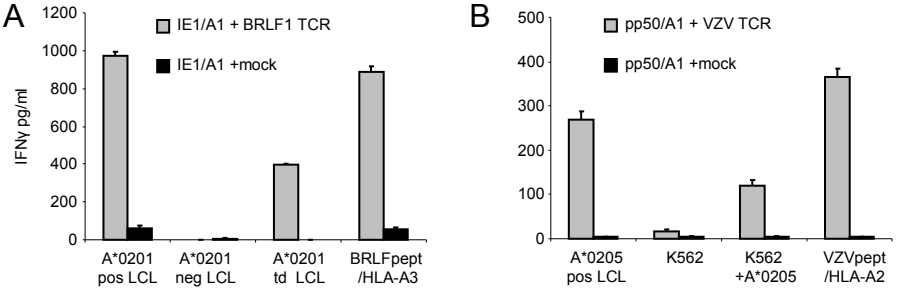


Figure 5. One TCR complex mediates both virus specificity and allo-HLA reactivity.
To exclude that allo-HLA reactivity was mediated via another TCR than the virus specific TCR, the TCR of two representative clones was transferred to T-cells with a different specificity. **(A)** IE1/A1 specific T-cells transduced with viral vectors encoding for the TCR of BRLF1/A3 specific clone 19 (IE1/A1 +BRLF1 TCR) and IE1/A1 specific T-cells transduced with a mock viral vector (IE1/A1 +mock) were tested for allo-HLA-A*0201 reactivity against HLA-A*0201 positive EBV-LCLs (A*0201 pos LCLs), HLA-A*0201 negative EBV-LCLs (A*0201 neg LCLs) and HLA-A*0201 negative EBV-LCLs transduced with HLA-A*0201 (A*0201 td LCLs) and for BRLF1 specificity against BRLF1 peptide loaded HLA-A*0301 positive EBV-LCLs (BRLF1pept /HLA-A3). **(B)** Pp50/A1 specific T-cells transduced with viral vectors encoding for the TCR of VZV clone 7 (pp50/A1 + VZV TCR) and pp50 specific T-cells transduced with a mock viral vector (pp50/A1 +mock) were tested for allo-HLA-A*0205 reactivity against HLA-A*0205 positive EBV-LCLs (A*0205 pos LCLs), K562 cells (K562), and K562 cells transduced with HLA-A*0205 (K562 +A*0205) and for VZV specificity against VZV-IE62 peptide loaded HLA-A*0201 positive EBV-LCLs (VZVpept /HLA-A2). The results demonstrate that virus specificity and allo-HLA reactivity exerted by virus specific T-cells were mediated by one TCR complex.

and β chains. The results shown in figure 5A demonstrate that the BRLF1-TCR transduced T-cells exerted reactivity against HLA-A*0201 expressing target cells cells as well as against EBV-BRFL1 peptide loaded HLA-A*0301+ target cells. No reactivity directed against peptide loaded or HLA-A*0201 expressing target cells was observed with mock transduced T-cells.
The VZV-IE62/HLA-A*0201 clone 7 expressed one TCRα transcript, AV6S1, and one TCRβ transcript, BV21S3. Pp50/A1 specific T-cells were transduced with retroviral vectors encoding for the VZV TCR chains. The results shown in figure 5B demonstrate that the VZV-TCR transduced T-cells exerted reactivity against HLA-A*0205 expressing target cells as well as against VZV-IE62 peptide loaded HLA-A*0201+ target cells. No reactivity directed against peptide loaded or HLA-A*0205 expressing target cells was observed with mock transduced T-cells. These results demonstrate that the virus specificity and the allo-HLA reactivity exerted by these virus specific T-cells were mediated via one TCR complex.

DISCUSSION

In this study, we demonstrated that a high percentage of virus specific memory T-cells exhibit cross-reactivity against allogeneic HLA molecules. CD8 as well as CD4 virus specific memory T-cells demonstrated to have allo-HLA reactive potential. In addition, we determined that the alloreactivity exerted by CD8 T-cells was directed against either HLA class I or HLA class II molecules, and that the alloreactivity of the T-cells was mediated by cytotoxicity and cytokine production. Furthermore, we demonstrate that virus specific T-cells can exert allo-HLA reactivity against normal cell subsets, indicating the potential clinical relevance of the response. By TCR transfer we confirmed that the allo-HLA reactivity and virus specificity were mediated via the same TCR.
Most virus specific T-cell lines and 45% of virus specific T-cell clones directed against EBV, CMV, VZV and FLU, exerted alloreactivity when tested against a panel of EBV-LCLs covering almost all common HLA molecules. The cross-reactivity exerted by the virus specific T-cells was confirmed to be based on allo-HLA recognition by testing the T-cell clones against K562 cells and EBV-LCLs transduced with single HLA molecules. Some of the alloreactive virus specific T-cell clones did not recognize K562 cells transduced with the specific allo-HLA molecules, but showed reactivity against EBV-LCLs transduced with the allo-HLA molecules. These data support the previous findings that allo-HLA reactivity is dependent on endogenous peptide.[38] Allo-HLA cross-reactivity was not only directed against EBV-LCLs, but also against PHA stimulated T-cells (figure 3B) indicating that the peptides responsible for allo-HLA reactivity were not EBV derived. Differential recognition of HLA transduced K562 cells and EBV-LCLs may indicate recognition of tissue specific peptides in allo-HLA molecules. Therefore it may be possible that we even underestimated the allo-HLA reactive repertoire of T-cells by initially screening only against an EBV-LCL panel.

Burrows et al. showed that EBV-EBNA3A specific HLA-B8 restricted T-cells derived from different HLA-B44 negative individuals all exert cross-reactivity against allo-HLA-B44[22;23]. Based on these finding it could be suggested that the allo-HLA reactivity of virus specific T-cells can be predicted. The EBV-EBNA3A specific T-cells however express an almost identical public TCR in all HLA-B8+ HLA-B44- individuals,[34] whereas most other virus responses are oligoclonal and the TCR usage of T-cells directed against the same viral epitope is variable between individuals[16;35] (table 3). We have demonstrated that virus specific T-cells with the same antigen specificity, but expressing different TCRs, exhibit cross-reactivity against different HLA molecules (figure 3). In addition, we have shown that three specific T-cell lines with the same specificity for CMV-pp50, but derived from different individuals, exerted a very variable pattern of allo-HLA reactivity ranging from no allo-HLA reactivity to very broad alloreactivity (figure 1). These results together illustrate that the cross-reactive potential of antigen specific T-cells against allo-HLA molecules is difficult to predict.

The alloreactivity exerted by the virus specific memory CD8 and CD4 T-cells was primarily directed against allo-HLA class I and II molecules, respectively, suggesting that the coreceptors expressed by the T-cells contributed to the affinity of the allo-HLA reactivity. However, we also demonstrated allo-HLA class II recognition by a small proportion of antigen specific CD8 T-cells, as was also shown recently by Rist et al.[39] HLA class II allorecognition by CD8+ T-cells could indicate an HLA class II-TCR interaction that is independent of CD8 co-receptor binding. It is however also possible that CD8 co-receptors bind to HLA class I molecules expressed on the target cell and thereby strengthen the TCR-HLA class II interaction, as was previously shown.[27] Although we did not observe HLA class I cross-reactive CD4 T-cells, only a limited number of CD4 T-cell clones were tested, and therefore we cannot exclude that CD4 T-cells may also cross-react with HLA class I complexes.

The results of our study illustrate that approximately 45% of all T-cells exert allo-HLA cross-reactivity. However, since T-cells were only analyzed for allo-HLA cross-reactivity against an EBV-LCL panel expressing most common HLA molecules, missing all infrequent HLA molecules as well as all tissue specific peptides presented in allo-HLA molecules, we speculate that virtually all T-cells may be allo-HLA reactive. Based on this assumption and the fact that the TCR repertoire of humans is highly diverse, after HLA mismatched transplantations sufficient allo-mismatched-HLA cross-reactive T-cells are likely to be present to induce acute GVHD or graft rejection. However, HLA mismatched stem cell transplantation or solid organ transplantations do not always lead to acute GVHD or graft rejection, indicating that other factors must be involved in these transplantation related complications. A range of acute viral infections have been linked to initiating GVHD and graft rejection following transplantation,[26] suggesting that virus specific T-cells may be mediators of GVHD and graft rejection. Since virus specific T-cell responses usually have a restricted TCR usage, high numbers of T-cells expressing an identical TCR can be found. In addition, herpes virus specific T-cell populations can remain present at high percentages for long periods of time in healthy individuals as

well as in patients,[18-21] and viral infections, leading to expansion of virus specific T-cells, are very common after HLA mismatched SCT or solid organ transplantation.[40-43] Therefore, given the high proportion of virus specific T-cells and the less stringent requirements for activation of memory T-cells,[12;13] it is tempting to speculate that if the HLA type of patient or the transplanted organ matches the cross-reactivity of the virus specific T-cells, these allo-HLA reactive virus specific memory T-cells may easily induce GVHD or graft rejection.

The ability of virus specific T-cells to exert allo-HLA reactivity might also have implications for the clinical applicability of virus specific T-cell lines. Because immune deficiency for viruses is a common complication after SCT, broad administration of virus specific T-cells lines over HLA barriers to SCT patients has been proposed.(44,45) The results of this study demonstrate that administration of virus specific T-cells over HLA barriers may increase risk of GVHD, and indicate that virus specific lines should be tested for alloreactivity against the patient before administration.

Based on our results we postulate that virtually all antigen specific T-cells will be cross-reactive against allo-HLA class I or II molecules. The high alloreactive potential of particularly virus specific memory T-cells might have important clinical implications in transplantation settings.

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4

ALLO-HLA REACTIVE T-CELLS INDUCING GRAFT VERSUS HOST DISEASE ARE SINGLE PEPTIDE SPECIFIC

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Blood. 2011 Dec 22;118(26):6733-42.



ABSTRACT

T-cell alloreactivity directed against non-self HLA molecules has been assumed to be less peptide specific than conventional T-cell reactivity. A large variation in degree of peptide specificity has previously been reported, including single peptide specificity, polyspecificity, and peptide degeneracy. Peptide polyspecificity was illustrated using synthetic peptides loaded target cells, but in the absence of confirmation against endogenously processed peptides this may represent low avidity T-cell reactivity. Peptide degeneracy was concluded based on recognition of antigen processing defective cells. In addition, since most investigated alloreactive T-cells were in vitro activated and expanded, the previously determined specificities may have not been representative for alloreactivity *in vivo*. To study the biologically relevant peptide specificity and avidity of alloreactivity, we investigated 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. All but one of the alloreactive T-cell clones, including those reactive against antigen processing defective T2 cells, recognized a single peptide allo-HLA complex, unique for each clone. Downregulation of the expression of the recognized antigens using silencing shRNAs confirmed single peptide specificity. Based on these results we conclude that biological relevant alloreactivity selected during *in vivo* immune response is peptide specific.

INTRODUCTION

Alloreactive T-cells directed against allogeneic HLA (allo-HLA) are involved in the development of graft versus host disease (GVHD) and graft rejection after HLA mismatched transplantation. In conventional T-cell reactivity directed against pathogens, each T-cell is specific for a single foreign peptide presented in a self HLA molecule.[1] During thymic development, T-cells that are reactive against self peptides presented in self HLA molecules undergo negative selection. Both T-cells reactive against a single peptide as well as T-cells exerting polyreactivity against more than one peptide presented in self HLA molecules will be deleted.[2;3] T-cells leaving the thymus are therefore tolerant for self-peptide-HLA complexes. Since T-cells never encounter allo-HLA molecules during thymic development, T-cells polyspecific for peptides presented in allogeneic HLA are not removed, and, therefore T-cell allo-HLA reactivity has been assumed to be less peptide specific than conventional T-cell reactivity.

The degree of peptide specificity of alloreactive T-cells has been extensively studied. 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 peptides in allorecognition.[4-11] In these studies, groups of alloreactive T-cell clones or lines were tested against these antigen processing deficient cells, unloaded or loaded with peptides. 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,[12] 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. Together these studies created the assumption that alloreactivity is a combination of different reactivities, ranging from peptide specific to peptide degenerate alloreactivity.

In several studies the degree of peptide specificity in allorecognition was determined by testing alloreactive T-cells against a limited number of defined peptides.[7;813-15] In these studies reactivity against target cells exogenously loaded with high concentrations of peptide was interpreted as biological relevant reactivity, and recognition of different peptides presented in allo-MHC by alloreactive T-cells was concluded to represent *bona fide* polyspecific allorecognition. However, since recognition of exogenously loaded peptides in the absence of reactivity against endogenously processed and presented antigen may represent low avidity T-cell recognition, biological relevance of reactivity against synthetic peptide loaded

target cells needs to be confirmed by investigating the T-cell reactivity against endogenously processed and presented antigen. In the absence of such confirmation, the polyspecific alloreactivity against synthetic peptides described in these studies might not be representative for alloreactivity that is exerted by T-cells during *in vivo* GVHD or graft rejection.

During *in vivo* immune responses T-cells with high avidity are selectively expanded, as indicated by the observation that the memory T-cell repertoire directed against microbial antigens comprises of T-cells with high avidity for the specific antigens, whereas the naïve T-cell repertoire specific for the same antigens contains a broad range of avidities.[16;17] In contrast, in vitro priming of T-cells results in a T-cell repertoire containing a large variety of avidities,[18;19] comparable to the repertoire of naïve T-cells, indicating that during in vitro activation and expansion no selection for high avidity occurs. Since under normal circumstances allo-HLA molecules are not encountered, alloreactive T-cells which can be present within the naïve as well as the memory T-cell pool,[20;21] will not have undergone selection for T-cells with high avidity for allo-HLA molecules. Therefore, the repertoires of alloreactive T-cells derived from individuals who have not been exposed to non-self HLA molecules, will contain a high variety of avidities. Most alloreactive T-cells used for the study of peptide specificity and avidity of allorecognition were activated and expanded *in vitro*, and, accordingly, many of these alloreactive T-cells have been reported to demonstrate a very broad range of avidities.[8;4;22] We hypothesize that only in circumstances in which *in vivo* selection does occur, like during *in vivo* allo-HLA directed immune responses including GVHD and graft rejection after HLA mismatched transplantations, T-cells with high avidity for allo-HLA molecules will be selected.

To study the biologically relevant degree of peptide specificity and avidity of T-cell alloreactivity, we investigated in detail the repertoire of allo-HLA reactive T-cells which were activated and expanded *in vivo*. From a patient experiencing severe GVHD after an HLA-A2 mismatched DLI, we isolated 50 different *in vivo* activated allo-HLA-A2 reactive CD8+ T-cell clones and investigated their peptide specific alloreactivity. All but one of the alloreactive T-cell clones showed reactivity against a single HPLC fraction of peptides eluted from HLA-A2, including 6 T-cell clones recognizing T2 cells without exogenously loaded peptides. Using multidimensional HPLC fractionation and mass spectrometry (MS), we identified the different peptides recognized by 7 alloreactive T-cell clones, including one T2 reactive T-cell clone, and demonstrated that the alloreactive T-cells exerted high avidity peptide specific alloreactivity. Downregulation of the expression of the recognized antigens using silencing shRNAs confirmed single peptide specificity of the alloreactive T-cell clones. These results demonstrate that T-cells exerting biological relevant alloreactivity *in vivo* exhibited peptide specificity. Based on these results we conclude that the biological relevant specificity which is selected during *in vivo* immune response is single peptide specific, as defined by recognition of endogenously processed and presented antigen.

MATERIALS AND METHODS

Cell collection and preparation

After informed consent, peripheral blood was obtained from different individuals, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Isopaque separation and cryopreserved. Stable Epstein–Barr virus (EBV)-transformed B-cell lines (EBV-LCLs) were generated using standard procedures. HLA-A2 negative donor EBV-LCLs and K562 cells were transduced with a retroviral vector encoding for HLA-A*0201 as previously described.[23] EBV-LCLs, T2 cells (obtained from the American Type Culture Collection (ATCC)), K562 cells, renal cell carcinoma cell line 1774 and melanoma cell line 1.14 were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Lonza, Basel, Switzerland) and 10% fetal bovine serum (FBS, Lonza). Fibroblasts were cultured from skin biopsies in Dulbecco's modified Eagle medium (DMEM; Lonza) and 10% FBS. Drosophila cells expressing HLA-A2, CD54 and CD80 were kindly provided by Dr. Hans Stauss (Department of Immunology and Molecular Pathology, Royal Free Hospital, University College London, London, UK) and cultured in Schneider's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS, at 24°C and atmospheric CO_2. 100 µM copper sulphate was added 48 hours prior to peptide-loading to induce expression of HLA-A2, CD54 and CD80.

Generation of allo-HLA-A2 reactive T-cell clones

Allo-HLA-A2 reactive T-cell clones were isolated from a patient experiencing acute GVHD after single HLA locus mismatch SCT and subsequent donor lymphocyte infusion (DLI). Based on a cross-over, the patient was HLA-A*0201 positive and the sibling donor was HLA-A*0201 negative, whereas all other HLA class I and II molecules were completely matched. To isolate the allo-HLA-A2 reactive T-cells, PBMCs collected during GVHD were stained with anti-HLA-A2 FITC (Pharmingen, San Jose, CA, USA), anti-HLA-DR APC (Pharmingen) and anti-CD8 PE mAbs (Carlsbad, CA, USA) at 4°C for 30 min and washed once. Activated (HLA-DR+), donor derived (HLA-A*0201-) CD8+ T-cells were sorted single cell per well into U-bottom microtiter plates containing 100 µl of feeder mixture consisting of IMDM, 5% FBS, 5% human serum (HS), IL-2 (120 IU/ml, Chiron, Amsterdam, The Netherlands), phytohemagglutinin (PHA, 0.8 µg/ml, Murex Biotec Limited, Dartford, UK) and 50 Gy irradiated allogeneic PBMCs ($0.5x10^6$ /ml). Proliferating T-cell clones were selected and further expanded non-specifically with PHA, IL-2 and irradiated allogeneic PBMCs. For frequency analyses, HLA-DR+ HLA-A*0201- CD8+ T-cells were sorted in bulk and non-specifically expanded with irradiated autologous PBMCs, IL-2 and PHA.

TCR-Vβ chain analysis

For determination of TCR Vβ usage of T-cell clones, the TCR Vβ kit (Beckman Coulter, Fullerton, CA) was used. TCRβ chains which could not be stained with mAbs were determined using multiplex amplification as previously described.[24] By sequence analyses the variable region and CDR3 region of the TCR Vβ chains of T-cell clones was determined.[25]

Peptide identification by peptide elution, high performance liquid chromatography (HPLC) and mass spectrometry

$3x10^{10}$ EBV-LCLs or T2 cells were lysed with lysisbuffer, composed of 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 and protease inhibitor mix (Complete; Roche). Lysates were centrifuged for 30 min at 11,000 x g and precleared for 60 min with CL4B sepharose beads. HLA-A2 immunoaffinity chromatography was performed with anti-HLA-A2 purified BB7.2 mAb covalently coupled to protein-A beads. Subsequently, HLA/peptide complexes were eluted with 10% acetic acid. High molecular mass material (HLA heavy chain and light chain) was removed by size filtration through Centriprep filtration units with a cutoff value of 10 kDa. After freeze drying, the peptide mixture was subjected to multidimentional HPLC fractionation, followed by peptide identification using MS. In the first round of the multidimentional HPLC fractionation the freeze dryed peptide mixture was subjected to C18 reversed-phase HPLC (RP-HPLC) on an Agilent 1100 series HPLC system. This first dimension was performed on a Reprosil-Pur C18-AQ 3µm 100 x 2 mm column using a water/acetonitrile/TFA gradient and fractions of 50 µl were collected. The non-T2 reactive T-cell clones were tested against T2 target cells loaded with a small sample of each HPLC fraction of peptides eluted from the HLA-A2 of EBV-LCLs. The T2 reactive T cell clones were tested against HLA-A2 positive drosophila cells loaded with a small sample of each HPLC fraction of peptides eluted from the HLA-A2 of T2 cells. For this purpose the T2 cells or drosophila cells were pre-incubated with the HPLC fractions for 1 hour at 20°C, before addition of T-cells. The highly recognized fractions were selected for a second round of RP-HPLC fractionation, which was performed on an Eclipse XDB C18 3,5 µm 150 x 1 mm column using a water/isopropanol/TFA gradient. Fractions were collected and tested for recognition. The recognized fractions were selected for a third round of RP-HPLC fractionation, which was performed on a 200 µm x 100 mm Phenomenex Aqua 5 µm column using a water/acetonitrile/formic acid gradient and fractions were collected and tested for recognition. The peptide masses present in the recognized fractions and in the neighboring unrecognized fractions were determined by on-line nano-HPLC-MS, using a LTQ-FT Ultra. The LTQ-FT Ultra (Thermo, Bremen, Germany) was operating in data dependent mode, automatically switching between MS and MS/MS acquisition. Full scan mass spectra were acquired in the FT-ICR with a resolution of 25,000 at a target value of 5,000,000. The two most intense ions were then isolated for accurate mass measurements by a selected ion monitoring scan in FT-ICR with a resolution of 50,000 at a target accumulation value of 50,000. The selected ions were then fragmented in the linear ion trap using collision-induced dissociation at a target value of 10,000. In a post analysis process, raw data were converted to peak lists using Bioworks Browser software, Version 3.1. For peptide identification, MS/MS data were submitted to the human IPI database using Mascot Version 2.2.04 (Matrix Science) with the following settings: 2 ppm and 0.8-Da deviation for precursor and fragment masses, respectively; no enzyme was specified. Peptide masses of which the abundance correlated with the recognition pattern of the T-cell clone were selected for tandem MS and peptide

identification was performed. Candidate peptides were synthesized by Fmoc-chemistry. To confirm peptide specificity the allo-HLA reactive T-cell clones were tested against T2 or HLA-A2+ drosophila cells loaded with titrated concentrations of characterized peptides. In addition, tandem mass spectrum of the synthetic peptides was recorded and compared to its eluted counterpart as additional confirmation.

Functional assays and flow cytometry

HLA-A2 restriction of allo-HLA reactive T-cell clones was determined in cytotoxicity assays in which T-cells clones were tested in a 4h ^{51}Cr-release assay against patient EBV-LCLs, donor EBV-LCLs and donor EBV-LCLs transduced with HLA-A*0201 at effector to target ratio10:1. In addition, blocking studies were performed using BB7.2 (anti-HLA-A2), W6.32 (anti-HLA class I) and B1.23.2 (anti-HLA-B and C) mAbs. Patient EBV-LCLs were pre-incubated with saturating concentrations of mAbs for 1h at 20°C before addition of T-cells.

Tetramer staining was performed at 37°C for 20 min using tetramers composed of HLA-A*0201 and peptides CMV-IE1-VLE, CMV-pp65-NLV, EBV-BMLF1-GLC, EBV-BRLF1-YVL, EBV-LMP2-CLG, EBV-LMP2-FLY, and FLU-IMP-GIL. In addition, tetramers composed of HLA-A*0201 and newly identified epitopes HLA-HLA-DRA-FID, ATXN10-QVF, VPS13B-SLW, FDPS-YLD, USP11-FTW, and SHIP-GFP were generated as described previously.[26] Functionality of tetramers was controlled by staining of the specific T-cell clones. All tetramers were functional with the exception of ATXN10-QVF.

Allo-HLA reactive T-cell clones were tested against T2 cells loaded with a mixture of HLA-A2 binding peptides derived from CMV, EBV, and Flu at a final concentration of 1 µg/ml. In the stimulation assays 5,000 T-cells were stimulated with 20,000 EBV-LCLs, T2 cells, K562+A2 cells, melanoma 1.14 cells or RCC1774 cells or with 5,000 fibroblasts or with 100,000 HLA-A2+ drosophila cells. All functional assays were performed in IMDM containing 5% FBS, 5% HS and 100 IU/ml IL-2 at a total volume of 150µl in 96 well plates. After 18h of incubation, supernatant was harvested and IFNγ production was measured by standard ELISA.

Alanine substitution experiment

Of the two peptides recognized by clone HSS11 (FIDKFTPPV derived from HLA-DRA and FLLKLTPLL derived from THRAP4) modified peptides were generated with alanine substitutions at the amino acid positions identical between the two peptides (1, 4, 6 and 7). In addition, modified peptides were synthesized in which the anchor residues at position 2 and 9 between the two peptides were exchanged. To determine the peptide concentrations required for half maximum IFNγ production (EC50), clone HSS11 was incubated with T2 cells loaded with titrated concentrations of wild type (wt) and modified peptides. EC50 of the modified peptides was compared to the EC50 of wt peptides.

Inhibition of gene expression by silencing short hairpin RNA

Lentiviral vectors encoding for short hairpin (sh) RNA sequences specific for the genes coding for the proteins from which the identified peptides were derived in combination with the puromycin resistance gene were used from the Sigma-Aldrich shRNA library (Sigma-Aldrich, St. Louis, MO, USA). For USP11 shRNA TRCN0000007360, for FDPS TRCN0000036294, for VPS13B TRCN0000083955, for ATXN10 TRCN0000084093, for HLA-DRA TRCN0000057309, and for THRAP4 TRCN0000019649 were used. Fibroblasts were transduced with the lentiviral vectors and after 3 days puromycin was added. Lentiviral transduced fibroblasts were cultured for 2 weeks with 4 µg/ml puromycin before testing. The lentiviral vector encoding the shRNAs for USP11, FDPS, VPS13B and ATXN10 were transduced into HLA-A*0201 fibroblasts derived from a healthy donor. The lentiviral vectors encoding the shRNAs for HLA-DRA and THRAP4 were transduced into SV40 transformed HLA-A*0201 fibroblasts derived from a Bare Lymphocyte Syndrome patient (BLS) (EBA) and its HLA identical healthy sibling (CBA), that were kindly provided by Dr. P. van den Elsen (IHB, LUMC). The fibroblasts from BLS patients were previously demonstrated to be HLA-DRA deficient.[27] Cells were tested in 384 well plates with 3,000 fibroblasts/well and 1,000 T-cells/well. The fibroblasts transduced with shRNA for HLA-DRA and THRAP were stimulated for 72h with 100 IU IFN-γ/ml to increase HLA-DRA expression.
To confirm shRNA downregulation of expression of the different genes, quantitative real-time PCR (qRT-PCR) (TaqMan) for the different genes was performed. Total RNA was isolated from cells using the micro RNaqueous kit (Ambion). First strand cDNA synthesis was performed with oligo dT primers using M-MLV reverse transcriptase (Invitrogen). Samples were run with EvaGreen on an ABI 7500. Primers specific for the different genes are depicted in table 1.

Table 1. Primers used for qRT-PCR

gene	position	sequence
USP11	forward	GGGCCTGTATAACGTCCTGA
	reverse	CTCTGGCTCCCAGTCGATAG
FDPS	forward	TGAGATCTGTGGGGGTCTTC
	reverse	TCCCGGAATGCTACTACCAC
VPS13B	forward	ATGATTGTGTGTGCCTTGGA
	reverse	TTGGCACTGAATGTTCCAGA
ATXN10	forward	CAAGCCATGTTTCCCAAACT
	reverse	ACCCGCAAAAGATCAATCAC
HLA-DRA	forward	CATCCAGGCCGAGTTCTATC
	reverse	CTGGTGGGGTGAACTTGTCT
THRAP4	forward	CTGCACATCGCCAAACTAGA
	reverse	TACCCTCGGGAGACTCAATG

RESULTS

Isolation and characterization of allo-HLA-A2 reactive T-cells activated during acute GVHD

To be able to characterize the peptide-HLA ligand specificity of allo-HLA reactive T-cells exerting allo-immune responses *in vivo*, we isolated activated donor derived CD8 T-cells from a patient experiencing severe acute GVHD after single HLA-A2 locus mismatch SCT and subsequent DLI. The activated, HLA-DR positive CD8 T-cells of donor origin (HLA-A2 negative) were sorted single cell per well from peripheral blood collected at the time of GVHD and expanded. Fifty of 56 isolated CD8 T-cell clones were shown to be alloreactive, since these T-cells recognized patient but not donor EBV-LCLs. All 50 alloreactive T-cell clones were allo-HLA-A2 reactive, since clones were reactive against donor EBV-LCL retrovirally transduced with HLA-A2, as shown for 8 representative clones (figure 1A). In addition recognition of HLA-A2 positive EBV-LCLs could be blocked by anti-HLA class I as well as anti-HLA-A2 mAbs (data not shown). By flow cytometric analysis using Vβ mAbs and multiplex PCR followed by sequencing of the different TCR Vβ chains (data not shown), we demonstrated that all 50 allo-HLA-A2 reactive CD8 T-cell clones were of different clonal origin. In addition, a high variety in TCR Vβ chain usage was observed, and no sequence homology between the allo-HLA reactive clones was detected.

The allo-HLA reactivity of GVHD inducing T-cells is dependent on peptide specific recognition.

To determine whether allo-HLA-A2 reactive T-cells recognized HLA-A2 irrespective of the peptide presented in the peptide binding groove, T-cell clones were stained with a mix of tetramers composed of HLA-A2 and different virus-specific peptides derived from CMV, EBV and Flu. None of the T-cell clones stained with the viral HLA-A2 tetramers (data not shown). Next, the 50 allo-HLA-A2 reactive T-cell clones were tested against T2 cells and T2 cells loaded with the different CMV, EBV and Flu HLA-A2 binding peptides. 44 of the 50 T-cell clones showed no reactivity against peptide loaded or unloaded T2 cells (figure 1A). 6 of the 50 T-cell clones were reactive against unloaded T2 cells (reactivity of 3 T-cell clones is shown in figure 2A) and showed comparable reactivity against peptide loaded T2 cells (data not shown). Since T2 cells express HLA-A2 peptide complexes, although with restricted peptides variation, the ability of allo-HLA reactive T-cells to recognize allo-HLA-A2 could still be based on peptide specific recognition. To determine whether these T-cell clones recognized allo-HLA-A2 independent of the peptide presented in HLA binding groove, the clones were tested against HLA-A2 expressing drosophila cells loaded with the different HLA-A2 binding peptides. The T2 reactive T-cell clones showed no reactivity against unloaded (figure 2A) or peptide loaded HLA-A2+ drosophila cells (data not shown). These results indicate that for all 50 alloreactive

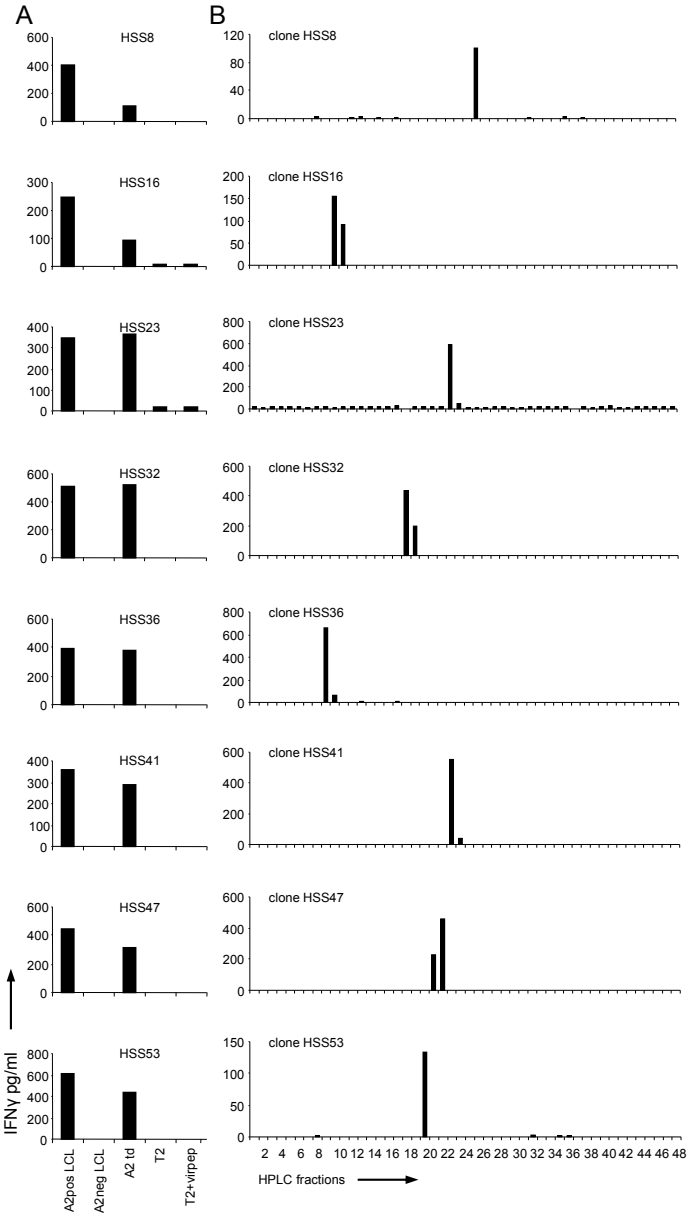


Figure 1. Recognition of HPLC fractions of HLA-A2 eluted peptides by the allo-HLA reactive T-cell clones.

(A) The allo-HLA reactive T-cell clones were stimulated with HLA-A2 positive EBV-LCLs (A2pos LCL), HLA-A2 negative EBV-LCLs (A2neg LCL), HLA-A2 negative EBV-LCLs transduced with HLA-A2 (A2 td), T2 cells (T2) and T2 cells loaded with a mixture of different HLA-A2 binding peptides from CMV, EBV and Flu (T2+virpep). Supernatants were harvested after 18 hours of stimulation and IFNγ was measured by standard ELISA. **(B)** Peptides were eluted from the HLA-A2 molecules of EBV-LCLs and fractionated by RP-HPLC using a water/acetonitrile/TFA gradient. 26 of the 44 non T2 recognizing allo-HLA-A2 reactive T-cell clones, of which 8 are shown in this figure, were stimulated with T2 cells loaded with the HPLC fractions.

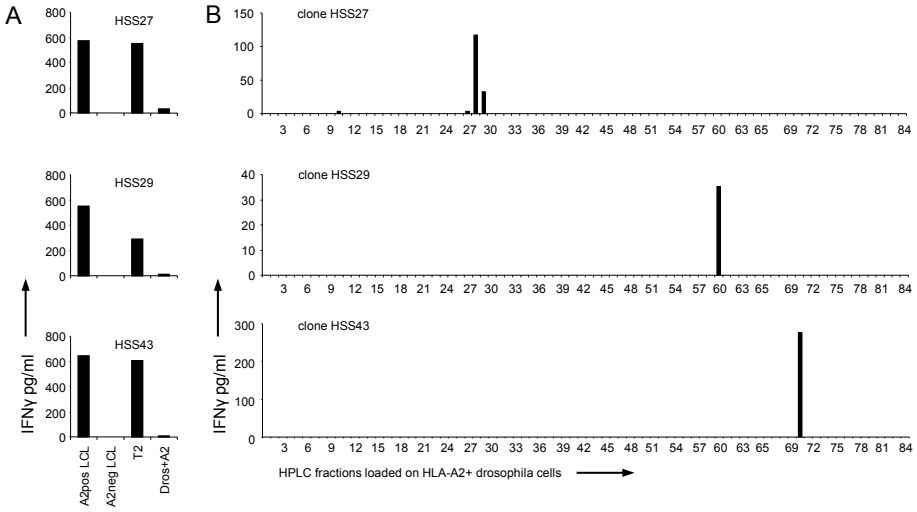


Figure 2. Investigation of the peptide specificity of T2 reactive T-cell clones.
(A) T2 reactive T-cell clones HSS27, HSS29 and HSS43 were stimulated with HLA-A2 positive EBV-LCLs (A2pos LCL), HLA-A2 negative EBV-LCLs (A2neg LCL), T2 cells (T2) and drosophila cells expressing HLA-A2, CD80 and CD54 (Dros+A2). **(B)** Peptides were eluted from the HLA-A2 molecules of T2 cells and fractionated by RP-HPLC. The six allo-HLA reactive T-cell clones which recognized T2 cells were tested for IFNγ production against the HPLC fractionations loaded on drosophila cells expressing HLA-A2, CD80 and CD54. Three representative clones are shown.

T-cell clones activation was not only dependent on the interaction of the TCR with allo-HLA-A2 molecule but that specific peptides were required.

To investigate the degree of peptide specificity exerted by the *in-vivo* selected allo-HLA-A2 reactive T-cells, 26 T-cell clones, randomly selected from the 44 allo-HLA-A2 reactive T-cell clones not recognizing T2 cells, were tested against T2 cells loaded with HPLC fractions of HLA-A2 eluted peptides. For this purpose HLA-A2 positive EBV-LCLs were lysed and their peptide-HLA-A2 complexes were purified. Subsequently, the peptides were eluted from HLA-A2, and separated from the HLA-A2 heavy chain and light chain. The peptide mixture was then subjected to RP-HPLC and fractions were collected. The T-cell clones were tested against T2 cells loaded with a small sample of each fraction, cultured for 18 h, after which IFNγ was measured by ELISA. 21 of the 26 tested T-cell clones, of which 8 are shown in figure 1B, showed recognition of one or two subsequent HPLC fractions. Almost all clones recognized a different HPLC fraction, indicative that different peptides presented in allo-HLA-A2 were recognized. One of 26 tested clones, clone HSS11, showed recognition of two not adjacent HPLC fractions (figure 4A). Four of 26 tested clones showed no reactivity against the HPLC fractions (data not shown).

To investigate the degree of peptide specificity exerted by the 6 allo-HLA reactive T-cell clones recognizing T2 cells, the clones were tested against HLA-A2 expressing drosophila cells loaded with HPLC fractions of HLA-A2 eluted peptides derived from T2 cells. All 6 clones,

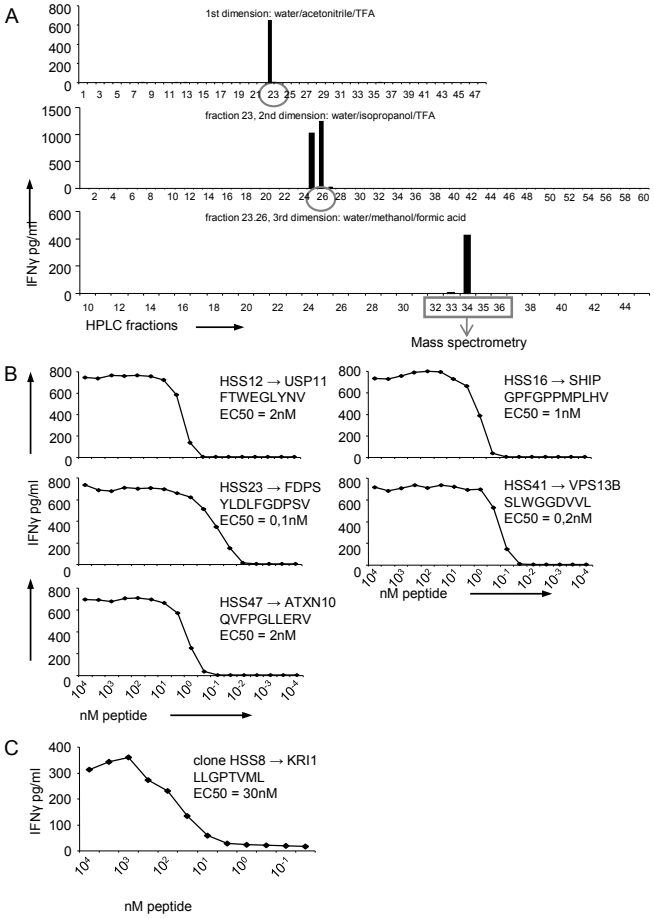


Figure 3. Identification of the peptides recognized by the allo-HLA reactive T-cell clones using multidimensional HPLC fractionation and MS.

(A) Five non-T2 recognizing allo-HLA reactive T-cell clones were stimulated for 18 h with T2 cells loaded with the first dimension HPLC fractions of peptides eluted from HLA-A2 derived from EBV-LCLs, and IFNγ was measured in the supernatant by standard ELISA. The recognized HPLC fractions were subjected to a second fractionation using a water/isopropanol/TFA gradient, loaded on T2 cells and subsequently tested for recognition by the T-cell clones. The recognized fractions were fractionated a third time, using a water/methanol/formic acid gradient and tested for recognition. After the third fractionation, the peptide masses present in the recognized and in the adjacent not recognized fractions were analyzed by MS and the sequences of the peptides were identified. The identification of the peptide recognized by clone HSS12, which is representative for the identification of the peptides recognized by the 4 other clones, is shown. **(B)** The 5 allo-HLA reactive T-cell clones, for which the recognized peptides were identified, were tested for their affinity for the respective peptides. The T-cell clones were stimulated with T2 cells loaded with titrated concentrations of the peptides for 18 h, and IFNγ was measured in the supernatant by standard ELISA. **(C)** By MS it was determined that the peptide recognized by the T2 reactive T cell clone HSS8 was derived from KRI1. To determine the affinity of the T-cell clone for this peptide, the clone was tested for IFNγ production against drosophila cells expressing HLA-A2, CD80 and CD54 loaded with titrated concentrations of the peptide. EC50 represents the peptide concentration needed for half of the maximum IFNγ production by the representative T-cell clone.

of which 3 are shown in figure 2B, showed recognition of one or two adjacent fractions, indicating that T2 reactive T-cell clones also exhibited peptide specific allo-HLA reactivity.

Identification of the peptides recognized in the context of allo-HLA-A2

To determine whether one or multiple peptides were recognized by the allo-HLA reactive T-cell clones, the peptides recognized by five randomly selected T-cell clones were identified using multidimensional RP-HPLC fractionation and MS. Identification of the peptide recognized by clone HSS12 is representative for the other 4 clones and is shown in figure 3A. All 5 clones recognized a single, but unique peptide (figure 3B and table 2).

Table 2. Identified peptides recognized by the alloreactive T-cell clones

Clone	Protein	Peptide	RefSeq ID
HSS8	KRI1	LLGPTVML	NM_023008
HSS11	HLA-DR1α	FIDKFTPPV	NM_019111
HSS11	THRAP4	FLLKLTPLL	NM_001079518
HSS12	USP11	FTWEGLYNV	NM_004651
HSS16	SHIP	GPFGPPMPLHV	NM_001017915
HSS23	FDPS	YLDLFGDPSV	NM_002004
HSS41	VPS13B	SLWGGDVVL	NM_181661
HSS47	ATXN10	QVFPGLLERV	NM_013236

For 7 T-cell clones (HSS8, 11, 12, 16, 23, 41 and 47), including one T2 reactive clone (HSS8) the recognized peptides were identified. Clone HSS11 showed recognition of peptides with sequence similarity.
The protein of which the peptides are derived from, the sequence of the peptides and the Reference Sequence identification number (http://srs.bioinformatics.nl/hspv/search.php) of the peptides are shown

To test the affinity of the 5 allo-HLA reactive T-cells for their respective recognized peptide presented in allo-HLA-A2, the allo-HLA reactive T-cell clones were tested against T2 cells loaded with titrated concentrations of the identified peptides. The concentration of peptide needed for half maximum IFNγ production (EC50) ranged between 3 nM to 100 pM for all T-cell clones.

In addition, for one of the T2 reactive T-cell clones, clone HSS8, peptide identification was performed with the HLA-A2 eluted peptides of T2 by multidimensional HPLC fractionation and MS (table 2). Peptide titration demonstrate that 30 nM of this peptide LLGPTVML derived from KRI-1 homolog, was needed for half maximum IFNγ production (EC50) by clone HSS8 (figure 3C). This illustrates that the recognition of TAP deficient T2 cells by allo-HLA reactive T-cells is also based on peptide specific allo-HLA recognition.

One of the allo-HLA reactive T-cell clones, clone HSS11, recognized two fractions after first HPLC fractionation (figure 4A). To determine whether this recognition pattern was based on recognition of two different peptides or based on recognition of different length variants of the same peptide, these two fractions were subsequently subjected to RP-HPLC for multidimensional fractionation, and peptides present in the positive fractions after three dimen-

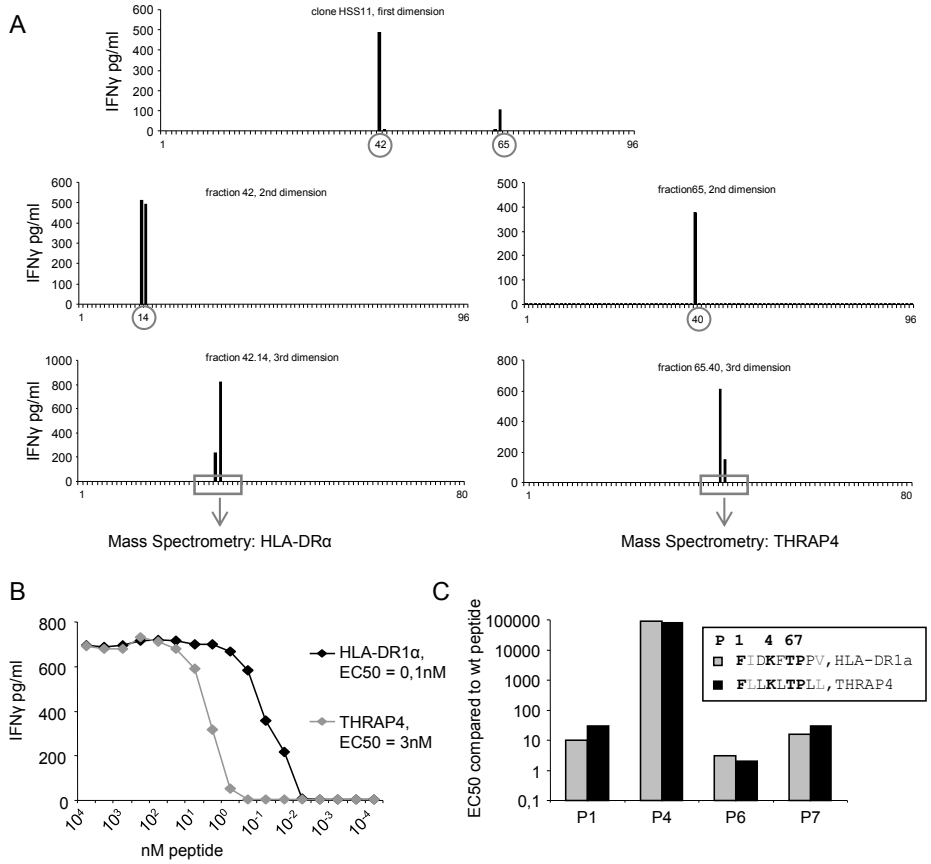


Figure 4. Identification of the peptides recognized by allo-HLA reactive T-cell clone HSS11.
(A) Clone HSS11 recognized two fractions after the first HPLC fractionation of peptides eluted from HLA-A2 derived from EBV-LCLs. These two recognized fractions were subjected to a second and a third fractionation. The peptide masses present in the fractions recognized by clone HSS11 after the third fractionation as well as in the adjacent not recognized fractions were determined by MS. Sequence analysis by tandem MS was performed on those peptide masses that correlated with the recognition pattern of the T cell clone. **(B)** To determine the affinity of clone HSS11 for the two identified peptides derived from HLA-DRA and THRAP4, the clone was stimulated with T2 cells loaded with titrated concentrations of the two peptides. EC50 represents the peptide concentration needed for half of the maximum IFNγ production by the T-cell clone. **(C)** To determine whether the 4 amino acids identical between the two peptides recognized by clone HSS11 at position P1, P4, P6 and P7, were involved in the TCR interaction, these amino acids were substituted by alanine residues. EC50 of the wt peptides and the modified peptides was determined by stimulating clone HSS11 with T2 cells loaded with titrated concentrations of the different peptides. The EC50 of the modified peptides was divided by the EC50 of the corresponding wt peptide.

sions were identified by MS (figure 4A, table 2). Testing the T-cell clone against the identified peptides loaded on T2 cells showed that clone HSS11 recognized epitope FIDKFTPPV derived from HLA-DRA, and epitope FLLKLTPLL derived from THRAP4. Peptide titration, shown in figure 4B, demonstrated that 100 pM of HLA-DRA peptide or 3 nM of THRAP4 peptides was needed for half maximum IFNγ production (EC50) of T-cell clone HSS11, illustrating that T-cell clone HSS11 was specific for two peptides.

The two recognized peptides share sequence homology. The amino acids at position 2 and 9 are different between the two peptides, however these amino acids form the anchor residues, which provide binding to HLA but do not interact with the TCR. Exchange of the amino acids at position 2 and 9 between the two peptides demonstrated no change in EC50 for both peptides (data not shown), indicating that the two anchor residues were similarly involved in HLA binding and did not influence the TCR interaction of the other amino acids. Of the 7 remaining amino acids 4 were identical. To investigate whether these identical amino acids at position 1, 4, 6 and 7 were involved in TCR interaction, these amino acids were substituted by an alanine residue. As illustrated in figure 4C all 4 amino acids were involved in TCR interaction, since alanine substitution at all positions increased EC50, demonstrating a lower binding affinity between the TCR and peptide/HLA-A2 complex. In addition, an identical change in EC50 for both peptides at all 4 positions was observed. Substitution at position 1 and 7 resulted in an intermediate 10-30 fold increase in EC50, substitution at position 4 resulted in a dramatic increase in EC50, and substitution at position 6 resulted only in a small change in EC50 of 3 fold. Together, these data suggest that the two peptide/HLA-A2 complexes recognized by clone HSS11 are conformational look-alikes. These results illustrate that allo-HLA reactive T-cells, including T2 reactive T-cells, derived from an *in vivo* GVH response recognize one or a restricted number of peptides with high affinity in the context of allo-HLA.

Confirmation of single peptide specificity using inhibition of gene expression by silencing shRNA

The allo-HLA-A2 reactive T-cell clones recognized EBV-LCLs as well as other HLA-A2 positive target cells including tumor cells and fibroblasts, as shown for one representative clone, clone HSS12 (figure 5A). To confirm the single peptide specificity of the allo-HLA reactive T-cells and biological relevance of the identified specificities, and to demonstrate that the reactivity directed against the other HLA-A2 positive target cells was also mediated by recognition of the same HLA-A2/peptide-complex, we transduced fibroblasts with silencing shRNAs specific for the respective recognized antigens of the characterized T-cell clones. By quantitative real-time PCR (qRT-PCR) we confirmed that all shRNAs downregulated the mRNA expression, varying between 5 to 31 fold (see legend figure 5B and 5C). The results in figure 5B demonstrate that silencing with the specific shRNA almost completely blocked the recognition of the corresponding allo-HLA-A2 reactive T-cell clones whereas the reactivity of other allo-HLA-A2 reactive T-cell clones was unaltered. In figure 5C we demonstrate

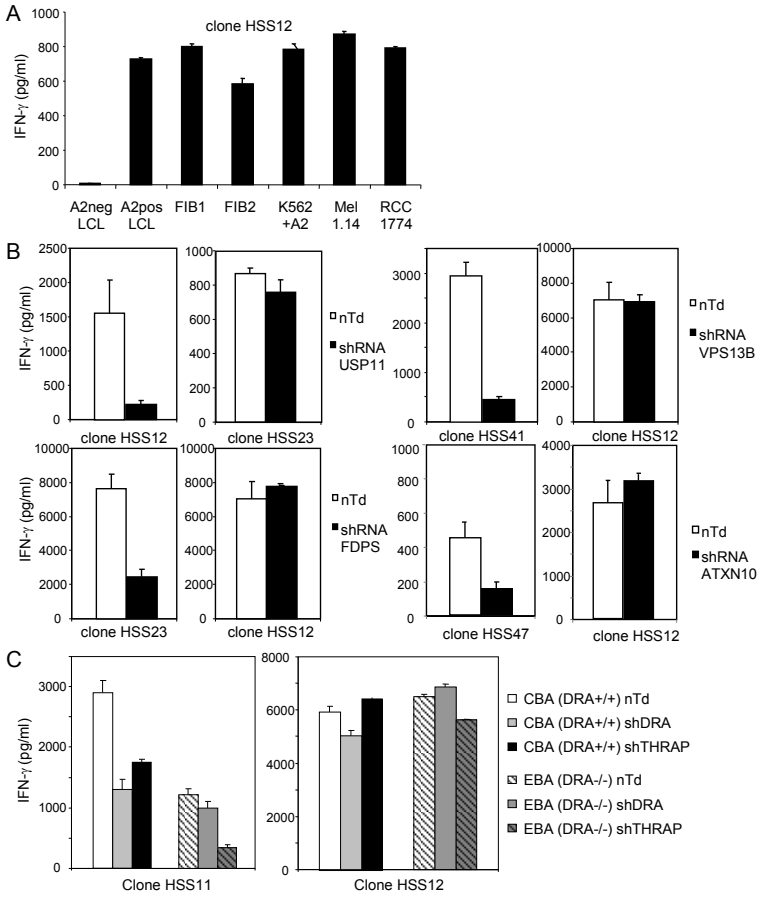


Figure 5. Recognition of different HLA-A2 positive target cells by allo-HLA reactive T-cell clones and confirmation of their single peptide specificity by transduction of shRNA specific for their respective recognized antigens.

(A) The allo-HLA reactive T-cell clones were stimulated with HLA-A2 negative EBV-LCLs (A2neg LCL), HLA-A2 positive EBV-LCLs (A2pos LCL), HLA-A2+ fibroblasts derived from two different individuals (FIB1 and FIB2), K562 cells transduced with HLA-A2 (K562+A2), HLA-A2 positive melanoma cell line 1.14 (Mel 1.14) and HLA-A2 positive renal cell carcinoma cell line 1774 (RCC 1774). Reactivity of one representative clone, clone HSS12, is shown. **(B)** Allo-HLA reactive T-cell clones HSS12, HSS23, HSS41 and HSS47 were tested against fibroblasts transduced with lentiviral vectors encoding shRNAs specific for USP11, FDPS, VPS13B or ATXN10 in combination with the puromycin resistance gene. shRNA transduced cells were cultured for 14 days with puromycin (4 ug/ml) and used as stimulator cells for the corresponding allo-reactive T cell clone and a control allo-reactive T cell clone. Non-transduced cells (nTd) were used as control stimulator cells. By qRT-PCR the downregulation of mRNA of the different genes compared to non-transduced cells was analyzed. The fold decrease for USP11= 5, FDPS=31, VPS13B= 5, ATXN10=10. **(C)** HSS11 and HSS12 were tested against HLA-DRA+/+ (CBA) and HLA-DRA-/- (EBA) fibroblasts transduced with lentiviral vectors encoding shRNAs specific for HLA-DRA or THRAP4 and subsequently selected for 14 days with puromycin and cultured for 72h with 100 IU IFNγ/ml. Downregulation of mRNA of HLA-DRA and THRAP4 was analyzed by qRT-PCR, the decrease for THRAP4 in CBA and EBA was 10 fold, the decrease for HLA-DRA in CBA was 4 fold. No expression of HLA-DRA was measured in EBA.

that the double peptide specificity of HSS11 directed against HLA-DRA and THRAP4 could be confirmed by transduction of fibroblast CBA with shRNA for either HLA-DRA or THRAP4. Both shRNAs were able to downregulate the recognition of clone HSS11, whereas the reactivity of control T-cell clone HSS12 was unaltered. To confirm that the reactivity of HSS11 was mediated by recognition of both HLA-DRA and THRAP4, HLA-DRA-/- fibroblasts (EBA) were transduced with shRNA for HLA-DRA or THRAP4. The reactivity of HSS11 directed against HLA-DRA-/- cells was reduced compared to HLA-DRA+/+ cells. Transduction with shRNA for THRAP4 almost completely blocked reactivity, and as expected, transduction with shRNA for HLA-DRA did not alter reactivity. These results clearly demonstrate that all allo-reactive T-cell clones recognized unique allo-HLA/peptide complexes.

Determination of the relative frequency of the identified allo-HLA/peptide specificities

To determine the frequency of the different allo-HLA-A2 specificities at the time of acute GVHD, the activated CD8+ donor T-cells were isolated, expanded and used for frequency analyses. Frequency analyses were performed by staining the pool of donor T-cells with tetramers composed of HLA-A2 in complex with the different newly identified allo-epitopes. As shown in figure 6 all allo-HLA reactive T-cell specificities could be observed, and the frequency of these different specificities varied between 0.07 and 1.16% of activated donor CD8+ T-cells.

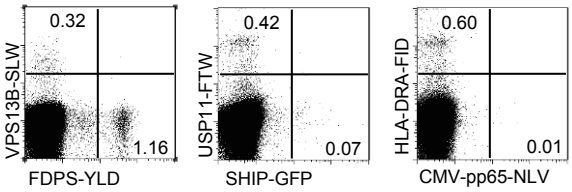


Figure 6. Variable frequencies of the different identified allo-HLA/peptide reactivities.
Activated CD8+ donor T cells were sorted from PBMCs at the time of GVHD, expanded non-specifically and stained with the different functional tetramers conjugated with either PE or APC, in combination with anti-CD8 Alexa700. As a control the CMV-pp65-NLV tetramer was included. In the dot-plots the CD8+ T cells are shown, and numbers in the different quadrants represent the percentage tetramer positive CD8+ T cells.

DISCUSSION

In this study we demonstrate that alloreactive T-cells isolated from a patient with GVHD after an HLA-A2 mismatched transplantation exerted peptide specific allo-HLA reactivity. The alloreactive T-cell clones derived from the *in vivo* allo-immune response, including those recognizing TAP deficient T2 cells, were reactive against one or two subsequent HPLC fractions

of peptides eluted from HLA-A2. Identification of the different peptides recognized in the context of the allo-HLA molecule followed by confirmation of the single peptide specificity by downregulation of the respective antigens using silencing shRNAs, demonstrated that the alloreactive T-cells exert high avidity recognition for a single endogenously processed and presented peptide.

Similar to other studies we observed that part of the allo-HLA reactive T-cells recognized TAP deficient T2 cells. Rammensee et al.[12] eluted and identified multiple peptides presented in the context of the HLA molecules expressed at the cell surfaces of TAP deficient T2 cells. Most of these peptides could also be found on TAP-expressing cells, indicating that many peptides can enter the ER and be presented in HLA molecules at the cell surface independent of TAP. It is therefore not surprising that part of the alloreactive T-cells recognized TAP independent peptides and were reactive against TAP deficient cells. Our results illustrate that high avidity reactivity against T2 cells is not based on peptide degenerate allorecognition, but on peptide specific allorecognition.

One of 26 T-cell clones tested against the HPLC fractions, clone HSS11, showed recognition of two different fractions, and peptide identification demonstrated that HSS11 recognized two different peptides. The two peptides share relevant sequence homology since 4 of 7 amino acids involved in interaction with the TCR are identical. Alanine substitutions of these 4 amino acids suggested that the two HLA-A2/peptide complexes are conformational look-alikes, most likely exhibiting a similar three dimensional structure. This degree of peptide cross-reactivity has also been described for T-cells specific for foreign peptides presented in self-HLA molecules.[28;29]

In most studies reporting polyspecific allorecognition, alloreactive T-cells were tested against a limited number of defined peptides loaded on allo-HLA expressing target cells.[2;8;13;14] Many of the alloreactive T-cells tested in this manner showed reactivity against several different peptides. However, since recognition of different peptides was not confirmed by testing the T-cells against endogenously processed and presented peptides, it is possible that the demonstrated T-cell reactivity directed against synthetic peptides loaded target cells represented low avidity T-cell reactivity. It was previously demonstrated that low avidity T-cell recognition does not lead to effective T-cell reactivity *in vivo*.[30] In our study, peptides recognized by the alloreactive T-cells were identified using multidimensional HPLC fractionations of peptides eluted from HLA-A2 of EBV-LCLs. In this method large numbers of peptides (10,000-20,000)[31] are tested at relatively low concentrations, especially after second and third fractionation. Therefore, by testing T-cells against HPLC fractions, only peptides recognized with high avidity were identified and low avidity T-cell recognition of other peptides was not determined. Downregulation of the identified antigens using silencing shRNAs almost completely blocked the reactivity of the corresponding allo-HLA reactive T-cells, demonstrating that the identified peptides, represented the actual biological relevant specificities of investigated alloreactive T-cells. During *in vivo* immune responses directed against pathogens, T-cells with high

avidity against a single antigenic peptide presented in the context of self-HLA molecules are selectively expanded.[16;17] Similarly, our results demonstrate that T-cells which are selectively activated and expanded during *in vivo* allo-HLA directed immune responses, apparently also exhibit high avidity recognition against single peptides presented in allo-HLA molecules.

Our results illustrate that high avidity allo-HLA recognition is as peptide specific as conventional T-cell recognition of foreign peptides presented in self-HLA molecules. These findings appear to be in conflict with the hypothesis that allorecognition is less peptide specific than conventional T-cell reactivity since allo-MHC molecules are not encountered during thymic development and therefore T-cells crossreactive against different peptides presented in allo-MHC molecules are not depleted.[2;3] This hypothesis is based on the assumption that the prethymic T-cell repertoire contains large numbers of MHC and peptide crossreactive T-cells and that thymic selection is responsible for removal of these T-cells. This assumption was already disputed by Zerrahn et al.,[32] who demonstrated that prethymic T-cells are as MHC crossreactive as T-cells after normal thymic selection, indicating that there may not be a large prethymic pool of highly MHC and peptide crossreactive T-cells which is removed during thymic selection. Our results demonstrating the peptide specific allo-HLA-A2 reactivity of 50 different *in vivo* activated and expanded alloreactive T-cells did also not provide evidence for the existence of T-cells crossreactive against multiple endogenously processed and presented peptides in the context of allo-HLA molecules not encountered during thymic development. We therefore speculate that highly crossreactive pre-thymic T-cells are either rare or non existent.

In summary, the results in this study demonstrate that T-cells exerting biological relevant alloreactivity *in vivo* exhibit high avidity recognition against single peptide-allo-HLA complexes. Based on the fact that downregulation of the identified antigens almost completely blocked the reactivity of the corresponding T-cells, we conclude that only these high avidity interactions, as defined by reactivity against endogenously processed and presented antigen, are biologically relevant *in vivo*. We hypothesize that during *in vivo* allo-HLA directed immune responses, only T-cells exhibiting high avidity recognition against single peptides presented in the context of allo-HLA molecules are selectively expanded.

ACKNOWLEDGEMENTS

We thank Guido de Roo and Menno van der Hoorn for expert technical assistance. We thank Martijn Rabelink and Prof. dr. Rob Hoeben for providing us the lentiviral constructs encoding the different shRNAs. Drosophila cells expressing HLA-A2, CD54 and CD80 were kindly provided by Prof. dr. Hans Stauss, and dr. Peter van den Elsen provided us with the HLA-DRA-/- fibroblasts derived from BLS patients.

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5

PRAME-SPECIFIC ALLO-HLA-RESTRICTED T-CELLS WITH POTENT ANTITUMOR REACTIVITY USEFUL FOR THERAPEUTIC T-CELL RECEPTOR GENE TRANSFER

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Clin Cancer Res. 2011 Sep 1;17(17):5615-25



ABSTRACT

Purpose: In HLA matched stem cell transplantation (SCT) it has been demonstrated that beneficial immune response mediating graft versus tumor (GVT) responses can be separated from graft versus host disease (GVHD) mediating immune responses. In this study we investigated whether it would be possible to dissect the beneficial immune response of allo-HLA reactive T-cells with potent anti-tumor reactivity from GVHD inducing T-cells present in the detrimental immune response after HLA mismatched SCT.
Experimental Design: The presence of specific tumor reactive T-cells in the allo-HLA repertoire was analyzed at the time of severe GVHD after HLA-mismatched SCT using tetramers composed of different tumor associated antigens (TAA).
Results: High avidity allo-HLA restricted T-cells specific for the TAA preferentially expressed antigen on melanomas (PRAME) were identified that exerted highly single peptide specific reactivity. The T-cells recognized multiple different tumor cell-lines and leukemic cells, whereas no reactivity against a large panel of non-malignant cells was observed. These T-cells however also exerted low reactivity against mature dendritic cells (DCs) and kidney epithelial cells, which was demonstrated to be due to low PRAME expression.
Conclusions: Based on potentially beneficial specificity and high reactivity, the T-cell receptors of these PRAME specific T-cells may be effective tools for adoptive T-cell therapy. Clinical studies have to determine the significance of the reactivity observed against mature DCs and kidney epithelial cells.

TRANSLATIONAL RELEVANCE

Adoptive cell therapy using T-cells expressing transgenic T-cell receptors (TCRs) with antitumor reactivity is a promising therapy for cancer patients. However, identification of high affinity TCRs specific for tumor associated antigens (TAAs) is a critical bottleneck in this strategy since high avidity TAA specific T-cells are deleted by negative selection in the thymus. Since allo-HLA reactivity of T-cells is not subjected to negative selection we investigated whether beneficial high avidity TAA specific T-cells could be identified within an allo-HLA directed immune response. Our search for TAA specific T-cells within a patient experiencing GVHD led to the discovery of two preferentially expressed antigen on melanomas (PRAME) specific T-cell clones. Based on their single peptide specificity and high antitumor reactivity, the TCRs of these PRAME specific T-cells may be effective tools for adoptive T-cell therapy.

INTRODUCTION

Alloreactive T-cells can mediate detrimental graft versus host disease (GVHD) as well as beneficial graft versus tumor (GVT) response after allogeneic stem cell transplantation (allo-SCT). The alloreactivity of T-cells after HLA matched allo-SCT is mainly directed against peptides derived from polymorphic proteins that differ between patient and donor, and are processed and presented in the context of HLA class I and II molecules. Previously, we and others have demonstrated that GVT reactivity can be separated from the GVH reactivity within polyclonal alloreactive immune responses mediating GVHD[1]. T-cells that are reactive against polymorphic peptides derived from proteins expressed in multiple tissues induce GVHD, whereas T-cells directed against polymorphic peptides derived from proteins exclusively expressed in hematopoietic cells steer the allo-immune response towards GVT[2] response. Analysis of the clinical responses in patients after allo-SCT and of the T-cell receptor (TCR) repertoires within these alloreactive immune responses demonstrated that beneficial GVT response occurs more frequently when the severity of the GVHD response increases, due to the activation of polyclonal responses[3]. This phenomenon was illustrated by the work of Goulmy *et al.* in which was demonstrated that a mismatch for the polymorphic peptide HA-1 was associated with a high risk of severe GVHD, initially suggesting that HA-1 specific T-cells may induce GVHD responses[4]. However, in later studies it was demonstrated that HA-1 specific immune responses also dominated in patients that experienced beneficial GVT responses[5], and that the expression profile of HA-1 was in accordance with a relative specific anti-leukemia reactivity of the T-cells, since HA-1 is exclusively expressed in cells of the hematopoietic lineage[6]. These results imply that despite an overall detrimental clinical phenotype, beneficial GVT reactivies can appear in GVHD, and that these beneficial immune reactivities can be isolated from GVHD responses.

Treatment of patients with HLA-mismatched allo-SCT is associated with GVHD. Also in this transplantation setting beneficial GVT alloreactivity is associated with GVHD, and, depending on the HLA mismatch, GVT is clinically observed[7]. The alloimmune responses after HLA-mismatched allo-SCT will mainly be directed against peptides derived from monomorphic proteins presented in the context of allo-HLA molecules. T-cells present in the polyclonal alloreactive immune response and directed against allo-HLA molecules presenting peptides derived from proteins that are ubiquitously expressed may induce GVHD. In contrast, T-cells directed against allo-HLA molecules presenting peptides derived from monomorphic proteins exclusively expressed in tumor cells, and therefore capable of inducing selective GVT reactivity may also be part of this immune response. This may imply that despite occurrence of a clinical detrimental immunological war after HLA mismatched SCT, characterization of the fine specificity of allo-reactive T-cells may result in the isolation of allo-HLA reactive T-cells that mediate beneficial GVT responses.

As allo-HLA molecules are not expressed within the thymic environment during lymphopoiesis, T-cells are able to recognize peptides with high affinity in the context of the allo-HLA, since allo-HLA reactivity of T-cells is not subjected to negative selection. Therefore, in contrast to self-restricted T-cells, allo-HLA reactive T-cells with anti-tumor reactivity may exert high avidity for the allo-HLA presenting tumor-associated antigens (TAA) derived from over-expressed or tissue specific self-proteins. Many research groups have tried to isolate or generate high affinity TAA specific TCRs. Stauss and colleagues[8], as well as other research groups have confirmed that high affinity T-cells specific for TAA can be derived from the allo-HLA repertoire[8-10]. However, these studies demonstrated that stringent selections have to be performed to identify high affinity single peptide specific T-cells from these *in-vitro* stimulations. In addition, selection from the murine repertoire can be alternative options to isolate high affinity TCRs[11;12]. Examples of over-expressed tumor antigens that may be of potential interest in human leukemia are Wilms' tumor 1 (WT-1), and proteinase 3 (Pr3). In addition, the preferentially expressed antigen on melanomas (PRAME) is of potential interest since it is highly expressed in many different cancers, including acute and chronic myeloid and lymphoid leukemia's[13], whereas normal tissues have low PRAME expression[14]. High affinity TCRs specific for TAA that are shared between various tumors would be attractive tools for TCR gene therapeutic strategies[15;16].

In this study we hypothesized that within the detrimental immunological war that is induced after HLA-mismatched SCT by donor T-cells reacting against patient allo-HLA, donor T-cells that mediate beneficial GVT responses may also selectively be activated and expanded. For this purpose we investigated whether high avidity T-cells directed against allo-HLA presenting TAAs could be observed in a patient experiencing severe GVHD after HLA-A2 mismatched SCT, using HLA-A2 tetramers composed of different TAA peptides. We observed polyclonal allo-HLA restricted T-cells specific for PRAME. These T-cells were demonstrated to exert single peptide specificity and recognition was strictly correlated with PRAME expression. The isolated allo-HLA restricted T-cell clones were in contrast to self-restricted T-cell clones highly reactive against multiple different PRAME positive tumor cell-lines as well as freshly isolated metastatic melanoma and primary leukemic cells, whereas no reactivity against a large panel of non-malignant cells was observed. However, the clones exerted limited on-target reactivity against mature dendritic cells (mDCs) and kidney epithelial cells. Based on their potential beneficial specificity and high reactivity against numerous different tumors, the high affinity TCRs from the allo-restricted PRAME specific T-cells may be effective tools for broad application of TCR gene therapy.

MATERIALS AND METHODS

Cell collection and preparation

Stable Epstein–Barr virus (EBV)-transformed B cell-lines (EBV-LCLs) were generated using standard procedures, and cultured in IMDM and 10% FBS. K562, COS, T2, renal cell carcinoma cell-lines (RCC 1257, RCC 1774, RCC 1851), lung carcinoma cell-lines (A549, NCI-H292), melanoma cell-lines (518A2, FM3, FM6, SK2.3, MI-3046, BML, 1.14), cervix carcinoma cell-lines (SIHA, HELA, CASKI), breast carcinoma cell-lines (MCF7, BT549, MDA231) and colon carcinoma cell-lines (SW480, HCT116, LS411, LS180) were cultured in IMDM and 10% FBS. K562, COS, H292, A549, SIHA and HELA not expressing HLA-A2 were transduced with a retroviral vector encoding for HLA-A2 as previously described[17]. In addition, melanoma cells were freshly isolated from an HLA-A2 positive patient with lymph node metastatic melanoma by ficol isolation of minced tumor cells and subsequent FACS sort of the CD45, CD3, CD19, CD14, CD56 negative cells. For selected experiments COS-A2 cells were transfected with pcDNA3.1 expression vector encoding for wild-type human PRAME. Peripheral blood of HLA-A2 positive patients with primary AML cells (>80% blasts) were cultured for 1 day in IMDM and 10% FCS and used as stimulator cells. Primary AML cells were activated for 1 day with GM-CSF (100 ng /ml; Novartis), TNFα (10 ng/ml; R&D Systems), IL-1b (10 ng/ml; Immunex), IL-6 (10 ng/ml; Cellgenix), PGE-2 (1 µg/ml; Sigma-Aldrich), and IFNγ (500 IU/ml; Immukine, Boehringer Ingelheim). HLA-A2 positive ALL cell-lines were generated as previously described[18]. B cells were isolated from PBMCs by MACS using anti-CD19 coated magnetic beads (Miltenyi Biotec). ALL cell-lines and freshly isolated B cells were activated by culturing the cells for 48 h at a concentration of 10^6 cells/ml in 24-well plates in the presence of IL-4 (500 U/ml; Schering-Plough), CpG oligodeoxynucleotide (10 µg/ml; Eurogentec) and $1x10^5$/ml murine fibroblasts transfected with the human CD40 ligand[19]. In-vivo activated B cells were derived from inflamed tonsils. T-cell blasts were generated by stimulation of PBMCs with PHA and IL-2 (120 IU/ml) for 7 days. Monocytes were isolated from PBMCs by MACS using anti-CD14 coated magnetic beads (Miltenyi Biotec). Macrophages (MØ) were generated by culturing CD14+ cells for 6 days in IMDM and 10% HS at a concentration of $0.5x10^6$cells/ml in 24-well plates. Pro-inflammatory macrophages (MØ1) were obtained by culture in the presence of GM-CSF (5 ng/ml) and anti-inflammatory macrophages (MØ2) cells were cultured with M-CSF (5 ng/ml, Cetus Corporation). Monocyte derived DCs were generated by culturing CD14+ cells for 48 h in IMDM and 10% HS at a concentration of $0.5x10^6$ cells/ml in 24-well plates in the presence of IL-4 (500 U/ml) and GM-CSF(100 ng/ml). For maturation of the CD14 DCs, cells were cultured for another 48 h in IMDM and 10% HS supplemented with GM-CSF (100 ng / ml), TNFα (10 ng/ml), IL-1b (10 ng/ml), IL-6 (10 ng/ml), PGE-2 (1 µg/ml), and IFNγ (500 IU/ml). CD34+ cells were isolated from peripheral blood stem cell grafts by MACS using anti-CD34 coated magnetic beads (Miltenyi Biotec). CD34 DCs were generated by culturing CD34 cells for 4 days in IMDM and 10% HS at a concentration of $0.25x10^6$ cells/ml in 24-well plates in

the presence of GM-CSF (100 ng/ml), SCF (20 ng/ml; kindly provided by Amgen), and TNFα (2 ng/ml), and subsequently for 3 days with additionally IL-4 (500 IU/ml). To maturate the CD34 DCs, the cells were cultured for another 48 h in IMDM and 10% HS supplemented with GM-CSF (100 ng /ml), SCF (20 ng/ml), TNFα (10 ng/ml), IL-1b (10 ng/ml), IL-6 (10 ng/ml), PGE-2 (1 ug/ml), and IFNγ (500 IU/ml). For the isolation of blood derived myeloid DCs (MDCs) and plasmacytoid DCs (PDCs), PBMCs were stained with anti-BDCA1-PE (Biolegend) or anti-BDCA2-PE mAbs (Miltenyi Biotec), respectively, and the BDCA1-PE or BDCA2-PE positive cells were isolated by MACS using anti-PE coated magnetic beads. The MACS isolated cells were stained with FITC conjugated anti-CD3, anti-CD14, anti-CD19 and anti-CD56 mAbs (BD) and the MDCs and PDCs were selected by cell sorting on bases of BDCA1 or BDCA2 positivity and the absence of lineage marker expression. To maturate the MDCs and PDCs, cells were cultured for 24 h in IMDM and 10% HS supplemented with either poly-IC (Amersham) or CpG (10 µg/ml) and IL-3 (50 ng/ml; kindly provided by Novartis), respectively. Fibroblasts were cultured from skin biopsies in Dulbecco's modified Eagle medium (DMEM; Lonza) with 1g/l glucose (BioWhittaker) and 10% FBS. Keratinocytes were cultured from skin biopsies in keratinocyte serum free medium supplemented with 30 µg/ml bovine pituitary extract and 2 ng/ml epithelial growth factor (EGF) (all components were purchased from Invitrogen). Fibroblasts and keratinocytes were cultured for 3 days in the presence or absence of IFNγ (200 IU/ml). Primary bronchial epithelial cells (PBEC) were derived and cultured as previously described[4]. Mesenchymal stromal cells (MSCs) were derived from bone marrow of healthy donors as previously described[5] and cultured in DMEM and 10% FBS. Colon epithelial cells were cultured in DMEM F12 (Lonza) and 10% FCS, and supplemented with EGF (10 ng/ml; Promega), T3 hormone (2nmol/l; Sigma), hydrocortisone (0,4ug/ml; Pharmacy LUMC), and insulin (5 ng/ml; Sigma). Hepatocytes and intrahepatic biliary epithelial cells (IHBEC) (both purchased from ScienCell) were cultured in RPMI (Lonza) and 10% FBS. Proximal tubular epithelial cells (PTEC) were isolated and cultured as previously described[20].

Isolation and analysis of PRAME specific T-cells

All studies were conducted with approval of the institutional review board at MACA. After informed consent, peripheral blood mononuclear cells (PBMCs) were collected from a patient suffering from AML that experienced acute GVHD after single HLA-A2 mismatched SCT and subsequent DLI. Based on a cross-over, the patient was HLA-A*0201 positive and the sibling donor was HLA-A*0201 negative, whereas all other HLA class I and II molecules were completely matched. Patient PBMCs collected during GVHD were stained with anti-HLA-A2-FITC (Pharmingen), anti-HLA-DR-APC (Pharmingen) and anti-CD8-PE (BD) for 30 min at 4°C, and activated (HLA-DRpos), donor derived (HLA-A2neg) CD8+ T-cells were isolated by cell sorting (FACSAria). Since PBMCs were limited, the sorted T-cells were first expanded with anti-CD3/CD28 and irradiated autologous PBMCs (0.5x106/ml) in T-cell medium. T-cell medium consist of Iscove's Modified Dulbecco's Medium (IMDM; Lonza) with 10% human serum (HS), IL-2 (120

IU/ml; Proleukin) and IL-15 (20 ng/ml; Peprotech). T-cells were stimulated non-specifically using irradiated allogeneic PBMCs ($0.5x10^6$ /ml), IL-2 (120 IU/ml), and phytohemagglutinin (PHA, 0.8 µg/ml; Murex Biotec Limited). After 14 days of culture, T-cells were labeled with anti-CD8-APC (BD Bioscience) and PE-conjugated HLA-A2 tetramers specific for the different TAA peptides[21-24]: for PRAME were tested: VLDGLDVLL (VLD), SLYSFPEPEA (SLY), ALYVDSLFFL (ALY), and SLLQHLIGL (SLL), for WT-1: RMFPNAPYL, for Pr-1: VLQELNVTV. For single cell sorting, T-cells were stained with APC conjugated tetramers in combination with TCR-Vβ repertoire kit staining (Beckman Coulter) for 1 h at 4°C, and SLL tetramer+ Vβ1+ and SLL tetramer+ Vβ3+ CD8+ T-cells were sorted and stimulated non-specifically.

Self-restricted PRAME specific T-cell clones were isolated from an HLA-A*0201 patient that was transplanted with a fully HLA-identical donor graft. PBMCs derived from the patient after SCT were labeled with PE-conjugated SLL tetramer for 1 h at 4 °C. Tetramer positive T-cells were isolated by MACS using anti-PE coated magnetic beads (Miltenyi Biotec) and were expanded for 10 days with anti-CD3/CD28 beads as described above. For subsequent sorting, T-cells were stained with PE-conjugated SLL tetramer and anti-CD8-APC for 1 h at 4°C, and tetramer positive CD8+ T-cells were sorted single cell per well and expanded. Three SLL tetramer positive T-cell clones were selected, and used for further analysis.

Functional reactivity of the PRAME specific T-cell clones

Stimulation assays were performed with 5,000 T-cells and 20,000 targets in 96-well plates in Iscoves Dulbecco Modified Medium (IMDM), supplemented with 10% human serum (HS) and 100 IU/ml interleukin 2 (IL-2). The different malignant and non-malignant cells were collected and prepared as described in cell collection and preparation. For peptide titrations, T2 cells were preincubated for 1 h with different concentrations of peptide, and washed. After 18 h of stimulation, supernatant was harvested and IFNγ production was measured by standard ELISA. In the cytotoxicity assays, T-cells were tested at different effector-target ratios against 1,000 51Cr labeled targets in 96-well plates in a standard 4h 51Cr-release assay. In these experiments a control HLA-A2 restricted T-cell clone HSS12 specific for a peptide derived from USP11 was included.

In the CD34 cell proliferation inhibition assay, CD34 cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described, and resuspended in progenitor cell culture medium[25].

Peptide elution, reverse phase high performance liquid chromatography (RP-HPLC) and mass spectrometry (MS)

Peptide elution, RP-HPLC and MS were performed as previously described[26]. Briefly, $3x10^{10}$ Epstein Barr Virus transformed B-cells (EBV-LCLs) were lysed and the peptide-HLA-A2 complexes were purified by affinity chromatography using HLA-A2 specific BB7.2 monoclonal antibody (mAb). Subsequently, peptides were eluted from HLA-A2 molecules, and separated

from the HLA monomers and ß2-microglobulin by size filtration. After freeze drying, the peptide mixture was subjected to a first round of RP-C18-HPLC using a water/acetonitrile/TFA, and fractions were collected. A small sample of each fraction was loaded on T2 cells and tested for recognition by the T-cell clones. The recognized fraction was subjected to a second and a third round of RP-C18-HPLC fractionation. In the second fractionation a water/isopropanol/TFA gradient was used, and in the third fractionation a water/methanol/formic acid gradient was used. After the third fractionation, the peptide masses present in the recognized fractions and in the adjacent non-recognized fractions were determined by MS. Peptides of which the abundance correlated with the recognition pattern of the T-cell clone were selected for tandem mass spectrometry and their sequences determined.

PRAME expression by quantitative real-time PCR, and inhibition of PRAME expression by silencing RNA

PRAME expression was quantified by real-time PCR (TaqMan). Total RNA was isolated from cells using a RNeasy mini kit (Qiagen) or the micro RNaqueous kit (Ambion). First strand cDNA synthesis was performed with oligo dT primers using M-MLV reverse transcriptase (Invitrogen) or with the Transcriptor reverse transcriptase (Roche). Samples were run on a 7900HT RT-PCR System of Applied Biosystems. The following PRAME primers were used, sense 5' CGTTTGTGGGGTTCCATTC 3', anti-sense 5' GCTCCCTGGGCAGCAAC 3' and for the anti-sense probe 5' CCTGCCAGCTCCACAAGTCTCCGTG 3'. The Probe used VIC as dye and TAMRA as quencher, both primers were chosen over an intron/exon boundary. Each sample was run in duplo with cDNA from 50 ng total RNA. The Porphobilinogen Deaminase (PBGD) gene was measured as housekeeping gene to ensure good quality of the cDNA.
Inhibition of PRAME was performed using retroviral vectors encoding for short hairpin (sh) RNA sequences specific for RAME in combination with the puromycin resistance gene that were kindly provided by Dr. R. Bernards, NKI, Amsterdam, The Netherlands[27]. Retrovirally transduced cells were cultured with different concentrations of puromycin for at least 1 week before testing. Proximal tubular epithelial cells (PTECs) were cultured with 3 μg/ml, renal cell carcinoma cell line RCC1257 with 4 μg/ml, and CD34+ derived dendritic cells (CD34DCs) with 0.4 μg/ml puromycin. CD34DCs were generated as described in cell collection and preparation, and were transduced on day 1 of culture.

TCR gene transfer

The TCRAV and TCRBV gene usage of clone HSS1 was determined using reverse transcriptase (RT)–PCR and sequencing[17]. HSS1 expressed TCR-AV1S1 and TCR-BV1S1. A retroviral vector was constructed with a codon optimized and cysteine modified TCRα and TCRβ chain linked by the T2A sequence in combination with the truncated nerve growth factor receptor (ΔNGF-R)[28;29]. Cytomegalovirus (CMV)-IE1 specific HLA-B8 restricted T-cells were sorted using CMV-IE1 tetramers, stimulated for 2 days with PHA and irradiated allogeneic PBMCs and

transduced with PRAME-TCR or mock. Transduced T-cells were sorted based on positivity for ΔNGF-R, and tested for functional reactivity.

RESULTS

Isolation of high avidity PRAME specific allo-restricted T-cells clones from a patient experiencing GVHD after HLA-mismatched SCT

The presence of allo-HLA restricted TAA specific T-cells was analyzed in a patient transplanted with a single HLA-A2 mismatched SCT that experienced GVHD after treatment with donor lymphocyte infusion (DLI) for relapsed acute myeloid leukemia (AML). The activated donor derived CD8+ T-cells at the time of GVHD were selected by FACS sort and expanded using CD3/CD28 expansion beads to be able to perform multiple reactivity screenings. T-cells were labeled with tetramers specific for the HLA-A2 restricted epitopes of different TAAs. The T-cells did not stain with most TAA tetramers, however 0.6% of CD8+ T-cells specifically stained with the PRAME-SLL tetramer (figure 1A). TCR-Vβ usage analysis using TCR-Vβ specific mAbs illustrated that the SLL tetramer positive T-cells consisted of minimally three different clonal populations, one dominant TCR Vβ1+ population (74%), a TCR Vβ3+ population (5%), and a T-cell population with unknown TCR Vβ usage (20%) (figure 1B). To analyze the specificity and avidity of the PRAME specific T-cells, the SLL tetramer positive Vβ1+ and Vβ3+ CD8+ T-cells were single cell sorted and expanded. Of both cell subsets one T-cell clone was selected for further analysis. Both T-cell clones HSS1, expressing TCR-Vβ1, and HSS3, expressing TCR-Vβ3, efficiently stained with the SLL tetramer, but not with control CMV-pp65 tetramer (figure 1C left part). FACS analysis with Vβ specific mAbs confirmed their difference in clonal origin (data not shown).

To determine whether the PRAME specific T-cell clones effectively recognized endogenously processed and presented PRAME, clone HSS1 was tested against K562, previously demonstrated to highly express PRAME[14], and K562 transduced with HLA-A2. To demonstrate that recognition was actually directed against PRAME derived peptide presented in HLA-A2, clone HSS1 was tested against COS cells, either only expressing HLA-A2 or PRAME, or expressing both HLA-A2 and PRAME. In addition, clone HSS1 was tested against different HLA-A2+ and HLA-A2- EBV-LCLs. As shown in figure 1D, HSS1 efficiently recognized K562+A2 and COS cells expressing both PRAME and HLA-A2, whereas untreated K562 and COS cells as well as COS cells expressing only HLA-A2 or PRAME were not recognized. These results indicate that HSS1 recognized endogenously processed and presented PRAME peptide in the context of HLA-A2. Interestingly, HSS1 also recognized all five different HLA-A2+ EBV-LCLs, although with variable strength, whereas HLA-A2- EBV-LCLs were not recognized (reactivity against two representative HLA-A2+ and one HLA-A2- EBV-LCL are shown in figure 1D).

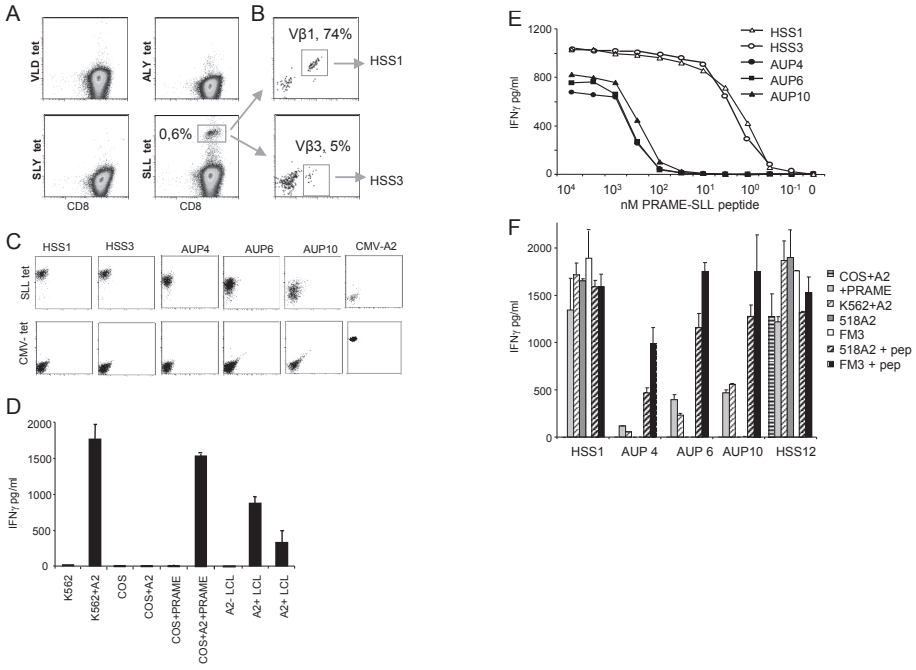


Figure 1. Isolation of allo-restricted and self-restricted PRAME specific T cell clones.
(A) Activated (HLA-DRpositive), donor derived (HLA-A2negative) CD8+ T cells were isolated from a patient experiencing GVHD after HLA-A2 mismatched SCT and subsequent DLI. The isolated T cells were expanded using CD3/CD28 beads and labeled with anti-CD8 and TAA tetramers, only the PRAME tetramers (VLD, SLY, ALY, and SLL) are shown. **(B)** To determine the TCR-Vβ usage, the T cells were labeled with anti-CD8, SLL tetramer and the Vβ repertoire kit. The Vβ1+ (74% of tet+) and Vβ3+ (5% of tet+) T cells were single cell sorted. **(C)** Allo-restricted PRAME-SLL clone HSS1, HSS3, CMV-A2 control clone, and the self-restricted PRAME-SLL clones AUP4, AUP6 and AUP10, were stained with SLL- and pp65-tetramer. **(D)** HSS1 was tested against K562, K562 transduced with HLA-A2 (K562+A2), untransduced COS cells (COS), COS transduced with HLA-A2 (COS+A2), COS+A2 transfected with PRAME (COS+PRAME), COS transduced with HLA-A2 and transfected with PRAME (COS+A2+PRAME), HLA-A2 negative EBV-LCL (A2- LCL), and 5 HLA-A2 positive EBV-LCLs of which two are shown (A2+ LCL). **(E)** HSS1, HSS3, AUP4, AUP6 and AUP10 were stimulated with T2 cells loaded with titrated concentrations of the PRAME-SLL peptide. **(F)** HSS1, AUP4, AUP6 and AUP10, and the USP11 specific T cell clone HSS12 used as a control, were tested for IFNγ production against COS+A2, COS+A2 transfected with PRAME (+PRAME), K562+A2, two HLA-A2+ melanomas (518A2 and FM3) unloaded, or loaded with PRAME peptide (+ pep).

As it has not yet been shown that EBV-LCLs express PRAME, and since these T-cell clones were allo-HLA reactive, we investigated whether clone HSS1 and HSS3, in addition to PRAME, also recognized other peptides presented in HLA-A2. For this purpose the HLA-A2+ EBV-LCLs recognized most prominently by the allo-restricted clones were expanded to 3 x 10^{10} cells, HLA-A2 was isolated by affinity chromatography, and peptides eluted from HLA-A2 were fractionated by multidimensional RP-HPLC, and loaded onto T2 cells. As shown in figure 2, HSS1 was reactive against T2 cells loaded with only one RP-HPLC fraction. This recognized

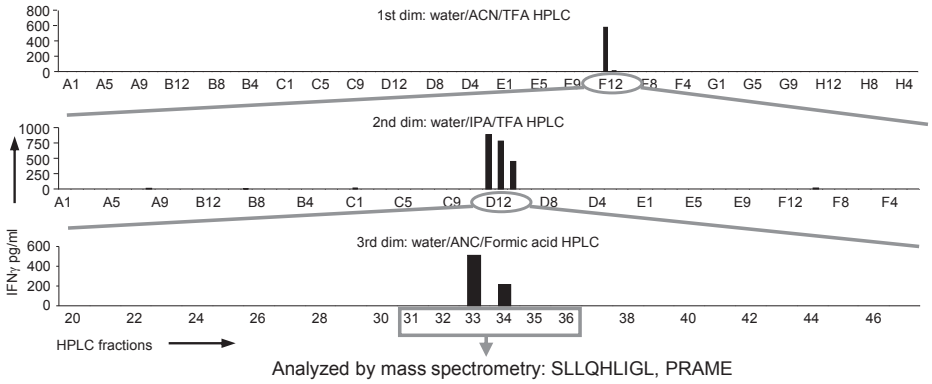


Figure 2. PRAME specific T cell clones are single peptide specific.
Peptides eluted from HLA-A2 were fractionated by RP-HPLC, loaded on T2, and used as stimulators for HSS1. The recognized fraction was fractionated a second and third time, using different organic solvents. After the third fractionation, MS analyses demonstrated that the recognized peptide was PRAME SLLQHLIGL.

fraction was subfractionated using a different HPLC gradient composition, and loaded on T2 cells. Again one fraction of the second RP-HPLC separation was efficiently recognized by HSS1, and this fraction was again further sub-fractionated. The peptide masses present in the recognized third dimension RP-HPLC fractions and in the adjacent not recognized fractions were determined by MS. Comparing the presence and abundance of these peptide masses to the recognition pattern of the T-cell clone resulted in one unique peptide candidate, which was analyzed by tandem MS to be the SLLQHLIGL peptide of PRAME, The synthetic PRAME peptide showed a fragmentation pattern identical to the fragmentation pattern of the natural eluted peptide. Similar results were obtained with clone HSS3 (data not shown). These results demonstrate that reactivity of the allo-reactive PRAME specific T-cell clones against EBV-LCLs was mediated by the recognition of PRAME presented in HLA-A2, and in addition demonstrate that the allo-reactive T-cell clones derived from GVHD exerted single peptide specific recognition.

Comparing the functional avidity of allo-HLA and self-HLA restricted PRAME specific T-cell clones

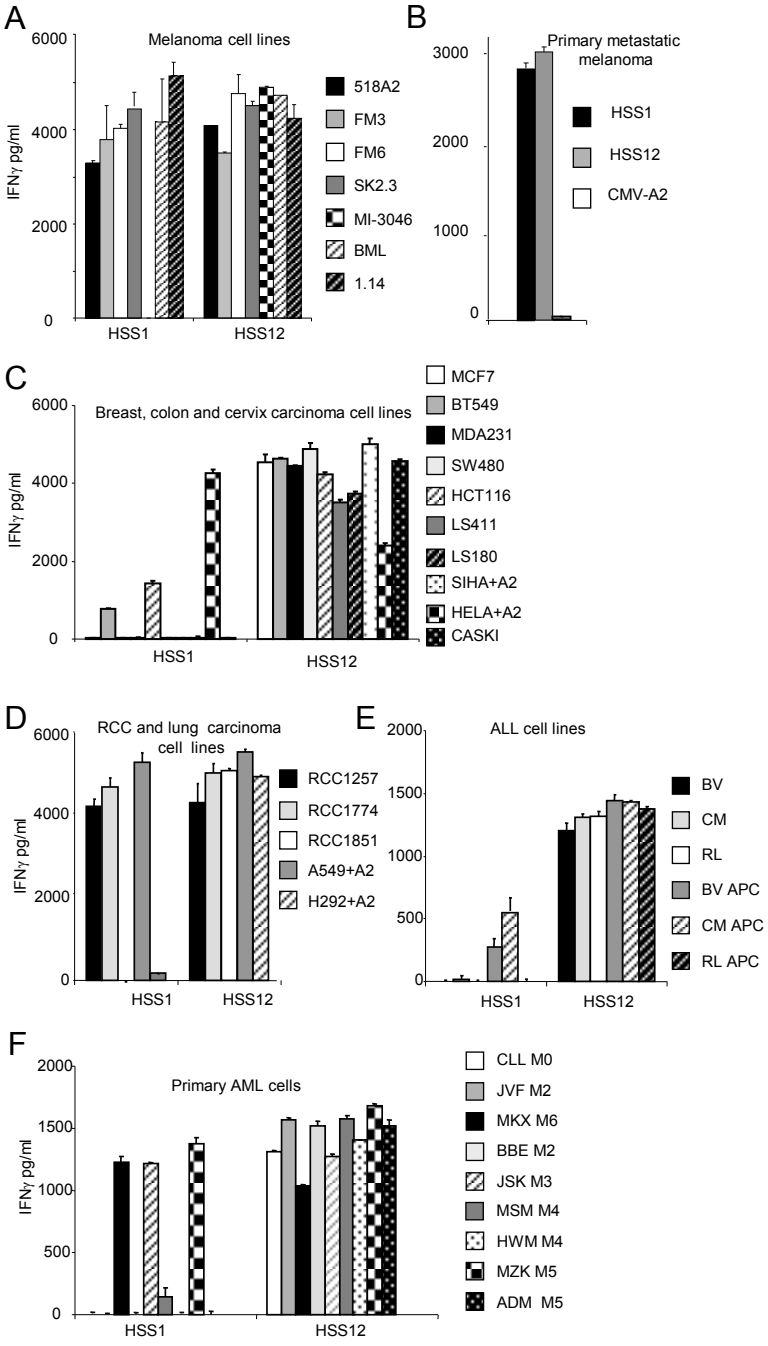
Since we speculated that the avidity of allo-restricted PRAME specific T-cells would be significantly higher compared to self-restricted PRAME specific T-cells, we isolated by flowcytometry using the SLL tetramer, three PRAME-SLL specific self-restricted T-cell clones (AUP4, AUP6 and AUP10) from an HLA-A2 positive patient after HLA-matched SCT. The self-restricted PRAME specific T-cell clones showed specific staining with the SLL tetramer, although with lower intensity compared to the allorestricted T-cell clones (figure 1C right part).

To compare the functional avidity of the self- and allo-restricted PRAME specific T-cell clones, AUP4, AUP6, AUP10, HSS1 and HSS3 were tested against T2 cells loaded with different concentrations of SLL peptide. Although all clones were able to recognize the SLL peptide, the peptide concentration needed for 50% of maximum IFN-γ production differed significantly between the self- and allo-restricted clones. As shown in figure 1E, HSS1 and HSS3 required only 1-2nM of peptide for half-maximal cytokine production, whereas AUP4, AUP6, and AUP10 needed approximately 300nM of peptide. These results demonstrate that in contrast to the allo-restricted PRAME specific T-cell clones, the self-restricted PRAME specific T-cell clones exerted low avidity recognition.

To investigate whether this large difference in avidity has consequences for reactivity of the clones against endogenously processed PRAME, the self- and allo-restricted clones were tested against COS+A2 transfected with PRAME, K562+A2, and 2 melanomas (figure 1F). As a positive control for the HLA-A2 expression and stimulatory capacity of the targets, the allo-HLA-A2 restricted control clone HSS12 specific for USP11 was also tested against all targets. Clone HSS1 efficiently recognized all PRAME expressing cell-lines. Although the self-restricted clones intermediately recognized PRAME transfected COS+A2 as well as K562+A2, the melanomas known to express approximately a 5-10 fold lower amount of PRAME compared to K562 were not recognized. The melanomas were strongly recognized by all four clones when exogenously loaded with SLL peptide. These results demonstrate that high avidity allo-restricted PRAME specific clones efficiently recognize melanoma, whereas the low avidity self-restricted PRAME clones exerted no reactivity against melanoma.

High avidity allo-HLA restricted PRAME specific T-cells effectively recognized a large proportion of tumors and a limited number of non-malignant cells

The PRAME gene is expressed at a high level in a large proportion of tumors, including melanomas, non-small-cell lung carcinomas, renal cell carcinoma (RCC), breast carcinoma, cervix carcinoma, colon carcinoma, as well as several types of leukemia[13;30-32]. We therefore analyzed whether clone HSS1 was able to recognize HLA-A2+ tumor cell-lines and primary tumor cells. Clone HSS12 was used as a control for the HLA-A2 expression of the targets and the ability of the targets to stimulate T-cells. Results shown in figure 3A demonstrate that in addition to the two previously tested melanomas, four different melanomas were also efficiently recognized by clone HSS1. In addition, from an HLA-A2 positive patient with lymph node metastatic melanoma we freshly isolated melanoma cells by ficolling the minced tumor cells and subsequently sorting the CD45, CD3, CD19, CD14, CD56 negative cells. HSS1 as well as the control HSS12 highly recognized the freshly isolated tumor cells, whereas a CMV-A2 control clone did not recognize the metastatic melanoma (figure 3B). As demonstrated in figure 3C and 3D clone HSS1 intermediately recognized 1 of 3 breast carcinomas, and 1 of 4 colon carcinomas, and efficiently recognized 1 of 3 cervix carcinomas, 2 of 3 RCCs, and 1 of 2 lung carcinomas. Three HLA-A2+ acute lymphoblastic leukemia's (ALLs) were not recognized, but two of these


A
Melanoma cell lines
IFNγ pg/ml
6000
4000
2000
0
HSS1
HSS12
518A2
FM3
FM6
SK2.3
MI-3046
BML
1.14
B
Primary metastatic melanoma
3000
2000
1000
0
HSS1
HSS12
CMV-A2
C
Breast, colon and cervix carcinoma cell lines
IFNγ pg/ml
6000
4000
2000
0
HSS1
HSS12
MCF7
BT549
MDA231
SW480
HCT116
LS411
LS180
SIHA+A2
HELA+A2
CASKI
D
RCC and lung carcinoma cell lines
IFNγ pg/ml
6000
4000
2000
0
HSS1
HSS12
RCC1257
RCC1774
RCC1851
A549+A2
H292+A2
E
ALL cell lines
2000
1500
1000
500
0
HSS1
HSS12
BV
CM
RL
BV APC
CM APC
RL APC
F
Primary AML cells
IFNγ pg/ml
2000
1500
1000
500
0
HSS1
HSS12
CLL M0
JVF M2
MKX M6
BBE M2
JSK M3
MSM M4
HWM M4
MZK M5
ADM M5

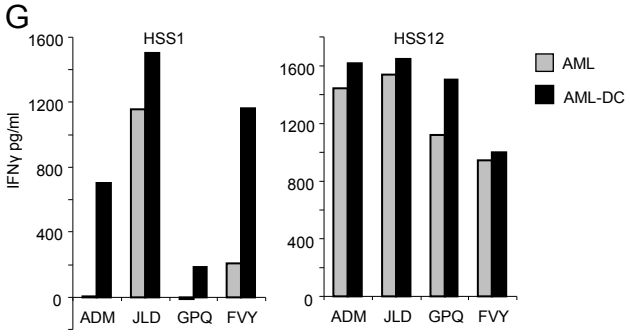
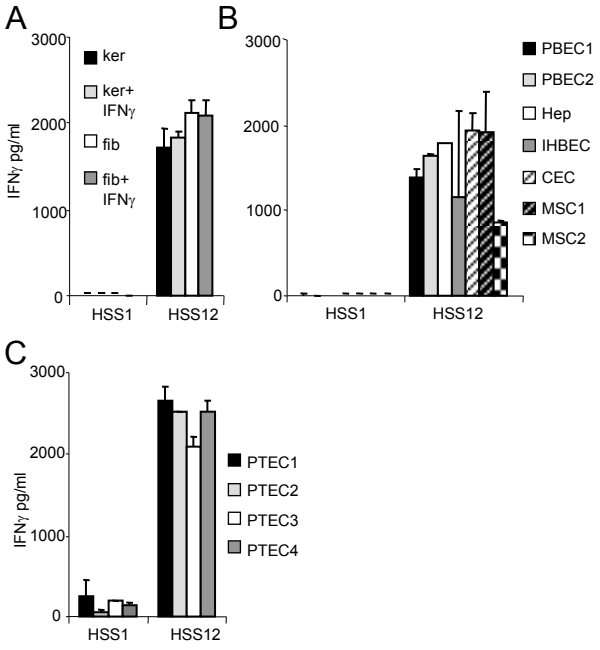


Figure 3. Allo-HLA restricted clone HSS1 specific for PRAME is able to recognize various different tumor cell-lines and primary malignancies.

HSS1 and control HSS12 were tested for IFNγ production against HLA-A2+ **(A)** melanoma cell lines: 518A2, FM3, FM6, SK2.3, MI-3046, BML, and 1.14, **(B)** primary metastasized melanoma (the CMV-A2 clone was included as negative control), **(C)** breast carcinoma cell lines: MCF-7, BT594 and MDA231, colon carcinoma cell lines: SW480, HCT116, LS411 and LS180 and cervix carcinoma cell lines: SIHA+A2, HELA+A2 and CASKI, **(D)** Renal cell carcinomas: RCC1257, RCC1774 and RCC1851, and lung carcinomas: A549+A2 and H292+A2, **(E)** 3 ALLs (BV, CM and RL) and , CD40L activated ALLs (APC), **(F)** 9 primary AMLs of different classification (as indicated), and **(G)** 4 primary AML samples not activated or activated (AML-DC) for 24h with GM-CSF, SCF, TNFα, IL-1b, IL-6, PGE-2, and IFNγ.



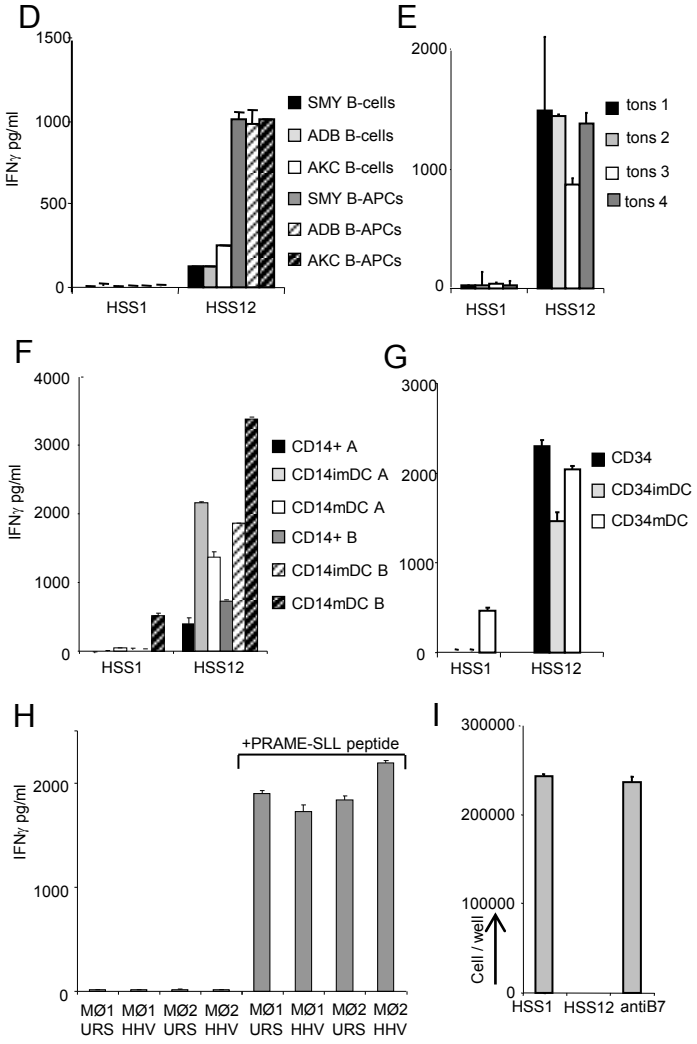


Figure 4. Recognition of non-malignant cells by the allo-HLA restricted PRAME T cells.
HSS1 and control HSS12 were tested for IFNγ production against HLA-A2+ **(A)** fibroblasts (fib) and keratinocytes (ker) untreated or treated with IFNγ (+IFNγ), **(B)** primary bronchus epithelial cells (PBEC1 and PBEC2), hepatocytes (Hep), intrahepatic biliary epithelial cells (IHBEC), colon epithelial cells (CEC) and mesenchymal stromal cells (MSC1 and MSC2), **(C)** 12 different PTECs, of which 4 are shown, **(D)** B cells and CD40L stimulated B cells (B-APC) from 3 individuals, **(E)** in vivo activated B cells derived from inflamed tonsils of 4 individuals (tons1-4), **(F)** CD14 cells, immature CD14 DCs (CD14imDCs) and mature CD14 DCs (CD14mDCs) derived from two individuals (A and B) and **(G)** CD34 cells, immature CD34 DCs (CD34imDC) and mature CD34 DCs (CD34mDC). **(H)** HSS1 was tested for IFNγ production against HLA-A2+ MØ1 and MØ2 macrophages (MØ1 and MØ2 from URS and HHV) unloaded or loaded with PRAME-SLL peptide. **(I)** HSS1, HSS12, and one HLA-B7 restricted T cell clone (negative control) were tested in a CFSE proliferation inhibition assay against HLA-A2+, HLA-B7- CD34 cells. The number of viable cells differentiated from CD34 cells was measured at 144h by FACS analysis.

3 ALLs were recognized after activation with CD40L and CpG (figure 3E). In addition, 4 of 9 primary AMLs were recognized by HSS1, of which 3 were strongly recognized (figure 3F). Also the patient AML was tested for recognition, however no recognition was seen (data not shown). Since no material of the patient was available anymore, we determined whether activated AML could be recognized using 4 primary AML samples from other patients with similar classification (M5). Results shown in figure 3G demonstrate that without activation 2 of 4 AML samples were recognized, whereas after activation all 4 tested AML samples were recognized.

Since PRAME has been described to be expressed at low and intermediate levels in certain normal tissues, we tested HSS1 against different HLA-A2+ non-malignant cell types. HSS1 showed no reactivity against fibroblasts and keratinocytes with or without pre-treatment with IFNγ (figure 4A), or against primary bronchus epithelial cells, hepatocytes, intrahepatic billiary epithelial cells, colon epithelial cells or mesenchymal stromal cells (figure 4B). In contrast, the clone showed low but significant recognition of all 12 tested HLA-A2+ proximal tubular epithelial cells (PTEC) derived from kidney tubules of which 4 are shown in figure 4C. Testing the clone against HLA-A2+ non-malignant cells of hematopoietic origin demonstrated that HSS1 did not recognize B cells, CD40L activated B cells or in vivo activated B cells derived from inflamed tonsils (figure 4D and 4E). In addition, T-cells and activated T-cell blasts were not recognized (data not shown). Monocytes or immature CD14 derived DCs (figure 4F), CD34 cells or immature CD34DCs (figure 4G), and MØ1 and MØ2 macrophages (figure 4H) were also not recognized by the PRAME specific T-cell clone. However, clone HSS1 showed low reactivity against mature DCs derived from CD14 and CD34 cells (figure 4F and 4G, respectively). To investigate whether the PRAME specific T-cells were also reactive against blood derived myeloid and plasmacytoid DCs (MDCs and PDCs, respectively), HSS1 was tested against MDCs and PDCs. No recognition of immature MDCs and PDCs and mature PDCs was observed. However, low reactivity against mature MDCs was observed (data not shown). To determine whether allo-restricted PRAME specific T-cells were also reactive against other myeloid cell-lineages, HSS1 was tested against proliferating and differentiating CD34 cells in a proliferation inhibition assay. As is shown in figure 4I, HSS1 was not able to inhibit the proliferation of CD34 cells, and progeny of CD34 cells.

To investigate whether the high avidity allo-restricted PRAME T-cells exerted antigen specific cytolytic activity, HSS1 and control HSS12 were tested at different E/T ratio against HLA-A2+ targets including 2 melanomas, 3 RCCs, CD14mDCs and 2 PTECs. Clone HSS1 exerted high cytolytic activity against the previously recognized HLA-A2+ malignant cell-lines, whereas only low reactivity against mature mDCs and PTECs was observed (figure 5A). The HSS12 control clone was highly reactive against all HLA-A2+ targets, including mDCs and PTECs (figure 5B).

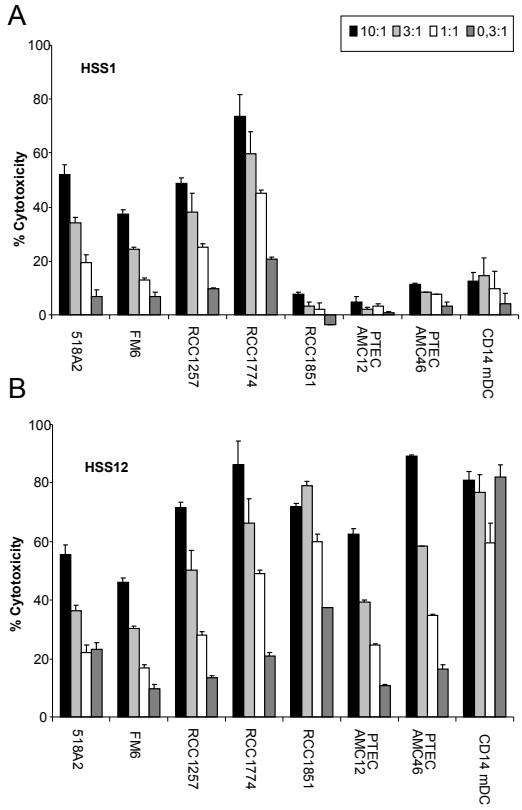


Figure 5. The allo-restricted PRAME specific T cell clone HSS1 exerts specific cytotoxic activity. HSS1 **(A)** and control clone HSS12 **(B)** were tested for cytotoxicity against HLA-A2+ melanoma's (518A2 and FM6), RCCs (RCC1257, RCC1774 and RCC1851), PTECs and CD14mDCs.

The results demonstrate that the allo-restricted PRAME T-cells are able to exert efficient reactivity against a large proportion of tumors. In addition, the results indicate that the PRAME T-cells exhibited low reactivity against non-malignant mDCs and PTECs.

Expression of PRAME gene, determined by quantitative RT-PCR, strictly correlates with recognition of PRAME specific T-cell clone

To establish whether the reactivity of the allo-HLA restricted PRAME T-cells correlated with expression of PRAME, we determined the mRNA expression level of PRAME by quantitative RT-PCR in the different cells. figure 6 illustrates that reactivity of HSS1 strictly correlated with PRAME expression. Cells with high or intermediate PRAME expression were effectively or intermediately recognized by the T-cells. Targets that did not express PRAME were not recognized. Targets expressing low levels of PRAME were also not recognized, with the exception of CD40L activated ALL, EBV-LCLs, and non-malignant PTECs and mDCs.

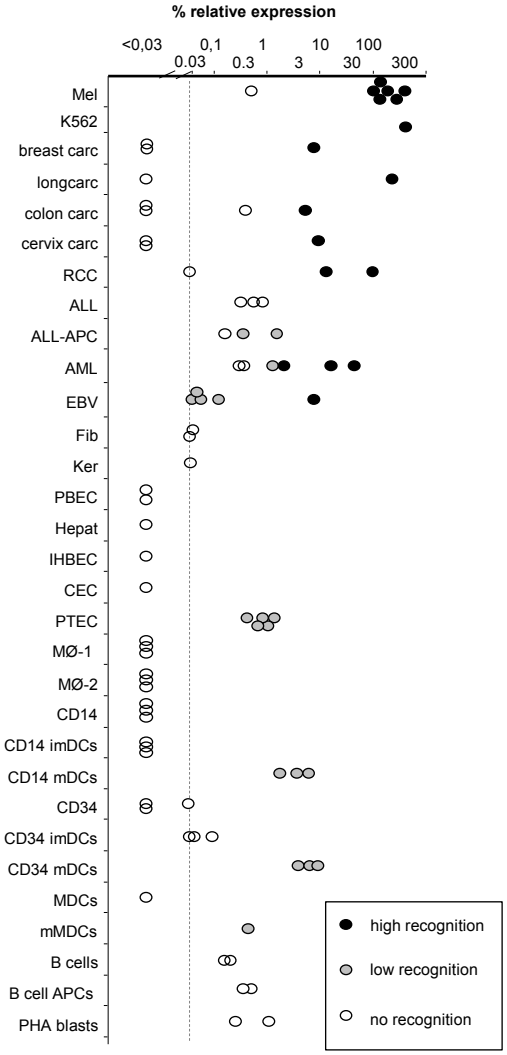


Figure 6. Correlation between functional reactivity of the PRAME specific T cell clone HSS1 and PRAME mRNA expression. The expression of PRAME mRNA in different cells was determined using quantitative RT-PCR, and referenced to the level of PRAME expression measured in Mel518. The limit of detection is indicated with the broken line. The black dots represent cells high or intermediate recognized by HSS1, gray dots represent cells low recognized by HSS1, and white dots represent cells not recognized by HSS1.

To confirm the strict correlation between PRAME expression and recognition, and to exclude that the reactivity was due to off-target toxicity mediated by crossreactivity against other peptide-HLA complexes, we transduced CD34mDCs, PTECs, and RCC1257 with PRAME silencing RNA (shRNA). The PRAME specific shRNA almost completely blocked the recognition of CD34mDCs, PTECs and RCC by HSS1, whereas the recognition of the targets by control HSS12

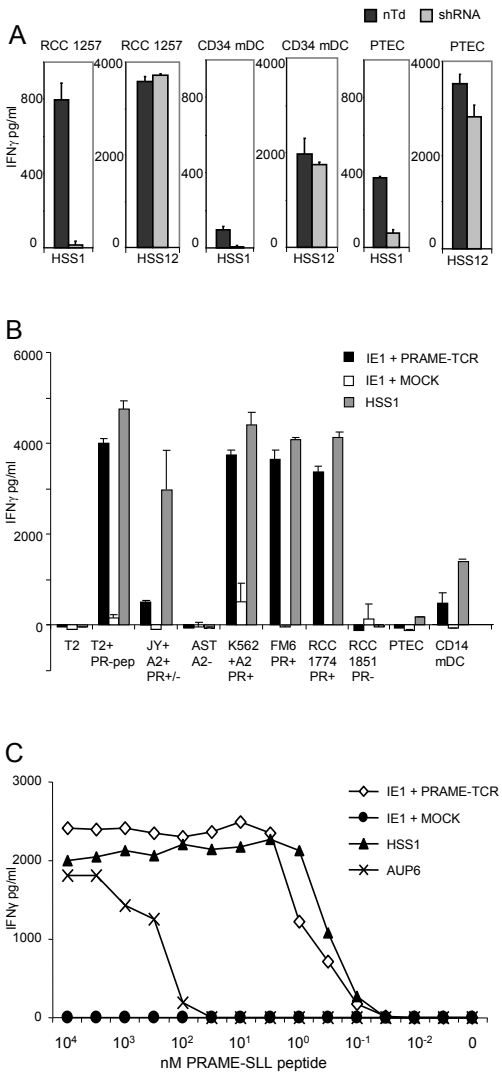


Figure 7. PRAME specific shRNA and PRAME-TCR transduction demonstrate that recognition of PTECs and mDCs is due to on-target toxicity, and tumor specific reactivity of PRAME-TCR can be efficiently transferred.

(A) HSS1 and control HSS12 were tested against HLA-A2+ RCC1257, CD34mDCs and PTECs transduced with a retroviral vector encoding for a PRAME specific shRNA and the puromycin selection gene. shRNA transduced cells were cultured for 7 days with puromycin (0.4-4 ug/ml) and used as stimulator cells. Non-transduced cells (nTd) were used as control stimulator cells. **(B)** CMV-IE1 specific T cells transduced with PRAME-TCR or mock transduced, and HSS1 were tested against T2 cells, T2 cells loaded with PRAME peptide, HLA-A2+ LCL-JY, K562+A2, FM6, RCC1774, RCC1851, PTECs, CD14mDCs, and HLA-A2 negative EBV-LCL (AST A2-) The expression of PRAME in the melanomas and RCCs is indicated (PR+ or PR-). **(C)** CMV-IE1 specific T cells transduced with the PRAME-TCR, mock transduced CMV-IE1 specific T cells, HSS1 and AUP6 were tested against T2 cells loaded with titrated concentrations of the PRAME-SLL peptide.

was unaltered (figure 7A). These results demonstrate that the reactivity against PTECs and mDCs by the allo-HLA restricted PRAME T-cells was based on PRAME recognition.

PRAME-TCR transduced T-cells exert clinically relevant PRAME specific reactivity

Based on the high reactivity against a large proportion of tumors and limited on-target toxicity exerted by the high avidity PRAME T-cells, their high affinity TCR may be effective tools for TCR gene therapeutic strategies[29;33]. Therefore, we investigated the functional activity of PRAME-TCR transduced T-cells. To prevent mixed TCR dimer formation and to optimize TCR expression the TCRα and β chain of HSS1 were codon optimized and cysteine modified[28;29]. Results shown in figure 7B demonstrate that the PRAME-TCR transduced T-cells exerted similar to the parental HSS1 high reactivity against PRAME peptide loaded T2 cells, and PRAME+ tumor cells, whereas PTECs and mDCs were only marginally recognized. To investigate the avidity of the PRAME-TCR transduced T-cells, these T-cells were compared to the parental high avidity PRAME specific T-cell clone HSS1 and to the low avidity self restricted T-cell clone AUP6 in a peptide titration experiment with the PRAME-SLL peptide loaded on T2 cells. As shown in figure 7C, PRAME-TCR transduced T-cells required comparable peptide concentration for 50% of maximum IFN-γ production as the parental clone HSS1, demonstrating that the avidity of PRAME-TCR transduced T-cells is similar to that of the parental high avidity PRAME specific T-cell clone. These results indicate that the PRAME-TCR of clone HSS1 can potentially be used for TCR gene therapy.

DISCUSSION

In this study, we succeeded in isolating beneficial GVT reactive T-cells restricted by allo-HLA-A2 and specific for PRAME from a detrimental allo-HLA directed immune response after HLA-A2 mismatched SCT. We demonstrated that the allo-restricted PRAME specific T-cells were single peptide specific and, in contrast to self-restricted T-cells, exerted high avidity reactivity against PRAME expressing tumor cells. The reactivity of the T-cells strictly correlated with PRAME expression, indicating that the allo-restricted PRAME T-cells were not cross-reactive against other peptides presented in the context of HLA-A2. The clones showed high reactivity against a large panel of tumor cell-lines, whereas a large panel of non-malignant cells was not recognized. The allo-restricted PRAME T-cells exerted limited on-target toxicity against mDCs and PTECs. Finally, we demonstrated high PRAME specific tumor reactivity of PRAME-TCR transduced T-cells, indicating that the PRAME-TCR can potentially be used in TCR gene therapeutic strategies.

In the patient at the time of severe GVHD approximately 85% of donor CD8 T-cells were highly activated. By isolating these activated polyclonal CD8 T-cells, we illustrated that 0.6%

of T-cells were PRAME-SLL specific, indicating that at the time of GVHD approximately 0.5% of the donor CD8 T-cells were directed against PRAME. Based on our results we speculate that the PRAME specific allo-restricted T-cells have likely been induced *in vivo* by activated patient derived HLA-A2+ mDCs or by the AML-DC. Although AML was not recognized in non-activated state, 4 primary AML samples with similar classification were recognized after activation (figure 3G), and may indicate that the AML-DC have induced the PRAME specific T-cell response. Based on the limited reactivity against PTECs even after IFN-γ activation, and the lack of expression of costimulatory molecules on these cells, we assume that it is unlikely that PTECs were the mediators of the PRAME specific allo-restricted T-cell response. Although the patient died of severe GVHD, at the time of the profound allo-HLA immune response in which a large variety of allo-HLA reactive T-cells including the PRAME specific T-cells were present no clinical signs of nephrotoxicity were observed.

The results demonstrating that mDCs express PRAME at levels able to activate high avidity PRAME specific T-cells, also explains why self-restricted T-cells exhibiting high avidity for the self-antigen PRAME can be deleted by negative selection during thymic development. The observation that we failed to isolate self-restricted PRAME specific T-cells exerting high avidity PRAME specific reactivity against tumor cells is in agreement with this hypothesis. In addition, although self-restricted PRAME specific T-cells were previously isolated that exhibited high peptide affinity, these T-cells exerted low anti-tumor reactivity[34-36]. Based on these findings we consider it unlikely that transfer of self-restricted PRAME specific T-cells[35;36] or vaccination with PRAME[35-39], even combined with optimal adjuvants, will induce high avidity PRAME specific T-cells exerting potent anti-tumor reactivity. In contrast, we isolated high avidity PRAME specific T-cells by circumventing immune tolerance, because negative selection is limited to self-HLA. The reactivity of the allo-restricted PRAME clones against multiple different tumor cell-lines and primary leukemia demonstrate that these PRAME-TCRs could potentially be useful in adoptive T-cell therapy with TCR engineered T-cells for the treatment of patients with many different malignancies.

The allo-restricted PRAME specific T-cell clones unfortunately also showed reactivity against mDCs and PTECs, due to low expression of PRAME. We hypothesize that the PRAME specific T-cells were induced in our patient after HLA-mismatched SCT and subsequent DLI by either the mDCs or the AML-DCs, and therefore we speculate that PRAME-TCR engineered T-cells may be reactive against autologous mDCs. The recognition and thereby eradication of mDCs by PRAME specific T-cells could lead to immune impairment, since mDCs play an important role in the initiation of new immune responses. In addition, the reactivity directed against PTECs could possibly lead to renal failure. It has indeed been previously demonstrated that recognition of specific non-malignant cells by administered high avidity TAA specific T-cells can result in toxicity. The infusion of high avidity T-cells directed against the RCC antigen carboxy anhydrase IX (CAIX) resulted in severe cholestasis, based on the CAIX expression by bile

duct epithelial cells[40]. In addition, patients who received high avidity gp100 T-cells developed uveitis and hearing loss due to expression of gp100 by melanocytes in eye and ear[15].
Based on the possible on-target toxicity induced by the PRAME-TCR a safety strategy may be necessary to co-transduce PRAME-TCR engineered T-cells with a suicide gene, which enables *in vivo* elimination of the engineered T-cells if serious adverse events occur[41;42]. Using this strategy a temporary loss of mDCs could be resolved, since imDCs, CD14+ and CD34+ progenitor cells as well as MDCs and PDCs from peripheral blood are not recognized by the PRAME T-cells. In fact, recognition of mDCs might also be beneficial since it could potentially boost anti-tumor responses and enhance persistence of the infused T-cells. In contrast, potential damage to the kidney powered by recognition of PTECs could be irreversible and therefore, co-transduction of a suicide gene might be necessary for protection against toxicity directed against the kidney. It is however also possible that the low reactivity observed against cultured PTECs even after IFN-γ activation *in vitro* does not necessarily indicate nephrotoxicity *in vivo,* as indicated by absence of renal dysfunction in our patient.
Based on these results we conclude that potentially beneficial high avidity TAAs specific T-cells can be isolated from detrimental allo-HLA immune responses after HLA mismatched SCT. The high affinity TCRs of these allo-restricted PRAME specific T-cells highly reactive against a large panel of tumors, may be effective tools to engineer T-cells with tumor-targeting TCRs that can be used for adoptive T-cell therapy of patients with metastasized cancer or relapsed leukemia.

ACKNOWLEDGEMENTS

The authors thank Menno van der Hoorn, Guido de Roo and Patrick van der Holst for expert technical assistance.

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6

SUMMARY AND GENERAL DISCUSSION



SUMMARY

Allo-HLA reactive T-cells can mediate graft versus host disease (GVHD) after HLA mismatched stem cell transplantation (SCT) and donor lymphocyte infusion (DLI). In addition, these T-cells can mediate graft rejection after HLA mismatched solid organ transplantation and SCT. Both GVHD and graft rejection can have detrimental consequences for patients. It has been demonstrated that in the HLA matched setting GVHD and graft versus tumor response can be separated by selection of T-cells directed against minor histocompatibility antigens (miHAs) exclusively expressed on hematopoietic cells. After HLA mismatched SCT, GVHD is often accompanied by graft versus leukemia response, indicating that allo-HLA reactive T-cells could potentially be useful for beneficial purposes. In this thesis we characterized the composition of an allo-HLA class I directed immune response occurring in vivo during GVHD and investigated which cells were possibly responsible for activating the response. We investigated which T-cells are responsible for allo-HLA reactivity and how these T-cells recognize allo-HLA. Finally we investigated whether allo-HLA reactive T-cells could potentially be used for adoptive T-cell therapy.

In **chapter 2** we characterized an allo-HLA class I directed immune response in a patient who developed severe graft versus host disease (GVHD) after the administration of HLA-A2 mismatched donor lymphocyte infusion (DLI) in the absence of inflammatory conditions. A previously administered DLI from the same donor did not lead to an immune response, excluding the presence of a substantial pool of CD8 T-cells crossreactive against HLA-A2 within the memory T-cell compartment of the donor, and suggesting a requirement for CD4 T-cell activation for initiation of the response. Using single cell sorting we isolated CD8 and CD4 donor derived T-cells activated at the time of the GVHD. Analysis of the clonal diversity, alloreactivity, HLA restriction and specificity of the isolated CD4 and CD8 T-cell clones demonstrated that a polyclonal CD8 T-cell response directed against the mismatched HLA-A2 as well as a polyclonal CD4 T-cell response recognizing peptides derived from HLA-A2 presented in HLA class II were present during the GVHD. Investigation of patient blood and bone marrow collected at the time of the two DLIs demonstrated that leukemic blasts were the only patient derived HLA class II positive cells present at the time of the second DLI leading to GVHD, and that these cells were absent at the time of the first DLI not leading to an immune response. Stimulation of alloreactive CD4 and CD8 T-cells clones with the leukemic blasts demonstrated that these cells were able to activate both the alloreactive CD8 and CD4 T-cells. The results in this study indicate that the GVHD was mediated by a coordinated CD4 and CD8 response directed against the mismatched HLA-A2 and suggest that leukemic blasts possibly activated the CD8 as well as the CD4 T-cell response.

Which T-cells are responsible for allo-HLA reactivity and whether this is a property of a specific subgroup of T-cells or of all T-cells is unknown. Although allo-HLA reactivity was shown to be present within naïve and memory T-cell populations, the ability of T-cells to exhibit allo-HLA

reactivity could especially have serious consequences when exerted by memory T-cells, since memory T-cells can be efficiently triggered by non-professional antigen presenting cells based on their lack of requirement for co-stimulation. In **chapter 3** the ability of virus specific T-cells to exhibit allo-HLA reactivity was investigated. Screening a large number of virus specific T-cells lines and clones against a panel of EBV transformed B-cells (EBV-LCLs), together covering almost all prevalent HLA class I and II molecules, demonstrated that 80% of the virus specific lines and 45% of the virus specific clones were allo-HLA reactive. This alloreactivity was demonstrated for Epstein Barr virus (EBV), cytomegalovirus (CMV), varicella zoster virus (VZV) and influenza virus (Flu) specific T-cells. Testing the alloreactive virus specific T-cell clones against single antigen transduced K562 cells confirmed that the alloreactivity was based on allo-HLA reactivity. TCR gene transfer demonstrated that the virus specificity and the HLA cross-reactivity were mediated by the same TCR. Three T-cell clones derived from the same individual and with the same specificity, but with different TCR usage demonstrated recognition of three different allo-HLA molecules, indicating that allo-HLA reactivity cannot be predicted on the basis of viral specificity. To extrapolate the results obtained with the EBV-LCLs and K562 cells to recognition of normal cell subsets in vivo, we tested virus specific T-cell clones against activated and non-activated blood derived cells and fibroblasts. Results demonstrated that virus specific T-cells can also be alloreactive against relevant normal cell subsets. The results in this study demonstrate that T-cells specific for different viruses exert cross-reactivity to allo-HLA molecules, and illustrate the high frequency of T-cells able to exert allo-HLA reactivity.

T-cell allo-HLA reactivity is assumed to be less peptide specific than conventional T-cell reactivity, based on the ability of allo-HLA reactive T-cells to recognize antigen processing deficient cells without exogenously loaded peptides and multiple different synthetic peptides loaded on target cells. However, peptide specificity of allo-HLA reactive T-cells against endogenously processed and presented peptides was not investigated. In addition, most of the studied allo-HLA reactive T-cells were in vitro activated and expanded. In **chapter 4** the degree of peptide specificity and avidity of allo-HLA reactive T-cells mediating biological relevant allo-immune responses was studies by investigating the peptide specificities of 50 different allo-HLA-A2 reactive T-cell clones activated and expanded in vivo during GVHD. Testing the allo-HLA reactive T-cell clones against HPLC fractions of peptides eluted from HLA-A2 and loaded on antigen processing deficient T2 cells demonstrated that each clone recognized a single or two subsequent fractions, indicating that the in vivo derived allo-HLA reactive T-cell clones were single peptide specific. Allo-HLA reactive T-cell clones recognizing T2 without exogenously loaded peptides were tested against peptides eluted from HLA-A2 of T2 cells loaded on HLA-A2 expressing drosophila cells and also demonstrated to be single peptide specific. The peptides recognized by the allo-HLA reactive T-cell clones, including one T2 reactive clone, were identified using multidimensional HPLC fractionation and mass spectrometry. One of the clones recognized two different HPLC fractions. Identification of the

two peptides recognized by this clone demonstrated sequence similarity between the two peptides and alanine substitution of the identical amino acids suggested that recognition of the two peptides was based on structural similarity. Finally, downregulation of the expression of the antigens recognized by the allo-HLA reactive T-cell clones using silencing RNA confirmed their single peptide specificity.

Since in vivo activated and expanded allo-HLA reactive T-cells were demonstrated to exert high avidity single peptides specific recognition, allo-HLA reactive T-cells directed against tumor associated antigens (TAAs) could be used for adoptive T-cell therapy. In **chapter 5**, the search for TAAs specific allo-HLA reactive T-cells within an allo-HLA-A2 directed immune response occurring during GVHD resulted in the isolation of two high avidity PRAME specific T-cell clones. These T-cells were demonstrated to exert single peptide specificity and recognition was strictly correlated with PRAME expression. The allo-HLA restricted T-cell clones were, in contrast to the self-restricted PRAME specific T-cell clones, highly reactive against multiple different tumor cell-lines as well as freshly isolated metastatic melanoma and primary leukemic cells, whereas no reactivity against a large panel of non-malignant cells was observed. However, the clones exerted limited reactivity against mature dendritic cells (mDCs) and kidney epithelial cells. Recognition of the different target cells correlated with PRAME expression, as was demonstrated by quantitative RT-PCR, and by downregulation of PRAME using silencing RNA the recognition of mDCs and kidney epithelial cells was demonstrated to be based on on-target toxicity. Finally, PRAME-TCR transduced T-cells were demonstrated to exert high PRAME specific tumor reactivity, indicating that the PRAME-TCR can potentially be used in TCR gene therapeutic strategies. Clinical studies will determine the effectiveness of tumor reactivity in vivo and the significance of the potential toxicity observed against mature DCs and kidney epithelial cells.

GENERAL DISCUSSION

AML blasts may have acted as antigen presenting cells in the initiation of the HLA class I directed immune response

In Chapter 2 we demonstrated that a coordinated response of CD8 T-cells directed against mismatched HLA-A2 and CD4 T-cells recognizing HLA-A2 derived peptide presented in HLA class II was likely to have been responsible for severe acute GVHD. In addition, we demonstrated that leukemic blasts were the only HLA-A2 and HLA class II positive cells present in blood and bone marrow at the time of emergence of GVHD which were able to activate the CD8 and CD4 T-cell clones. The patient had received a SCT and two DLIs from the same donor, of which the first DLI did not lead to an immune response and the second DLI led to a severe acute GVHD. The leukemic blasts were absent at the time of the first DLI and relapsed before the second DLI. The remaining non-leukemic HLA-A2 positive cells present in patients

blood and bone marrow at the time of the GVHD, consisted mostly of patient T-cells which were present at the time of both DLIs but showed a marginal expression of HLA class II. We therefore hypothesize that the leukemic blasts acting as antigen presenting cells may have been required for initiation of the immune response responsible for GVHD. The absence of an immune response after the first DLI indicated that there was not a substantial pool of CD8 T-cells crossreactive against HLA-A2 within the memory T-cell compartment of the donor and thus suggests that the CD8 immune response required priming by activated antigen presenting cells for initiation[1]. Investigating the leukemic blasts for the expression of co-stimulatory and adhesion molecules demonstrated that these cells did not express the surface molecules CD80, CD86 and CD54 previously shown to be relevant for initiation of primary immune responses[2;3]. However, even professional antigen presenting cells, such as B-cells and dendritic cells need to be activated in order to express co-stimulatory and adhesion molecules and to be able to efficiently prime naïve T-cells. The activation of professional APCs can occur via the inflammatory signals of pathogens[4;5]. Alternatively, in the absence of inflammatory signals, CD4 T-cells can activate APCs by CD40-CD40L interaction and cytokine production[6-8]. This indicates that recognition of antigen by naïve CD4 T-cells on non-activated antigen presenting cells can lead to activation of APCs, which can subsequently prime other CD4 T-cells and CD8 T-cells. Stevanovic et al.[9] demonstrated that also leukemic blasts can be activated by CD4 T-cells, by showing that leukemic blasts which in a non-activated state, did not express co-stimulatory and adhesion molecules and did not function as APCs, differentiated into APCs with expression of appropriate surface molecules after infusion of allogeneic CD4 T-cells in mice. In chapter 5 we demonstrated that the PRAME specific T-cell clones, which were derived from the same patient experiencing GVHD as is described in chapter 2, showed no or low reactivity against HLA-A2+ AML blasts but high reactivity against the same AML blasts activated by cytokines. The high reactivity against activated AML blasts by the PRAME specific clones could indicate that the leukemic blasts expressed PRAME higher after activation, but it could also be due to a more efficient antigen presentation and higher expression of co-stimulatory and adhesion molecules by the activated AML blasts. We speculate that part of the CD4 T-cells recognizing HLA-A2 derived peptides presented by HLA class II expressed on the leukemic blasts activated the leukemic blasts and thereby enabled them to prime both the CD4 and CD8 T-cell response.

Allo-HLA reactivity is a common property of all T-cells

In chapter 3 we used virus specific T-cells as a model for T-cells with a known specificity to investigate which T-cells are responsible for allo-HLA reactivity. 45% of the virus specific T-cells clones were demonstrated to be allo-HLA reactive, indicating that at least a substantial part of all T-cells is able to exert reactivity against specific allo-HLA molecules. However, since T-cells were analyzed for allo-HLA cross-reactivity against an EBV-LCL panel covering all prevalent HLA class I and class II molecules, missing all infrequent HLA molecules as well

as all tissue specific peptides presented in allo-HLA molecules, we speculate that virtually all T-cells may be allo-HLA reactive. It was previously hypothesized that each TCR needs to be able to react with structurally different peptide-HLA (pHLA) ligands[10]. This property of 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[11]. In addition, HLA cross-reactivity is thought to be essential for enabling the TCR to briefly dock and 'scan' the peptide contents of many different HLA molecules[12]. Combining these hypotheses with our results in chapter 3 we conclude that the ability to form high avidity interactions with structurally different pHLA complexes is not an aberration of a certain subgroup of T-cells, but a normal property of T-cells.

Peptide specificity of allo-HLA reactive virus specific T-cells

In chapter 4 we demonstrated that biologically relevant allo-HLA reactivity, as defined by reactivity against endogenously processed and presented antigen in the context of an allo-HLA molecule, is based on high avidity single peptide specificity. We assume that the allo-HLA reactivity of virus specific T-cells, as demonstrated in chapter 3, is high avidity and single peptide specific, since allo-HLA reactivity was demonstrated against EBV-LCLs expressing endogenously processed and presented antigens in the context of allo-HLA molecules. In addition, for some of the alloreactive virus specific T-cell clones dependency on recognition of endogenous peptide for allo-HLA reactivity was demonstrated by showing reactivity against EBV-LCLs and PHA blasts expressing the recognized allo-HLA molecules and absence of reactivity against K562 cells transduced with the same allo-HLA molecules. Moreover, testing the virus specific T-cell clone, specific for EBV-BRLF/HLA-A3 and cross reactive against HLA-A2, against peptides eluted from HLA-A2 and loaded on T2 cells, demonstrated reactivity against one HPLC fraction, indicating peptide specific recognition by this clone. By multidimensional HPLC fractionation and mass spectrometry, as described in chapter 4, the single peptide recognized by the BRLF/A3 specific T-cell clone in the context of allo-HLA-A2 could be identified (data not shown in this thesis). The CMV-IPS/HLA-B35 specific T-cell clone cross reactive against HLA-DR4 also demonstrated recognition of a single peptide when tested against a recombinant bacteria cDNA library[13] expressed by HLA-DR4 positive target cells (data not shown in this thesis).

In the future more peptides recognized in the context of allo-HLA molecules by virus specific T-cells may be identified using multidimensional HPLC fractionations and mass spectrometry or by bacteria cDNA library screening[13]. The identification of peptide/allo-HLA complexes recognized by virus specific T-cells offers the possibility to compare the TCR interactions with the viral peptide in self HLA and with the endogenous peptide in allo-HLA. Comparing the two interactions on a molecular level using crystalstructures may lead to more insight into the molecular mechanism of TCR cross reactivity[14;15]. In addition, crystalstructures of the two TCR-pHLA interactions of the CMV-IPS/HLA-B35 specific T-cell clone cross reactive against

HLA-DR4, may give more insight into TCR interaction with HLA class I and class II and into co-receptor dependency and independency of TCR/pHLA interaction[16].

The potential risk of allo-HLA reactivity of HLA mismatched virus specific T-cell lines

The results in chapter 3, demonstrating that allo-HLA reactivity of virus specific T-cells is common, suggest that administration of virus specific T-cells over HLA barriers may induce GVHD and therefore virus specific lines should be tested for alloreactivity against the patient prior to administration. However, Melenhorst and colleagues recently reported that partially mismatched virus specific T-cell lines did not lead to de novo development of GVHD in any of the 73 studied patients[17]. As we demonstrated in chapter 3, the reactivity exerted by each of the allo-HLA reactive virus specific T-cell clones was specifically directed against one or a few allo-HLA molecules. It is therefore possible that the virus specific T-cells described by Melenhorst et al. were not alloreactive against the HLA molecules expressed by the recipients, especially since a selection criterion for infusion was a low or absent reactivity exerted by the virus specific line when tested against recipient APCs. In an attempt to exclude that the lack of GVHD was due to fortuitous choice of CTLs lacking recipient-specific allo-HLA reactivity, they demonstrated alloreactivity of the lines by testing them against panels of APCs expressing different HLA molecules. However, the alloreactivity observed against APCs expressing recipient allo-HLA molecules was either marginal of absent. In addition, it is not confirmed that the alloreactivity observed was directed against allo-HLA molecules, since reactivity against single antigen expressing cells or even against a few APCs sharing the same allo-HLA molecule is not described. Another possible explanation for the lack of GVHD in vivo could be that the T-cells lines are immunogenic targets for the immune system of the patient, based on their HLA mismatch, and are therefore quickly lost after transfusion. However, it was demonstrated that these lines were effective against viruses in vivo, indicating that they were at least not immediately lost and were able to establish an effective immune response.
In contrast to the results of Melenhorst et al., other groups using partially HLA mismatched virus-specific T-cells lines did report GVHD after CTL infusion. In an extension of a previously reported study of Micklethwaite et al., 10 of 33 adults receiving bivirus-specific T-cells after allogeneic transplantation developed GVHD, de novo in 9 patients[18]. Three patients developed grade III or IV GVHD. Peggs et al. reported similar results with 11 of 30 patients developing acute GVHD after CMV-specific T-cell infusions with grade III disease in 3 of the patients[19]. Randomized studies of both prophylactic and preemptive CMV-specific T-cells currently running in the United Kingdom will provide further information on the risk of GVHD related to virus-specific T-cells in patients receiving uniform conditioning[20].

Difference in degree of peptide specificity and affinity between in vivo and in vitro activated and expanded alloreactive T-cells

In chapter 4 we demonstrated that in vivo activated and expanded alloreactive T-cells exert high avidity single peptide specific reactivity. In contrast, other research groups studying in vitro activated and expanded alloreactive T-cells demonstrated a large variation of avidity and degree of peptide specificity between the different T-cells[21-27]. The difference between our findings and those of other groups could possibly be explained by the differences between in vivo and in vitro induced immune responses. It has been shown that during in vivo immune responses only T-cells with high avidity are selected[28;29]. Zehn et al. demonstrated that low avidity T-cells stop expanding, exit lymphoid organs and start contraction earlier than high avidity T-cells, possibly explaining the mechanisms by which selection of high avidity T-cells occurs[30]. Several factors might influence the selection of high avidity T-cells during in vivo immune responses. First of all, the quantity of antigen could play a role, since it has been shown that in vivo, in the absence of excessive antigen low avidity T-cells undergo apoptosis, whereas high avidity T-cells survive and expand[31]. In addition, the number of activated APCs presenting the antigen could be of influence. Kedl et al. demonstrated that in vivo T-cells have to compete for APCs presenting the antigen, indicating selection of T-cells forming high avidity interactions with APCs[32]. Finally, cytokines like IL2 and IL7 have been shown to play a role in T-cell expansion and survival during contraction phase[33-35], suggesting that the available amount of cytokines, which is most likely limited in most in vivo immune responses, also influences the selection of high avidity T-cells. In contrast to the priming conditions of in vivo induced immune responses, during in vitro activation and expansion antigen specific T-cells are usually in the presence of an abundance of antigen, antigen presenting cells and cytokines. Due to this lack of stringent selection, not only high avidity but also intermediate and low avidity T-cells are able to activate and expand during in vitro stimulations, explaining the documented findings of research groups studying in vitro induced alloreactive T-cells[21-27]. Since in these studies recognition of low avidity T-cells was not confirmed by testing the T-cells against endogenously processed and presented antigen, we suspect that the reported low avidity reactivity directed against synthetic peptides loaded target cells was not biologically relevant recognition. In other terms, these T-cells would probably not have been activated and expanded and therefore not effective in vivo.

Thymic selection

In chapter 4 we demonstrated that allo-HLA recognition is as peptide specific as conventional T-cell recognition of foreign peptides presented in self HLA. These results appear to be in conflict with a currently accepted hypothesis that allorecognition is less peptide specific than

conventional T-cell reactivity, since allo-MHC molecules are not encountered during thymic development. It is assumed that the prethymic T-cell repertoire contains a large number of MHC and peptide crossreactive T-cells and that thymic selection is responsible for the removal of these T-cells. This assumption is based on the studies of Husbey et al., amongst others, in which T-cells selected in mice expressing MHC linked to a single peptide were demonstrated to exert high peptide and MHC crossreactivity[36;37]. Thymic selection of T-cells with one pMHC was in these studies interpreted as impaired negative selection and the hereby obtained T-cells repertoire was therefore thought to resemble the prethymic T-cell repertoire. Husbey et al. however ignored the fact that T-cells also undergo positive selection in the thymus. In mice expressing only one pMHC complex, the T-cells have to be able to form a low avidity interaction with this specific pMHC complex in order to be positively selected. It is therefore possible that selection with one pMHC complex does not represent impaired selection, but instead a very stringent selection of TCRs, not representative for the prethymic TCR repertoire. In contrast to Husbey et al. who used expression of a single pMHC as a model for lack of thymic selection, Zerrahn et al. used T-cells selected in MHC negative mice as a model for T-cells without thymic selection and compared them to T-cells selected in normal mice[38]. With this method Zerrahn et al. demonstrated that prethymic T-cells are as MHC crossreactive as T-cells after normal thymic selection. In addition, TCRs which were self engineered by combining the α-chain and the β-chain of two different TCRs and therefore really representing T-cells which have not undergone thymic selection, were also able to interact with one or a few pHLA complexes, but reactivity against multiple pHLA complexes has not been found[39]. These results indicate that the ability to specifically interact with pHLA complexes is not a property selected for in the thymus, but is most likely a germline property of TCRs

Adoptive T-cell therapy

The single peptide specificity of almost all investigated in vivo activated and expanded allo-HLA reactive T-cells (chapters 4 and 5), opens the door for the use of allo-HLA reactive T-cells in adoptive cell therapy. In contrast to adoptive cell therapy using T-cells directed against miHAs, in which a difference in the single nucleotide polymorphism (SNP) coding for the specific MiHA between patient and donor is necessary for application in a patient, adoptive cell therapy using allo-HLA reactive TAA specific T-cells could be applied in all patients positive for the targeted HLA and suffering from malignancies expressing the targeted TAA. This therapy could therefore potentially be applied in many patients, suffering from many different types of cancer. The TCRs of the in chapter 5 reported PRAME / allo-HLA-A2 specific T-cells could be applied in all HLA-A2+ patients, which are 50% of the West European and North American population, suffering from PRAME expressing melanoma (88% of melanoma's are PRAME positive) renal cell carcinoma, leukemia and other malignancies[40]. However, the application of allo-HLA reactive T-cells in the clinic could also lead to severe toxicity in patients. Therefore,

for every TCR potentially useful for adoptive cell therapy, a balance should be made of the expected benefits and risks, prior to application in the clinic.

For adoptive T-cell therapy high avidity TAA specific T-cells are required to mediate an effective anti-tumor response after infusion. High avidity TAA specific T-cells have been discovered before by different research groups [27;30;41-53]. Nevertheless, only a few of these discoveries have actually led to clinical application[43;45;47]. Quintarelli et al.[48] and Griffioen et al.[42] reported high avidity T-cell reactivity against PRAME derived peptides (P435-443 and P100-108 respectively) presented in self HLA. Both groups could demonstrate high reactivity against peptide loaded target cells and high peptide affinity in peptide titration experiments. However, in both studies T-cell reactivity against PRAME positive target cells was minimal and unconvincing. In our hands, the previously reported (griffioen et al.) autologous PRAME P100-108/HLA-A2 specific T-cell clone demonstrated high peptide affinity (EC50 = 0,3 nM) in peptide titration experiments, comparable to the peptide affinity of the alloreactive PRAME specific T-cells HSS1 and HSS3 (chapter 5). However, this autologous PRAME specific T-cell clone demonstrated no significant reactivity against endogenously PRAME expressing target cells (data not shown) which were highly recognized by our alloreactive PRAME specific T-cell clones. Since PRAME is a self-antigen, which is, as we demonstrated in chapter 5, expressed by mature DCs, autologous PRAME specific T-cells are most likely deleted in the thymus. This indicates the PRAME derived peptides identified in the two studies are actually not efficiently processed and presented in cells and are therefore not expressed in the thymus, explaining the demonstrated high reactivity against synthetic PRAME peptide loaded target cells but absent reactivity against endogenous PRAME positive target cells by the described autologous PRAME specific T-cells. By using allo-HLA recognition, and thereby circumventing thymic deletion, efficiently processed and presented peptides derived from tumor associated antigens could be targeted despite the fact that these are self-antigens.

Of the previously reported allo-HLA reactive TAA specific T-cells, most were derived from in vitro activations and expansions[27;41;44;49-51;53;54]. Since, in the majority of these studies, single peptide specificity of the TAAs specific T-cells was not confirmed, we suspect that at least some of these T-cells could also recognize other peptides, and thereby could possibly lead to off-target toxicity when applied in the clinic. The use of in vivo activated and expanded allo-HLA reactive T-cells could, based on their single peptide specificity, limit the risk of off target toxicity. However, as we demonstrated in chapter 4, also in vivo activated and expanded T-cells could be specific for more than one peptide (as clone HSS11 in chapter 5). Recognition of another look alike peptide with a broader expression pattern than the targeted peptide would most likely lead to severe toxicity. Therefore, single peptide specificity should in any case be confirmed before the use of allo-HLA reactive T-cells for clinical purposes.

Another obvious risk of the use of high avidity tumor associated self-antigen specific T-cells in the clinic is the occurrence of on-target toxicity, by the potential recognition of non-malignant cells expressing the tumor associated antigen. Several research groups who

applied adoptive celle therapy have indeed found organ specific toxicity. Infusion of high avidity T-cells directed against the RCC antigen carboxy anhydrase IX (CAIX) resulted in severe cholestasis, based on the CAIX expression by bile duct epithelial cells[45] and patients who received high avidity MART-1 or gp100 T-cells developed uveitis and hearing loss due to expression of MART-1 and gp100 by melanocytes in eye and ear[43]. The one patient who received T-cells expressing chimeric antigen receptor (CAR) specific for the TAA HER-2/neu died of respiratory distress based on HER-2/neu expression in the lungs[47]. The disabling or mortal on-target toxicity observed in these studies suggests the need for validated cell type specific screening of the high avidity TAAs specific T-cells before application in the clinic. As described in chapter 5, we screened the high avidity PRAME specific T-cells against multiple different cells derived from different organs, and found unexpected and not yet described reactivity against kidney tubular epithelial cells and mature DCs. Downregulation of PRAME by siRNA demonstrated that this reactivity was indeed based on on-target recognition. Based on these results, we propose that high avidity tumor associated self-antigen specific T-cells destined for use in the clinic, should be thoroughly screened against multiple cell lines or primary cells derived from different organs, in order to assess the on-target toxicity that can be expected when these T-cells are infused in patients. Additionally, expression arrays including the targeted tumor associated antigen could be performed on tissue samples derived from multiple different organs.

The discovered reactivity of the high avidity PRAME specific T-cells against kidney tubular epithelial cells and mature DCs could possibly lead to nephrotoxicity and to reduced immunity, respectively. Co-transduction of the PRAME-TCR engineered T-cells with a suicide gene could resolve the temporary loss of mDCs. In addition, the low reactivity observed against cultured PTECs *in vitro* does not necessarily indicate nephrotoxicity *in vivo,* as indicated by absence of renal dysfunction in the patient. Therefore, since the toxicity of high avidity PRAME specific T-cells could also be very limited or absent, and because the large potential benefits of this therapy, we decided to continue our development of clinical applicable PRAME-TCR viral constructs and aim at applying adoptive cell therapy using high avidity PRAME specific TCR transduced T-cells in the clinic.

Besides the PRAME specific T-cells, other allo-HLA reactive TAAs specific T-cells could be searched for in PBMCs derived from patients undergoing GVHD and / or GVL response after HLA mismatched transplantations. Using tetramers of all known TAAs, in vivo activated and thereby probably single peptide specific allo-HLA reactive TAAs specific T-cells could be searched for. Alternatively, allo-HLA reactive T-cells activated in an in vivo immune response leading to GVHD or GVL could be screened against non-malignant and malignant cells, and the specificities of those cells only recognizing malignant cells could be identified. Thereby, a whole arsenal of high avidity TAAs specific T-cells could be created.

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NEDERLANDSE SAMENVATTING

Stamceltransplantaties worden toegepast bij de behandeling van hematologische maligniteiten zoals leukemie. Voorafgaand aan stamceltransplantaties worden patiënten behandeld met chemotherapie en radiotherapie met als doel zoveel mogelijk leukemiecellen te vernietigen. Behandeling met chemo- en radiotherapie resulteert echter ook in vernietiging van gezonde stamcellen verantwoordelijk voor de aanmaak van bloedcellen (hematopoietische stamcellen), waardoor alle verschillende hematopoietische cellen niet meer gevormd kunnen worden. Om te voorkomen dat de patiënt daaraan overlijdt, worden door een stamceltransplantatie (SCT) de hematopoietische stamcellen van de patiënt vervangen door gezonde stamcellen (graft). Behalve het vervangen van de patiënt stamcellen, kan een stamceltransplantatie van een donor leiden tot een graft versus leukemie (GVL) reactie, waarbij de afweercellen (T-cellen) van de donor de leukemiecellen herkennen en opruimen. De donor T-cellen kunnen echter ook gezonde weefselcellen van de patiënt als vreemd herkennen en afstoten, wat kan leiden tot graft versus host disease (GVHD). Hierbij zijn vaak huid, darmen, lever of longen aangedaan. Verwijdering van de T-cellen uit het stamceltransplantaat verkleint de kans op GVHD, maar het vergroot de kans op het recidiveren van de leukemie. Om een recidief van de leukemie te voorkomen, worden patiënten enkele maanden na de SCT behandeld met een donor lymfocyten infusie (DLI). Door eerst T-cellen uit de SCT te depleteren en pas na enkele maanden een DLI toe te dienen, wordt een pauze gecreëerd tussen de weefsel beschadigende chemo- en radiotherapie en de toediening van T-cellen. Hierdoor is de kans op het ontstaan van GVHD na een T-cel gedepleteerde SCT, gevolgd door DLI, kleiner dan na een niet T-cel gedepleteerde SCT. Echter, ook na DLI blijft er een aanzienlijke kans op het ontstaan van GVHD.

Stamceltransplantatie van een donor die dezelfde HLA typering heeft als de patiënt, reduceert de kans op het ontstaan van GVHD. HLA-moleculen zijn eiwitten die aanwezig zijn op het celmembraan van alle kernhoudende cellen. Het doel van HLA-moleculen is het presenteren van peptiden op de oppervlakte van cellen die vervolgens door T-cellen herkend kunnen worden. De peptiden die gepresenteerd worden, zijn afkomstig van eiwitten van binnen of buiten de cel en van lichaamseigen of van pathogeen afkomstige eiwitten. De HLA-moleculen die betrokken zijn bij conventionele immuunresponsen bestaan uit twee groepen: HLA-klasse I en HLA-klasse II. HLA- klasse I bestaat weer uit 3 groepen: A, B en C. HLA-klasse II bestaat uit de groepen DR, DQ, DP. De genen die coderen voor deze HLA-moleculen behoren tot de meest polymorfe genen van het menselijk genoom. Van elke HLA-groep (A,B,C,DR,DQ en DP) bestaan er tientallen tot honderden typen die allemaal verschillend zijn tussen verschillende individuen. Het is daarom zeer onwaarschijnlijk dat twee willekeurig geselecteerde individuen dezelfde HLA-typering zullen hebben.

Omdat complete HLA-matching tussen patiënt en donor de kans op het ontstaan van GVHD verkleint, hebben HLA-identieke SCT's de voorkeur boven HLA niet-identieke SCT's. Helaas

heeft elke broer of zus van de patiënt maar 25% kans op een volledige HLA-match met de patiënt. Bij de afwezigheid van een HLA-identieke broer of zus, kan een donor gezocht worden in de internationale donordatabank, die 9.000.000 donoren bevat. Voor ongeveer 70% van de West-Europese en Noord-Amerikaanse Caucasische patiënten kan een HLA-identieke donor worden gevonden in de donordatabank. De kans op het vinden van een HLA-identieke donor daalt echter tot 10-15% voor niet-Caucasische patiënten. Patiënten voor wie geen HLA-identieke donor kan worden gevonden, kunnen getransplanteerd worden met stamcellen van een HLA-niet-identieke donor. Het is aangetoond dat een mismatch tussen patiënt en donor in een van de A, B, C, DRB1 of DQB1 loci een negatief effect heeft op het resultaat van de SCT.

Zowel GVHD als GVL respons worden hoofdzakelijk veroorzaakt door T-cellen. T-cellen zijn een deel van het adaptieve immuunsysteem en spelen een belangrijke rol in immuunresponsen tegen verschillende pathogenen zoals virussen en parasieten. T-cellen brengen T-cel receptoren (TCR) tot expressie waarmee ze zeer specifiek antigenen in de vorm van vreemde peptiden gepresenteerd in HLA kunnen herkennen. CD8 T-cellen herkennen peptiden in de context van HLA-klasse I en CD4 T-cellen herkennen peptiden in de context van HLA-klasse II. In de thymus, waar de T-cellen zich ontwikkelen, vindt een negatieve selectie plaats van T-cellen die gericht zijn tegen eigen peptiden gepresenteerd in HLA moleculen. Als resultaat hiervan is het T-cel-repertoire in een individu tolerant voor alle zelf-peptide-HLA-complexen. Bij immuunresponsen na HLA-identieke SCT en DLI herkennen de alloreactieve T-cellen peptiden die polymorf zijn tussen patiënt en donor op basis van enkel nucleotide polymorfismen, genaamd minor histocompatibility antigenen (MiHA). T-cellen die gericht zijn tegen MiHA die specifiek tot expressie komen op hematopoietische cellen of op tumorcellen zorgen voor GVL, terwijl GVHD wordt veroorzaakt door T-cellen die MiHA herkennen die tot expressie komen in de verschillende hematopoietische en niet-hematopoietische weefsels. Het is aangetoond dat in een HLA-identieke setting GVHD- en GVL- response van elkaar gescheiden kunnen worden door de T-cellen te selecteren die gericht zijn tegen hematopoietisch of tumor-specifieke MiHA.

Bij GVHD en GVL na HLA niet-identieke SCT en DLI zijn de alloreactieve T-cellen gericht tegen vreemde HLA-moleculen, waarop ze niet zijn geselecteerd tijdens hun ontwikkeling in de thymus. Net als na HLA-identieke SCT, gaat GVHD na HLA-niet-identieke SCT ook vaak gepaard met GVL-responsen, wat erop wijst dat allo-HLA reactieve T-cellen potentieel gebruikt zouden kunnen worden voor nuttige doeleinden. In het promotieonderzoek, beschreven in dit proefschrift, is onderzocht hoe een allo-HLA gerichte immuunrespons geïnitieerd wordt en welke cellen verantwoordelijk zijn voor de activatie van zo'n respons. Daarnaast is onderzocht welke T-cellen verantwoordelijk zijn voor allo-HLA reactiviteit en op welke manier vreemd HLA herkend wordt door allo-HLA reactieve T-cellen. Ten slotte is onderzoek gedaan naar het bestaan en de mogelijke effectiviteit van tumor specifieke allo-HLA reactieve T-cellen en vervolgens geanalyseerd of deze T-cellen veilig zouden zijn voor klinische doeleinden.

In de studie beschreven in **hoofdstuk 2** is een immuunrespons gericht tegen HLA klasse I gekarakteriseerd die plaats had gevonden in een patiënt tijdens GVHD. De GVHD was ontstaan na een HLA-A2 niet-identieke DLI, in de afwezigheid van inflammatoire condities. Een eerder toegediende DLI afkomstig uit dezelfde donor had niet geleid tot een immuunrespons. Dit toonde aan dat het memory T-cel compartiment van de donor geen substantiële groep CD8 T-cellen bevatte die kruisreactief was tegen HLA-A2, wat er op wijst dat CD4 T-cellen nodig waren voor de initiatie van de allo-immuunrespons. CD8 en CD4 donor T-cellen die geactiveerd waren tijdens de GVHD werden door middel van single cel sorteren individueel geïsoleerd en opgegroeid tot T-cel klonen. Analyse van de klonale diversiteit en alloreactiviteit van de geïsoleerde CD4 en CD8 T-cel klonen toonde aan dat tijdens de GVHD zowel een polyklonale CD8-respons als een polyklonale CD4-respons aanwezig was. Analyse van de HLA-restrictie en specificiteit van de alloreactieve CD8 en CD4T-cellen toonde vervolgens aan dat de CD8-respons gericht was tegen het allo-HLA-A2-molecuul en dat de CD4-respons gericht was tegen peptiden afkomstig van het allo-HLA-A2-molecuul gepresenteerd in HLA-klasse II. Analyse van het bloed en beenmerg afgenomen ten tijde van de twee DLI's toonde aan dat de leukemische blasten de enige HLA-klasse II positieve patiëntcellen waren die aanwezig waren tijdens de GVHD-inducerende tweede DLI, en niet aanwezig waren tijdens de eerste DLI, die niet tot een immuunrespons had geleid. Stimulatie van de alloreactieve T-cellen door de leukemische blasten toonde aan dat deze cellen in staat waren om zowel de alloreactieve CD8 als CD4T-cellen te activeren. De resultaten van deze studie laten zien dat de GVHD werd veroorzaakt door een gecoördineerde CD4- en CD8-respons gericht tegen het allo-HLA-A2-molecuul en suggereren dat de leukemische blasten zowel de CD8- als de CD4-respons hebben geactiveerd.

Welke T-cellen verantwoordelijk zijn voor allo-HLA-reactiviteit en of dit een eigenschap is van een specifieke subgroep van T-cellen of van alle T-cellen is onbekend. Het is aangetoond dat allo-HLA-reactiviteit zowel in de naïve als in de memory T-cel-populaties aanwezig is. Echter, allo-HLA- reactiviteit zou vooral ernstige consequenties kunnen hebben als het wordt uitgeoefend door memory-T-cellen, omdat memory-T-cellen efficiënt geactiveerd kunnen worden door niet-professionele antigen presenterende cellen doordat ze geen co-stimulatie vereisen. In de studie beschreven in **hoofdstuk 3** is onderzocht of virusspecifieke T-cellen allo-HLA-reactiviteit kunnen uitoefenen. Het screenen van een groot aantal virusspecifieke T-cel-lijnen en -klonen tegen een panel van EBV-getransformeerde B-cellen (EBV-LCLs), die samen bijna alle veel voorkomende HLA-klasse I en II moleculen tot expressie brachten, toonde aan dat 80% van de virusspecifieke lijnen en 45% van de virusspecifieke klonen allo-HLA reactief waren. Deze alloreactiviteit is aangetoond voor Epstein-Barrvirus, Cytomegalovirus, Varicella zostervirus en Influenzavirus specifieke T-cellen. Het testen van de alloreactieve virusspecifieke T-cel klonen tegen targetcellen positief voor 1 HLA-molecuul (HLA getransduceerde K562-cellen) bevestigde dat de alloreactiviteit gebaseerd was op allo-HLA reactiviteit. Het overzetten van de TCR in andere T-cellen met behulp van gentransfer toonde aan dat

zowel de virusspecificiteit als de allo-HLA-reactiviteit uitgevoerd werden door dezelfde TCR. Drie T-cel klonen met dezelfde specificiteit en afkomstig uit hetzelfde individu, maar met verschillend TCR gebruik, toonden herkenning van drie verschillende allo-HLA-moleculen, wat er op wijst dat allo-HLA-reactiviteit niet kan worden voorspeld op basis van virusspecificiteit. Om de resultaten verkregen met de EBV-LCL's en K562-cellen te kunnen extrapoleren naar herkenning van gewone cel subgroepen in vivo, zijn allo-HLA reactieve virusspecifieke T-cel klonen getest tegen geactiveerde en niet geactiveerde normale celsubgroepen. De resultaten in deze studie tonen aan dat T-cellen specifiek voor verschillende virussen kruisreactiviteit vertonen tegen allo-HLA-moleculen, en wijst op de hoge frequentie van T-cellen die allo-HLA-reactiviteit kunnen uitoefenen.

T-cel-reactiviteit tegen allo-HLA wordt verondersteld minder peptidespecifiek te zijn dan conventionele T-cel-reactiviteit. Deze veronderstelling is gebaseerd op het vermogen van allo-HLA reactieve T-cellen om targetcellen deficiënt in het verwerken van peptiden in HLA (TAP-deficiënte T2 cellen) te herkennen en om multipele synthetische peptiden beladen op T2-cellen te herkennen. Echter, de specificiteit van allo-HLA reactieve T-cellen tegen endogeen verwerkte en gepresenteerde peptiden is niet onderzocht. Daarnaast waren de voorheen bestudeerde allo-HLA reactieve T-cellen voornamelijk in vitro geactiveerd en geëxpandeerd. In de studie beschreven in **hoofdstuk 4** is onderzocht wat de mate is van peptidespecificiteit en aviditeit van de biologisch relevante allo-HLA-reactiviteit van T-cellen. Hiervoor werden vijftig verschillende allo-HLA-A2 reactieve T-cel klonen, die in vivo geactiveerd en geëxpandeerd waren tijdens GVHD, getest tegen HPLC-fracties van peptiden geëlueerd uit HLA-A2 en beladen op TAP-deficiënte T2-cellen. Elke kloon toonde herkenning van één enkele fractie, wat aantoonde dat de in vivo geactiveerde allo-HLA reactieve T-cellen single peptide specifiek waren. Allo-HLA reactieve T-cel klonen die T2-cellen herkenden zonder exogeen beladen peptiden, werden getest tegen peptiden die geëlueerd waren uit HLA-A2 van T2-cellen en beladen op HLA-A2-expresserende drosophila cellen, en bleken ook single peptide specifiek te zijn. De door de T-cel klonen herkende peptiden werden geïdentificeerd door middel van multidimensioneel HPLC-fractionering en mass spectrometry. Peptide verdunningen toonden vervolgens aan dat de geïdentificeerde peptiden met hoge affiniteit werden herkend door de desbetreffende allo-HLA reactieve T-cel klonen. De single peptide specificiteit van de allo-HLA reactieve T-cellen werd ten slotte bevestigd door het verlagen van de expressie van de herkende antigenen door middel van silencing RNA. De resultaten van deze studie tonen aan dat de biologische relevante allo-HLA reactiviteit van in vivo geactiveerde en geëxpandeerde T-cellen single peptide specifiek is.

Omdat er in de thymus geen negatieve selectie plaatsvind van T-cellen die zelf-peptiden herkennen in vreemd HLA, kunnen allo-HLA reactieve T-cellen hoog avide reactiviteit uitoefenen tegen zelf-peptiden die hoog tot expressie komen op tumoren (tumor geassocieerde antigenen, TAA's) gepresenteerd in allo-HLA. Hierdoor kunnen allo-HLA reactieve T-cellen effectief zijn tegen tumorcellen waardoor ze potentieel bruikbaar zijn voor adoptieve T-

celtherapie. Op basis van de in hoofdstuk 4 aangetoonde single peptide specificiteit van in vivo geactiveerde en geëxpandeerde T-cellen, zouden TAA specifieke allo-HLA reactieve T-cellen daarnaast veilig kunnen zijn voor gebruik in adoptieve T-celtherapie. In de studie beschreven in **hoofdstuk 5** zijn TAAs specifieke allo-HLA reactieve T-cellen gezocht in een allo-HLA-A2 gerichte immuunrespons leidend tot GVHD, wat resulteerde in de isolatie van twee hoog avide PRAME-specifieke T-cel klonen. PRAME (preferentially expressed antigen in melanoma) is een TAA die hoog tot expressie komt in verschillende maligniteiten waaronder melanoom, niercelcarcinoom en verschillende leukemieën. De PRAME-specifieke T-cellen toonden aan single peptide specifiek te zijn en hun herkenning was strikt gecorreleerd met PRAME-expressie. In tegenstelling tot zelf-HLA gerichte PRAME specifieke T-cel klonen, vertoonden de allo-HLA gerichte PRAME specifieke T-cel klonen hoge reactiviteit tegen veel verschillende tumorcellijnen en tegen primaire melanoom- en leukemiecellen. Daarentegen vertoonden ze geen reactiviteit tegen veel verschillende niet maligne cellen. Echter, de klonen waren wel reactief tegen gematureerde dendritische cellen en nierepitheelcellen. T-cellen getransduceerd met PRAME-TCR toonden hoge PRAME-specifieke tumorreactiviteit, wat bevestigde dat de PRAME-TCR gebruikt kan worden voor TCR-gentherapie. Klinische studies bij patiënten met gemetastaseerde PRAME positieve maligniteiten, zoals melanoom en niercelcarcinoom, zullen uitwijzen wat de effectiviteit is van de tumorreactiviteit van de PRAME specifieke TCR'en in vivo en wat de klinische relevantie is van de mogelijke toxiciteit tegen gematureerde dendritische cellen en nierepitheelcellen.

De resultaten beschreven in dit proefschrift laten zien dat ook binnen allo-HLA reactiviteit T-cellen zich houden aan de algemene wetten van T-cel immunologie. Zo vond in een allo-HLA gerichte immuunrespons coördinatie plaats tussen een CD4- en een CD8-respons. Het uitoefenen van allo-HLA-reactiviteit door virusspecifieke T-cellen toonde aan dat dit een normale eigenschap van T-cellen is. Daarnaast is aangetoond dat biologisch relevante allo-HLA reactiviteit single peptide specifiek is. Door dit begrip over de biologie van T-cellen binnen allo-HLA reactiviteit hebben we kunnen concluderen dat allo-HLA reactieve T-cellen, behalve effectief, ook veilig zouden kunnen zijn voor adaptieve T-celtherapie. Onze zoektocht naar tumor antigen specifieke T-cellen heeft geleid tot de identificatie van twee PRAME/HLA-A2 specifieke TCR'en die gebruikt zullen worden in klinische studies.

NAWOORD

Promoveren doe je alleen. Je verdedigt je in je eentje tegenover de commissie en alleen jij krijgt alle eer en felicitaties. Vooral dat laatste voelt niet rechtvaardig, want promotieonderzoek doe je niet alleen.

Twee mensen die een zeer grote bijdrage aan mijn onderzoek hebben geleverd en met wie ik daarom alle eer zou willen delen, zijn Dirk van de Steen en Renate Hagendoorn. Dirk heeft de HPLC-fractioneringen gedaan. Dankzij zijn eigenwijsheid en doorzettingsvermogen zijn onder andere de PRAME-specifieke klonen geïdentificeerd. Renate heeft me aan het begin van de promotie bijgestaan door de CDR3-gebieden van alle TCR'en te sequensen, waarmee we hebben kunnen aantonen dat we beschikten over 50 verschillende alloreactieve CD8 T-cel klonen. Later was ze ook onmisbaar met de silencing RNA-experimenten en de kwantitatieve PCR's, waarmee we de single peptide specificiteit en on-target PRAME specificiteit hebben kunnen bevestigen.

Daarnaast zijn er nog veel meer collega's van de afdeling Experimentele Hematologie die ik voor hun bijdrage aan mijn onderzoek of proefschrift zou willen bedanken. Zoals meestal aan het begin van een promotie, was het ook aan het begin van mijn promotie niet helemaal duidelijk waar het heen zou gaan en wat we precies zouden gaan doen. Ik kwam er toen snel achter dat om inspiratie en goede ideeën op te doen je met Michel op vrijdagmiddag koffie moest gaan drinken. Tijdens één van die vrijdagmiddag-brainstormsessies stelde Michel als eerste voor om "gewoon de peptiden van de allo-klonen te identificeren". Zijn voorstel heeft uiteindelijk geleid tot hoofdstuk 4 en 5 van dit proefschrift. Daarnaast heeft Michel veel geholpen met het opzetten en uitvoeren van de HPLC-fractioneringen. Renate de Boer heeft, met haar gave om grote en ingewikkelde experimenten zonder stress en binnen de tijd uit te voeren, de TCR gentransfer-experimenten op zich genomen, samen met Marleen de EBV-panel gemaakt en mij geholpen met veel andere experimenten. Aan Marieke heb ik veel te danken vanwege al haar PRAME-gerelateerde kennis en materialen die ze met ons heeft gedeeld. Daarnaast heeft Marieke veel goede voorstellen, ideeën en inzichten geleverd en veel gezelligheid en vertrouwen zowel in Leiden als tijdens congressen. Kees heeft "de klonen van Avital" opgegroeid en ingevroren, waar veel experimenten mee zijn uitgevoerd. Daarnaast was hij ook betrokken bij de HPLC-fractioneringen. Simone heeft zonder mij de MLR's uitgevoerd omdat ik net zwanger was en dat keurig voor zich gehouden. Alwine stond altijd klaar met keratinocyten en fibroblasten en met medium en groeifactoren voor deze cellen. De peptide-identificatie zijn we begonnen met cDNA library screening, waarmee Willie en Edith mij veel hebben geholpen. Guido en Menno hebben mij geholpen bij het celsorteren. Nelleke en Henriette hebben natuurlijk al het bovenstaande mogelijk gemaakt.

Deel uit te maken van "Mirjam's groepje" was heel prettig. De andere deelnemers daarvan, Dirk, Renate, Renate, Esther, Pleun, Marleen, Monique en Hetty wil ik bedanken voor het meedenken tijdens de wekelijkse werkbesprekingen en voor de gezelligheid op kamer 37.

Op C5 was het meestal vrij stil, want er werd goed doorgewerkt. Toch was het daar met elkaar erg gezellig en vertrouwd, bijna knus. De collega's met wie ik de werkplek op C5 heb gedeeld wil ik bedanken voor de leuke sfeer en gezelligheid. Daarnaast heeft Sanja, tegenover wie ik op C5 zat, altijd de tijd en aandacht opgebracht om mijn presentaties aan te horen en mijn abstracts, inleidingen en discussies door te lezen en daarover bijdragende kritiek te geven.
Ook aan aantal mensen van buiten de afdeling experimentele hematologie wil ik bedanken. Dankzij Peter van Veelen en Arnoud de Ru zijn de door de allo-klonen herkende peptiden geïdentificeerd. Voor hoofdstuk 3 van dit proefschrift heb ik samengewerkt met Lloyd D'Orsogna, Dave Roelen, Ilias Doxiadis en Frans Claas van de IHB. Deze samenwerking was behalve prettig ook erg vruchtbaar.
Op kantoor bij Wietse en Michel heb ik, tussen het kolven op de serverkamer door, mijn proefschrift afgeschreven. Ik wil Wietse, Michel en de andere medewerkers van i2i bedanken voor deze plek en voor de gezellige en gezonde lunches. Carla wil ik bedanken voor het reviewen van het Nederlandse deel van het proefschrift. Yoshua van de Huurlingen bedank ik voor het ontwerpen van de kaft.
Ten slotte zou zonder mijn ouders en schoonouders het proefschrift waarschijnlijk nooit zijn afgekomen. Ik bedank ze voor alle tijd, geduld en liefde die zij aan Sara en Simcha hebben gegeven waardoor ik zonder zorgen kon schrijven.

CURRICULUM VITAE

Avital Amir is geboren op 12 september 1979 in Hadera, Israël. Het eerste deel van haar jeugd heeft ze doorgebracht in Israël in een kibboets genaamd Ein Hahoresh en in 1990 verhuisde zij naar Nederland. Na het cum laude behalen van haar VWO diploma aan het Jan van Echmond college in Purmerend in 1998, is zij in datzelfde jaar begonnen met de studie Geneeskunde aan de Universiteit van Amsterdam. Voor haar wetenschappelijke stage ging zij naar Boston, USA, waar ze in een laboratorium van het aan Harvard geaffilieerde Beth Israel Deaconess Medical Center onder leiding van Prof. Dr. Steven Balk onderzoek heeft gedaan naar het ontstaan van prostaatkanker. De stage is afgerond met een publicatie als eerste auteur in the Journal of Biological Chemistry. Een tweede stage heeft ze gelopen aan het Hubrecht Laboratorium in Utrecht waar ze onder leiding van Prof. Dr. Hans Clevers onderzoek heeft gedaan naar het ontstaan van darmkanker. Tijdens de co-schappen liep ze haar keuze co-schap op de afdeling oncologie en hematologie in het Onze Lieve Vrouwen Gasthuis in Amsterdam en haar oudste co-schap in het kankercentrum Antoni van Leeuwenhoek in Amsterdam. In 2005 behaalde ze haar artsexamen en begon ze aan het in dit proefschrift beschreven promotieonderzoek onder leiding van Dr. Mirjam Heemskerk en Prof. Dr. Fred Falkenburg op het laboratorium voor experimentele hematologie in het Leids Universitair Medisch Centrum. In 2010 begon ze met de opleiding interne geneeskunde in het Rijnland ziekenhuis in Leiderdorp, wat ze echter in april 2012 verruilde voor de opleiding pathologie in het AMC. Avital Amir is partner van Samuel Smits en moeder van Sara en Simcha Smits.

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