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The human minor histocompatibility antigen HA-1 : its processing, presentation and recognition

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The human minor
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its processing, presentation and recognition

Mommaas Bregje, Leiden, The Netherlands

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The human minor
histocompatibility antigen HA-1
its processing, presentation and recognition

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*Voor mijzelf
Aan mijn ouders*

CONTENTS

CHAPTER 1	General introduction	9
CHAPTER 2	Extreme instability of HLA-A2/HA-1 ^R peptide complexes explains the absence of cell surface expression of minor histocompatibility antigen HA-1 ^R in HLA-A2 <i>Manuscript in preparation</i>	31
CHAPTER 3	Competition-based cellular peptide binding assays for 13 prevalent HLA class I alleles using fluorescein-labeled synthetic peptides <i>Human Immunology. 2003;64:245-255</i>	49
CHAPTER 4	Identification of a novel HLA-B60 restricted T cell epitope of the minor histocompatibility antigen HA-1 locus <i>The Journal of Immunology. 2002;169: 3131-3136</i>	69
CHAPTER 5	Cord blood comprises antigen-experienced T cells specific for maternal minor histocompatibility antigen HA-1 <i>Blood. 2005;105:1823-1827</i>	85
CHAPTER 6	Adult and cord blood T cells can acquire HA-1 specificity through HA-1 T cell receptor gene transfer <i>Haematologica/The Hematology Journal. 2005;90:1415-1421</i>	101
CHAPTER 7	General discussion	119
	Abbreviations	135
	Summary	139
	Samenvatting	145
	Nawoord	151
	Curriculum vitae	155



CHAPTER 1

General introduction



Contents

- 1.1 Definition of minor histocompatibility antigens
- 1.2 Characteristics of minor histocompatibility antigens
 - 1.2.1 mH antigens are peptides presented by MHC molecules
 - 1.2.2 The origin of mH antigens
 - 1.2.3 Mechanisms for generation of mH antigens
 - 1.2.3.1 mH antigens due to gene polymorphisms
 - 1.2.3.2 mH antigens due to gene deletion
 - 1.2.3.3 mH antigens encoded by an unconventional ORF
- 1.3 Processing and presentation for the generation of minor histocompatibility antigens in complex with MHC class I
 - 1.3.1 Antigen processing and presentation by MHC class I molecules
 - 1.3.2 T cell recognition of MHC presented mH peptides
- 1.4 Clinical relevance of minor histocompatibility antigens
 - 1.4.1 GvHD and GvL
 - 1.4.2 Tissue distribution
 - 1.4.3 Population frequency
 - 1.4.4 Clinical importance of mH antigen HA-1 association with GvHD and GvL
- 1.5 The minor histocompatibility antigen HA-1 as a tool for immunotherapy
 - 1.5.1 Identification of HA-1
 - 1.5.2 HA-1^H directed immunotherapy
 - 1.5.3 Alternative strategies for HA-1^H specific CTL generation
- 1.6 Aim of the study

1.1 Definition of minor histocompatibility antigens

Allogeneic stem cell transplantation (SCT) from a related or unrelated donor is a well-established and effective therapy for advanced hematological malignancies¹. Besides the required graft versus leukemia (GvL) response, donor cells may initiate graft versus host disease (GvHD). GvHD is caused by disparities between tissue antigens expressed by patient and donor. Donor T cells recognize tissue antigens expressed by patient cells but not by donor cells, resulting in a vigorous T cell response. Likewise, following tissue or organ transplantation, tissue antigen disparities can cause graft rejection by patient T cells recognizing tissue antigens expressed by the graft but not by patient cells. In the beginning of the 20th century, Little and Tyzzer² discovered these tissue antigens by the observation that the rejection of tumour grafts by inbred mouse strains was regulated by independently segregating gene loci. The congenic mouse strains generated by Snell in the 1940s³, made identification possible of single loci responsible for tumour graft rejection. The chromosomal segment determining susceptibility or resistance to tumour or tissue transplants was said to contain a Histocompatibility (H) locus. H loci strongly differed in the speed with which tumour graft rejection was induced. Snell identified thirteen H loci. The H-2 locus was characterized by the strongest rejection and therefore was later named Major Histocompatibility Complex (MHC)^{4,5}. All other Histocompatibility loci were grouped under the name Minor Histocompatibility (mH) loci, although incompatibility for a number of mH loci could be as strong as MHC incompatibility⁶. This is illustrated by the fact that polymorphic mH loci are capable of inducing mH specific MHC restricted T cell responses^{7,8}. The total number of murine mH loci is estimated to be over several hundreds^{6,9}. Murine mH antigens have been shown to be encoded by almost every autosomal chromosome, on the Y chromosome and on the mitochondrial genome^{10,11}.

In human the existence of mH antigens was found after the discovery of the Human Leucocyte Antigens (HLA); the human analogue of the murine MHC¹². In HLA identical settings, bone marrow transplantations still resulted in GvHD^{13,14} indicating histo-incompatibility beyond HLA. T cells obtained after HLA identical marrow transplantations indeed defined human mH antigens. Generation of MHC class I restricted cytotoxic T lymphocyte (CTL) lines or -clones has made *in vitro* definition of single human mH antigens possible¹⁵⁻¹⁷. The generation of multiple human mH specific T cell clones within one donor recipient combination indicated the existence of a high number of different human mH antigens^{18,19}. In addition to HY specific T cell clones, T cell clones specific for autosomally encoded mH antigens were found^{15,16}. Furthermore, family segregation studies demonstrated that several mH antigens were encoded by germline genes²⁰⁻²⁵.

1.2 Characteristics of minor histocompatibility antigens

1.2.1 mH antigens are peptides presented by MHC molecules

In the 1980s it became clear that T cells recognize peptides in the context of MHC molecules. To determine whether mH antigens are also peptides in complex with MHC, cells expressing a defined mH antigen have been enzymatically digested into protein extracts after which the fragments were separated by reverse phase HPLC. Then the peptides were loaded on mH antigen negative target cells and tested by mH antigen specific CTLs. These CTLs recognized material from a distinct position of the HPLC elution profile, while their activity was absent when the material was treated with proteases of broad specificity, which shows the peptidic nature of the mH antigens²⁴. Furthermore, the CTLs were only active when the restricting MHC molecule was expressed by the target cell, which indicates that the isolated peptides were naturally presented in the context of MHC^{25,26}. Similarly, human mH antigens were also shown to be peptides presented by MHC molecules. This was demonstrated by immunoprecipitation of the mH antigen presenting MHC class I molecules followed by acid treatment, separation of the peptides from the MHC molecules and fractionation of the peptides on reverse phase HPLC²⁷⁻²⁹. The Goulmy group was the first to purify HLA-A2 molecules that contain the natural human mH peptide HA-2³⁰. Using these and other sophisticated techniques, our group and others have subsequently identified an as yet relative small number of human mH loci²⁹⁻⁴⁵ encoded by genes on the Y chromosome^{51,52,54-57,40,44,45} and by autosomal genes i.e.: HA-1, HB-1, HA-8, BCL2A1, UGT2B17 and HA-3^{29,35,58,41-43}.

1.2.2 The origin of mH antigens

The small number of human mH antigens identified to date revealed to consist of peptides derived from naturally processed intracellular proteins. These peptides are encoded by the Y chromosome or autosomal chromosomes and are presented by MHC class I and class II molecules. The peptide sequences of mH antigens HA-1, HA-2, HA-3, HA-8, HB-1, BCL2A1, UGT2B17 and several human HY mH antigens have been elucidated^{29-31,34,35,58,42,45,45-48}. HY antigens were found to be derived from the genes SMCY, DFFRY, DBY, UTY, RPS4Y and TMSB4Y, located on the Y-chromosome with an homologous gene on the X-chromosome^{51,52,54,55,57,40,44,45}. These HY antigens are all derived from household or transcription proteins except for the TMSB4Y antigen (described in section 1.2.5). The SMCY gene encodes an hypothetical transcription factor, the gene is involved in protein synthesis and the DFFRY, DBY and UTY genes are all involved in spermatogenesis⁴⁹. In contrast, the mH antigens HA-1, HA-2, HA-3, HA-8, HB-1, BCL2A1 and TMSB4Y are encoded by onco-related genes^{45,49}. HA-1 is derived from the di-allelic KIAA0223 gene located on chromosome 19²⁹, en-

coding a Rho-like GTPase-activating protein (GAP) while HA-2 is derived from a di-allelic gene encoding a novel human class I myosin protein³⁹. HA-3 is a product of the lymphoid blast crisis (Lbc) oncoprotein⁴⁵. The HA-1, HA-2 and HA-3 proteins are potentially linked together in one signaling cascade involved in cytoskeletal rearrangement⁴⁹. The HA-8-containing protein contains a Pumilio repeat region implicated in translational regression. Deletion of one of the major members of the Pumilio family (PUF) leads to disturbance of primary spermatocytes, which then develop into rapidly growing tumours⁵⁰. The gene encoding HB-1 shows only significant expression in acute lymphoblastic leukemia B cells and not in mature non-malignant B cells⁵⁵. BCL2A1 is a member of the anti-apoptotic BCL-2 protein family that suppresses apoptosis induced by the p53 tumour suppressor protein⁵¹. Finally, the X-chromosome homologue of TMSB4Y, known as thymosin β 4, encodes a protein that plays an important role in the organization of the cytoskeleton and is highly expressed in metastatic melanoma cells^{52,55}. All T cells specific for mH antigens encoded by onco-related genes except HA-2, were isolated from patients with hematological malignancies. In contrast, T cells specific for the mH antigens derived from household or transcription proteins have generally been isolated from patients suffering from an hematological disorder; not a malignancy⁴⁹.

1.2.3 Mechanisms for generation of mH antigens

1.2.3.1 mH antigens due to gene polymorphisms

Minor H antigens generally originate from amino acid polymorphisms in self-proteins. The first self protein derived mH antigen determined was maternally transmitted antigen (MTA)⁵⁴. This murine mH antigen was maternally inherited and thus of mitochondrial origin. CTLs were generated against four different alleles of MTA which differed from each other by one amino acid in the presented peptide⁵⁵. In several human MHC class I restricted mH antigens single, dual or triple amino acid polymorphisms have been found^{29,51,54,55,58,59,43,56}. These amino acid polymorphisms result in two allelic counterparts of the mH antigen, of which generally only one is capable of generating a CTL response. However, recognition of both alleles from the same mH locus has also been described^{56,57}. The two allelic counterparts of the HA-1 locus that have been previously described, result from a Histidine (H) to Arginine (R) substitution. Recently, two additional polymorphisms in the HA-1 locus have been reported. Namely an Aspartic acid (D) to Glutamic acid (E) substitution and a Glycine (G) to Serine (S) substitution at different positions (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=23526). These additional amino acid polymorphisms may lead to additional HA-1 antigens. To date, only the HA-1^H allele has been found to be immunogenic.

1.2.3.2 mH antigens due to gene deletion

The fact that deletion of a gene can give rise to a mH antigen disparity was first illustrated by Speiser et al. who showed that the nuclear myxovirus resistance protein Mx in mice could act as a mH antigen⁵⁸. In Mx⁻ mice both Mx⁺ skin graft rejection and Mx directed CTL response was observed. The allelism was a consequence of the absence or presence of the Mx protein, since in the Mx⁻ mice a part of the Mx gene was deleted^{59,60}. cDNA expression cloning provided the first evidence that gene deletion can be a mechanism for generating human mH antigens. Using this technique, a novel human mH antigen was identified encoded by UGT2B17, an autosomal gene in the multigene UDP-glycosyltransferase 2 family that is selectively expressed in liver, intestine, and antigen-presenting cells. The UGT2B17 antigen is immunogenic because of differential protein expression in donor and recipient cells as a consequence of homozygous gene deletion in UGT2B17 negative individuals⁴².

1.2.3.3 mH antigens encoded by an unconventional ORF

Evidence has been accumulating that cryptic polypeptides derived from non-coding regions or encoded in alternative open reading frames (ORF) occasionally encode CTL epitopes for tumour or viral antigens in humans or mice⁶¹. All as yet identified mH epitopes are encoded by conventional ORFs. However, a novel HY antigen encoded in the 5'-untranslated region of the TMSB4Y gene was identified recently⁴⁵. Probably, this novel HLA-A35 restricted mH antigen TMSB4Y is not derived from a functional polypeptide, but is a subsidiary translation product of the TMSB4Y transcript. This is the first demonstration of a mH antigen encoded outside a conventional ORF of a nonmutated gene.

1.3 Processing and presentation for the generation of minor histocompatibility antigens in complex with MHC class I

1.3.1 Antigen processing and presentation by MHC class I molecules

To be presented on the cell surface, intracellular proteins must be degraded into small peptides, which is generally known as antigen processing. Since this thesis focuses on mH antigen HA-1 presented by MHC class I, the processing and presentation of MHC class I binding peptides (figure 1) will be discussed only. MHC class I presented peptides are mainly generated in the cytosol by the 26S proteasome⁶², an approximately 2000 kDa multiprotein. The intracellular protein turnover is controlled by the 26S proteasome by a nonlysosomal, ATP- and ubiquitin-dependent pathway. Proteins are recognized and unfolded by the 19S caps of the proteasome after being marked for degradation by conjugation with ubiquitin. Subsequently, the 20S catalytic core of the 26S proteasome degrades

the unfolded proteins. This degradation results in peptides of mainly hepta- to nonameric lengths. The 20S proteasome consists of 2 outer rings of 7 α subunits and 2 inner rings of 7 β subunits. The β subunits preferentially cleave the C-terminus of aromatic, aliphatic, basic and glutamic acid residues. Following proteasomal peptide cleavage and TAP translocation (described below), further N-terminal trimming by aminopeptidases takes place in the endoplasmatic reticulum (ER)⁶⁵⁻⁶⁵. C-terminal trimming in the ER has not been demonstrated. The proper generation of the correct COOH terminus by an early major proteasome cleavage site is thus indispensable for efficient epitope generation⁶⁶⁻⁶⁹. The fact that the positions of certain amino acid residues in the protein can influence peptide processing in various manners is utilized by several peptide processing prediction programs⁷⁰ (<http://www.cbs.dtu.dk/services/NetChop/>; <http://www.paproc.de/>). Furthermore, peptide degradation takes place by proteases in the cytosol⁷¹⁻⁷⁵.

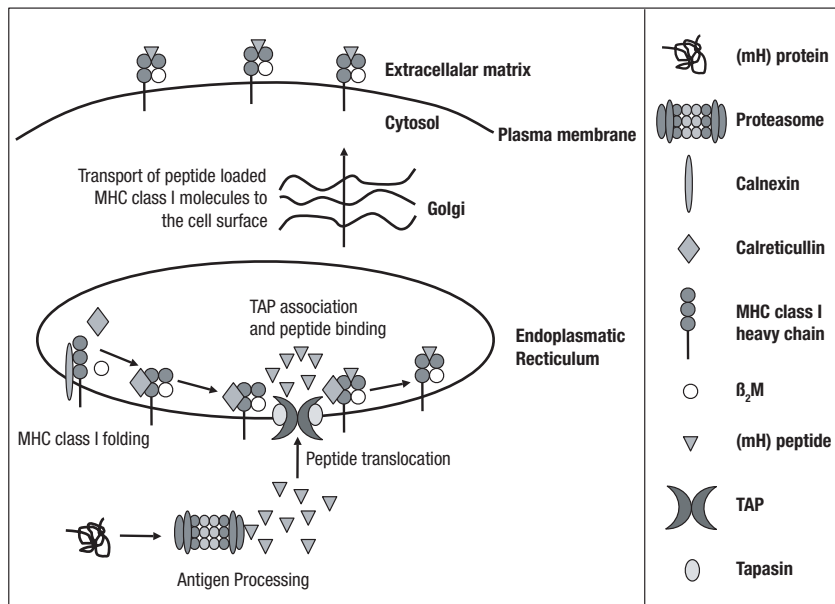


Figure 1
The MHC class I processing and presentation pathway

The transporter associated with antigen processing (TAP) is a heterodimer of the subunits TAP1 and TAP2, which are encoded in the MHC complex. Following peptide generation, TAP transports preferentially 8-12 amino acids long peptides, via an ATP-dependent process to the lumen of the ER^{74,75}. Like protein degradation, this TAP translocation is dependent on the positions of certain amino acid residues in the peptides to be transported⁷⁶. In the ER human

MHC class I heavy chain and β_2M assemble to an unstable heterodimer, which is facilitated by chaperones such as calnexin⁷⁷. Subsequently, the heterodimers dissociate from these chaperones and bind to calreticulin, another chaperone. This complex then binds to tapasin and TAP, after which MHC-peptide binding takes place⁷⁸. The resulting stable trimolecular complex of peptide, β_2M and MHC class I heavy chain then dissociates from TAP, moves to the Golgi apparatus and is eventually expressed on the cell surface. Generally, MHC class I molecules bind 9 amino acid long peptides in their groove, however, shorter and longer peptides have been described. The restricted peptide length is caused by the conserved end residues of the heavy chain- $\alpha 1$ and - $\alpha 2$ helices that close the ends of the MHC class I groove and interact with the N- and C-terminus of the peptide. Furthermore, the MHC class I groove consists of six pockets named A to F. Pockets A and F respectively accommodate the N- and C-terminus of the peptide in all MHC alleles, whereas the pockets B to E vary in size and hydrophobicity for the different MHC alleles. This leads to different requirements for the amino acid sequence of the peptide in order to bind to the MHC molecule^{79,80}. The MHC binding amino acid anchors are generally positioned at the second and/or third position and the ninth or last position of the peptide^{81,82}, forcing the peptide to bulge in the middle. This in turn leads to further variation of peptide conformation and thereby to variation of specific T cell receptor (TCR) interactions. The different sequence characteristics known to be involved in MHC-peptide binding are taken into account by binding prediction algorithms⁸³⁻⁸⁵ (<http://bimas.dcrf.nih.gov/>; <http://syfpeithi.bmi-heidelberg.com/>).

Since the positions of specific amino acid residues in proteins can influence peptide processing and TAP translocation, single amino acid substitutions of reciprocal mH alleles can interfere with cell surface expression. Causes of such "immunological null allelism" have been demonstrated for the allelic counterparts of mH antigens HA-3, which is destroyed by proteasomes, and HA-8, which is poorly translocated into the ER^{58,45}. The formation of mH antigens is thus dependent on correct antigen processing. Furthermore, MHC-peptide binding and dissociation rates are dependent on peptide motives. The peptide motives thus determine the MHC-peptide complex stability, which is important for epitope recognition⁸⁶.

1.3.2 T cell recognition of MHC presented mH peptides

The generation and loading of antigenic peptides takes place in different compartments of the cell, resulting in different sets of peptides presented by MHC class I or MHC class II molecules. MHC class I molecules present peptides derived from intracellular proteins such as mH proteins, whereas MHC class II molecules generally present peptides derived from exogenous proteins and transmembrane proteins. TCRs recognize their antigens as peptide fragments in

the groove of the MHC molecules. CD8⁺ cytotoxic T cells generally recognize antigen in the context of MHC class I molecules which are expressed on the cell surface of virtually all nucleated cells. CD4⁺ T helper cells generally recognize antigen in the context of MHC class II molecules, which are only expressed by cells such as (amongst others) macrophages, dendritic cells, and B lymphocytes that possess a specialized antigen presenting function. Both CD8⁺ and CD4⁺ T cell responses against mH antigens presented by respectively MHC class I and MHC class II molecules have been described⁸⁷.

The TCR heterodimer consists of two transmembrane glycoprotein chains, α - and β , which are attached by a disulfide bond. The extracellular portion of each chain consists of domains resembling the immunoglobulin variable (V) binding domain, -joining (J) domain and -conserved (C) domain. The crystal structures of both TCR subunits have been elucidated^{88,89}. The V binding sites of the TCR chains determine the antigen specificity of the T lymphocytes. For instance, all TCRs specific for HA-1^H in the context of HLA-A2 described thus far, express the same TCR β -chain BV7S9, illustrating the important role of this chain in HA-1^H specificity^{90,91}. On the contrary, several different TCR α -chains have been found in separate HA-1^H specific HLA-A2 restricted T cell clones⁹⁰ (chapter 6). The TCR repertoire is formed by selection in the thymus. Throughout thymic development, maturing thymocytes are continuously interrogated by their TCR to ensure proper expression of TCRs specific for non-self antigens and to exclude TCRs specific for self-antigens. Furthermore, the strength of TCR signals transmitted in a given thymocyte influences survival or elimination. *In utero*, the antigens encountered during thymic development may include maternal antigens not inherited by the child. Therefore, the TCR repertoire against non-inherited maternal antigens (NIMAs) may be debilitated⁹². However, possibly the encounter of maternal mH antigens in the thymus is too low to result in the elimination of their specific TCRs. Subsequently, T cell priming against mH NIMAs may take place *in utero*.

CTL recognition of a mH epitope in context of MHC class I is not only dependent on the immunogenicity of the mH peptide but also on its processing and presentation, as described in section 1.3.1. For instance, CTLs specific for either HA-2 in the context of HLA-A2, HA-3 in the context of HLA-A1 or HA-8 in the context of HLA-A2 are capable of recognizing both concerned allelic counterparts if the mH peptides are administered synthetically^{30,38,39,45}. Both allelic peptides from each mH locus contain a common amino acid motive since they are both recognized by the same TCR. However, the mH epitopes are presented by MHC class I molecules, whereas their allelic counterparts are not or at very low concentration present on the cell surface. Thus, the HA-2, HA-3 and HA-8 specific CTLs recognize those mH peptides that are correctly processed and presented.

1.4 Clinical relevance of minor histocompatibility antigens

1.4.1 GvHD and GvL

As previously mentioned, allogeneic stem cell transplantation (SCT) from a related or unrelated donor is a well-established and effective therapy for advanced hematological malignancies¹. Following transplantation, donor T cells may initiate graft versus host disease (GvHD). If the donor is HLA mismatched this is caused by cytotoxic T cell responses to patient HLA, disparate from donor HLA. Two pathologically distinct types of GvHD are known, namely acute- and chronic GvHD. The acute form may occur during approximately the first 100 days following SCT whereas the chronic form may occur after the 3rd month following SCT. The latter may develop de novo or follow acute GvHD. Both types of GvHD occur in different grades dependent on the severity of the disease. Following SCT from an HLA-genotypically identical sibling donor, patients still develop acute or chronic GvHD in 35–65% of the cases^{95,94}. The suggestion that incompatibility for mH antigens thus plays an important role in causing GvHD, was confirmed by the observation that GvHD following HLA-identical SCT significantly correlates with the disparity for a single mH antigen⁹⁵. Both T cells recognizing mH antigens and recognizing leukemia associated antigens were isolated from SCT patients^{96,97}. In addition, several studies described T cells that recognized mH antigens expressed by leukemic cells^{98,99}, indicating that mH specific T cells are not only important in the induction of GvHD but also in the induction of the Graft versus Leukemia (GvL) effect. In agreement with this finding, the occurrence of GvHD was associated with a decreased leukemia relapse rate¹⁰⁰, which is the result of donor T cells eliminating residual leukemic cells¹⁰¹. In order to prevent GvHD, T cell depletion of human bone marrow was introduced. This not only resulted in a dramatic reduction of GvHD, but unfortunately also in an increase of graft rejection and leukemia relapse rates^{102,105}. Relapsed leukemia patients can be successfully treated with donor lymphocyte infusion (DLI). The association of the GvL effect with GvHD after DLI further indicates the important role of mH antigens in both the GvL effect and GvHD^{104–106}. Donor CTLs specific for an immunogenic hematopoietic restricted mH allele will specifically recognize malignant recipient cells and induce a GvL response with only a low risk of GvHD.

1.4.2 Tissue distribution

To determine whether mH antigen expression is restricted to certain areas of the body, tissue distribution has been evaluated for several murine and human mH antigens, using mH antigen specific T cell clones^{107,108}. Several mH antigens were found to be expressed only by certain cell types, while others are expressed ubiquitously. Epa-1 is a murine mH antigen expressed only by macrophages, epidermal cells and fibroblasts¹⁰⁹, whereas the mH antigens H-4 and HY are

expressed by all murine organs²⁶. CTL-mediated lysis of tissue-derived cells and cultured cell lines was used as an *in vitro* assay for mH antigen expression of several human tissues. The human mH antigens HA-1 and HA-2 were found to be expressed only by cells of hematopoietic origin, including leukemic cells, hematopoietic progenitor cells, dendritic cells and Langerhans cells^{98,99,107,110}. In contrast, the human mH antigens HA-3, HA-4 and HY were expressed by all cells tested, which included cells of hematopoietic origin, immature thymocytes, fibroblasts, keratinocytes, melanocytes, epithelial cells and endothelial cells¹⁰⁷.

Cell surface presentation of mH antigens has been found to be an important cause for GvHD induction. mH antigens presented by different cell types might play different roles in the induction of GvHD. Mainly epithelial cells are affected during GvHD, however, only half of the murine mH antigens causing GvHD were found to be present on skin cells, whereas all of these mH antigens were present on lymphocytes or monocytes¹⁰⁸. Possibly these hematopoietic cells are sited in peripheral tissues which are, as a consequence, destroyed during GvHD¹¹¹. Differential tissue distribution of mH antigens may also be an important feature in targeting specific cell types using mH specific donor CTLs. For instance, the human mH antigens HA-1 and HA-2, expressed by cells of hematopoietic origin only, may be targeted specifically to induce a GvL response. When all remaining patient hematopoietic cells have migrated out of the peripheral tissues prior to administration of the mH specific donor CTLs, this GvL response can be generated without causing GvHD.

1.4.3 Population frequency

To map the prevalence of mH disparities between recipient and donor, defining population frequencies of the immunogenic mH alleles is important. The mH antigens HA-1, HA-2, HA-4 and HA-5 were shown to be the product of single Mendelian genes segregating independently from the HLA complex²¹. The frequencies of a number of mH antigens have been determined by the frequencies of T cell clones recognizing the relevant mH antigens. For instance, the leukemia associated HLA-B44 restricted mH antigen HB-1 is expressed by 28% of the HLA-B44 positive individuals⁴⁷. Furthermore, the HLA-A1 and HLA-A2 restricted mH antigens HA-1, HA-2, HA-3, HA-4 and HA-5 were found at frequencies of 69%, 95%, 88%, 16% and 7% respectively¹⁷. These mH allele population frequencies determine the chance of finding recipient-donor combinations expressing mH disparities that are suitable to avoid GvHD but perhaps induce a GvL response.

1.4.4 Clinical importance of mH antigen HA-1 association with GvHD and GvL

Among all mH antigens identified so far the mH antigen HA-1 is the most ideal candidate for immunotherapy of hematological malignancies. This because HA-1 is highly immunogenic, hematopoietic restricted, expressed in high levels on

leukemic cells and its frequency is 69% among the HLA-A2 positive population. Recently several *in vitro* and clinical studies indicated its role in the GvL effect. For instance, HA-1 specific CTLs effectively lyse all types of leukemic cells including CML, CLL, ALL and AML cells. In clinical studies it has been shown that the emergence of HA-1 specific CTLs after DLI shows a close association with the clinical response¹¹². Similarly, HA-1 specific CTLs were isolated from CML patients with complete remission after DLI¹¹³.

HA-1 is also associated with GvHD^{95,114-116}. This is illustrated by the fact that HA-1 specific CTL clones have been isolated from patients with severe GvHD^{15,17}. These HA-1 specific CTL clones may be part of a general immune response, which does not exclude that they contribute to a GvL effect.

1.5 The minor histocompatibility antigen HA-1 as a tool for immunotherapy

1.5.1 Identification of HA-1

The HA-1 specific CTL clones originally isolated from patients with severe GvHD were restricted by HLA-A2¹⁷. Subsequently, using HA-1 specific CTLs it was shown that the mH antigen HA-1 was present in 69% of the HLA-A2 positive population¹⁷ and was a product of single Mendelian genes segregating independently from the HLA complex²¹. To identify the mH antigen HA-1, HLA-A2 molecules were purified from an HA-1 expressing EBV-transformed B lymphoblastoid cell line (EBV-LCL). The HLA-A2 bound peptides were isolated and fractionated after which the fractions were analyzed for their capacity to reconstitute HA-1 specific lysis. The results demonstrated that the HLA-A2 restricted mH antigen HA-1 was the nonameric peptide VLHDDLLEA. Database searches revealed that the peptide VLHDDLLEA is identical at 8 out of 9 amino acids with the peptide VLRDDLLEA encoded by the cDNA sequence KIAA0223. Subsequent analyses revealed indeed that the HA-1 antigen is derived from the di-allelic KIAA0223 gene. It appeared that two nucleotide differences in the KIAA0223 sequence result in the single amino acid sequence difference between the immunogenic CTL epitope VLHDDLLEA and the VLRDDLLEA counterpart. These two nucleotide differences are positioned at the 3' end of exon A; the H to R substitution is located at amino acid position 139 of the HA-1 locus. Recently, two additional polymorphisms in the HA-1 locus have been found. Namely a Aspartic acid (D) to Glutamic acid (E) substitution at amino acid position 259 and a Glycine (G) to Serine (S) substitution at amino acid position 439 (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=23526). Other nucleotide differences found within the HA-1 locus do not lead to amino acid change. Genomic identification of the mH antigen HA-1 locus by allele specific PCR revealed that the polymorphic KIAA0223 gene is located on chromosome 19. Thus the identification of the gene encoding the HA-1 protein revealed the

first example of a human non-sex linked mH antigen that is derived from a polymorphic gene.

Binding studies of HA-1 peptides to recombinant HLA-A2 molecules revealed that peptide VLRDDLLEA has a 10 to 12 fold lower binding affinity compared to immunogenic peptide VLHDDLLEA. Furthermore, peptide VLRDDLLEA could not be detected by peptide elution from HLA-A2 molecules of HA-1^R homozygous EBV-LCLs, indicating absent or very low cell surface expression of HA-1^R in HLA-A2²⁹.

1.5.2 HA-1^H directed immunotherapy

Since the polymorphic mH antigen HA-1 is exclusively expressed on hematopoietic cells¹⁰⁷ including leukemic cells⁹⁸, adoptive immunotherapy by selectively infusing HA-1 specific donor CTLs may mediate a strong GvL effect with a low risk for GvHD^{96,117}. Since HA-1^H can be presented by HLA-A2^{17,29}, this approach would be feasible following an HLA-A2 matched HA-1 mismatched SCT. The identification of the nonameric HA-1^H peptide VLHDDLLEA has made the *in vitro* generation of large numbers of HA-1^H specific donor CTLs possible for the use of adoptive immunotherapy. HLA-A2 restricted HA-1^H specific CTLs have been generated from HLA-A2 HA-1^{RR} peripheral blood mononuclear cells (PBMCs) *in vitro*, using autologous dendritic cells (DCs) pulsed with HA-1^H peptide as antigen presenting cells (APCs). These HA-1 specific CTLs efficiently lysed HA-1^H expressing leukemic cells but not the fibroblasts from the same individual. Furthermore, autologous DCs retrovirally transduced with a sequence of the HA-1^H allele, have been used as APC to successfully induce HLA-A2 restricted HA-1^H specific CTLs from HLA-A2 HA-1^{RR} PBMCs *in vitro*¹¹⁸.

However, upon transfer the HA-1 specific donor CTLs may eliminate not only patient leukemic cells but also residual HA-1 expressing patient hematopoietic cells residing in peripheral tissues. These residual HA-1 expressing patient hematopoietic cells may thus be responsible for the destruction of the peripheral tissues in which they remain and therewith for the induction of HA-1 related GvHD⁹⁵. A time lapse between SCT and DLI is required for the remaining patient's autologous hematopoietic cells to migrate out of the peripheral tissues. The main response of the subsequently infused donor HA-1 specific CTLs will then be directed towards the remaining patient leukemic cells specifically without destroying the peripheral tissues, inducing the desired GvL effect without causing GvHD.

Thus in an HLA-A2 HA-1^{RR} donor / HLA-A2 HA-1^{HH} or HA-1^{HR} patient SCT setting, adoptive immunotherapy with generated HLA-A2 restricted HA-1^H specific donor CTLs, specifically targets the hematopoietic cells from the patient, including leukemic cells. This approach provides a feasible option for adoptive immunotherapy of relapsed leukemia with a low risk of GvHD as has been described^{112,119,120}. Since the mH antigen HA-1 was further found to be aberrantly expressed in epithelial cancer cells¹²¹ it may also be a useful carcinoma

cell-specific target antigen following SCT. Patient/donor pairs expressing the useful HA-1 mismatch can be identified and selected using prospective genomic typing for the HA-1 alleles¹²².

1.5.3 Alternative strategies for HA-1^H specific CTL generation

Next to the *in vitro* generation of sufficient numbers of HA-1^H specific CTLs for the use of leukemia specific immunotherapy, alternative strategies may be used. Boosting the HLA-A2 HA-1^{RR} stem cell donor with an HA-1^H peptide vaccination prior to transplantation may be a useful method to induce the generation of large numbers of HLA-A2 restricted HA-1^H specific donor CTLs *in vivo*¹²⁵. Boosting the HLA-A2 HA-1^H patient with HA-1^H peptide following subsequent SCT may however result in the induction of a local GvH response since the peptide may be bound by HLA-A2 molecules expressed by non-hematopoietic patient cells.

Furthermore, genetic transfer of the HLA-A2 restricted HA-1^H specific TCR chains may be a less time consuming strategy compared to *in vivo* or *in vitro* HA-1^H specific CTL induction, to introduce HA-1^H specificity into large numbers of cytotoxic T cells of the donor (chapter 6). This approach has already been described for the mH antigen HA-2¹²⁴. When HLA-A2 restricted HA-2 TCRs were transferred into T cells from HLA-A2-negative donors, the HA-2 TCR-modified T cells exerted antileukemic reactivity without signs of anti-HLA-A2 alloreactivity. Therefore, the option of mH antigen specific TCR transfer has been previously proposed as a strategy to circumvent the undesired induction of allo-HLA-specific T cells in HLA-A2 mismatched SCT settings¹²⁴. In addition, several other studies describing successful redirection of recipient T cell specificity by TCR transfer have been published previously¹²⁵⁻¹²⁸. Introduction of an MHC class I-restricted TCR into CD8⁺ peripheral T cells resulted in antigen specific cytolytic activity and cytokine secretion by these T cells. Furthermore, TCR-modified T cells displayed the avidity and fine specificity of the transferred TCR^{129,150} and were capable of eradicating tumour cells *in vivo*¹⁵¹.

1.6 Aim of the study

As described, HA-1 directed adoptive immunotherapy may separate GvHD from GvL response¹¹⁹. The HA-1^H specific immunotherapy is currently feasible only for HLA-A2 HA-1^{HH} or HA-1^{HR} patients who relapsed following an SCT from an HLA-A2 HA-1^{RR} donor. Thus the feasibility of this therapy depends both on the presence of recipient-donor disparity for the mH antigen HA-1¹⁵² and on HLA-A2 expression. HLA-A2 is expressed by approximately 45% of the population¹⁵³. Of the HLA-A2 positive population 16% is HA-1^H homozygous and 54% is HA-1 heterozygous, whereas 30% is HA-1^R homozygous^{17,122}. This suggests that approximately 70% of the HLA-A2 positive- or 32% of the total

patient population could be candidates for HA-1^H specific immunotherapy if they have an HLA matched HA-1^{RR} stem cell donor. However, the therapy is not guaranteed for all of these patients. For instance, the anonymous UCB donors cannot be traced again for use of DLI or adoptive immunotherapy following transplantation. Moreover, the success rate of HA-1^H specific HLA-A2 restricted CTL induction is donor dependent¹¹⁸.

This thesis describes several attempts and possibilities to extend the patient population that can benefit from HA-1 specific immunotherapy. First, the development of a feasible immunotherapy directed towards the HA-1^R counterpart would significantly broaden this patient population. However, HLA-A2/HA-1^R expression on the cell surface could not be found by peptide elution²⁹. We investigated several possible causes for the absence of cell surface HLA-A2/HA-1^R expression in order to evaluate the option of HA-1^R directed immunotherapy (chapter 2).

Another possibility to extend the patient population that can benefit from HA-1 directed immunotherapy is to investigate whether the HA-1^{H/R} polymorphic region contains peptides that can be presented by MHC class I molecules other than HLA-A2. The generation of CTLs specific for HA-1 in the context of various MHC class I molecules would extend this population to patients not expressing HLA-A2. In order to find novel HA-1 epitopes in MHC class I both from the HA-1^H and HA-1^R allele, competition-based cellular peptide binding assays were performed (chapters 3 and 4). All possible nonameric and several decameric HA-1 peptides containing the H to R polymorphism were synthesized and tested for binding to MHC class I molecules.

Because of the reduced severity and reduced incidence of GvHD following umbilical cord blood (UCB) transplantation, UCB SCT is becoming a popular alternative treatment in case of hematological malignancies when no HLA identical SCT donor is available¹⁵⁴⁻¹⁵⁶. Relapse rates following UCB SCT do not seem to be higher than relapse rates following adult SCT¹⁵⁵. However, since donor cells cannot be obtained following anonymous UCB transplantation, no specific immunotherapy is yet available for patients who relapsed following an UCB SCT. Chapter 5 investigates the feasibility of generation of HA-1 specific CTLs from UCB.

In general, CTL induction for adoptive immunotherapy is time-consuming, requires well trained personnel and specialized centers. In order to increase the success rate of HA-1 specific immunotherapy, HA-1 specific TCR transfer is proposed as an alternative method to generate HA-1^H specific HLA-A2 restricted CTLs. Chapter 6 describes attempts to transfer HA-1^H specificity by transduction of HA-1^H specific TCRs to T lymphocytes both from adult donors and from cord blood.

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CHAPTER 2

Extreme instability of HLA-A2/HA-1^R peptide complexes explains the absence of cell surface expression of minor histocompatibility antigen HA-1^R in HLA-A2

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ABSTRACT

The polymorphic minor histocompatibility antigen (mHag) HA-1 locus encodes two nonameric peptides that differ only in one amino acid (aa) i.e. Histidine (H) versus Arginine (R) from each other at aa position 139 of the HA-1 locus. The HA-1^H peptide is presented on the cell surface in HLA-A2 molecules and induces strong cytotoxic T cell (CTL) responses from HA-1^R individuals. Though the HA-1^R peptide binds to the HLA-A2 molecules, it cannot be detected on the cell surface. We searched for the mechanisms that could explain the loss of cell surface expression of the HA-1^R peptide. Intracellular antigen processing by proteasomes and translocation of both HA-1 peptides into the Endoplasmic Reticulum (ER) were identical. Namely, equal amounts of HA-1^H and HA-1^R nonameric peptides were generated by *in vitro* digestion of 28 aa long HA-1^H and HA-1^R peptides with 20S immuno- or constitutive-proteasomes. Both HA-1^H and HA-1^R peptides were translocated equally well into ER by TAP molecules. A discrepancy was observed in peptide binding, where the HA-1^R peptide showed a 10 fold lower binding affinity to TAP-associated HLA-A2 molecules. The most striking difference between the two peptides was found in their dissociation rates, where HA-1^R dissociated from HLA-A2 more than 10 fold faster than HA-1^H. Our results indicate that the lack of cell surface expression of HA-1^R is not due to interference with antigen processing and presentation by MHC class I but to the extreme instability of cell surface HLA-A2/HA1^R peptide complexes.

INTRODUCTION

Disparities in minor histocompatibility antigens (mHag) between Stem Cell (SC) donor and recipient give rise to Graft versus Host Disease (GvHD) and Graft versus Leukemia (GvL) activities after HLA identical SC transplantation (SCT)¹. mHags are polymorphic peptides presented on the cell surface by MHC class I or II molecules capable of inducing strong cellular immune responses from mHag negative individuals. The polymorphic mH peptides are derived from allelic cellular proteins encoded by autosomal genes or by the genes located on the Y chromosome². The immunogenicity of mHags is determined by their MHC binding capacity and by the capability of the intracellular antigen processing machinery to digest these polypeptides into proper sizes that can be translocated into the endoplasmic reticulum (ER) where they can bind to MHC molecules.

The mHag HA-1 is an hematopoietic system-specific antigen that has been identified as a nonameric peptide eluted from HLA-A2 molecules³ which induces HLA-A2 restricted cytotoxic T cell (CTL) responses⁴. The HA-1 peptide is derived from an intracellular protein encoded by a di-allelic gene on chromosome 19⁵. The difference between the HA-1 CTL epitope and its allelic counterpart comprises a single Histidine (H) to Arginine (R) substitution at aa position 3 of the nonameric peptide. Peptide elution studies from HA-1^H and HA1^R homozygous cell lines have revealed that the HA-1^H peptide is expressed

at approximately 80 copies per cell. In contrast, HA-1^R peptide could not be detected on the cell surface⁵. The HA-1^R peptide displays 10 fold less binding as compared to the HA-1^H peptide^{5,5}. However, this relative binding difference may not fully account for the absence of cell surface expression of HA-1^R. We therefore investigated the impact of the HA-1^{H/R} polymorphism on molecular and cellular mechanisms important for the intracellular generation and cell surface expression of MHC class I bound peptides. To this end we analyzed a) the *in vitro* digestion of 28-32 aa long HA1^{H/R} peptides by 20S immuno- and household-proteasomes, b) the *in vitro* TAP translocation of 9-13 aa long HA-1^{H/R} peptides, c) the HA-1^{H/R} peptide binding affinity to TAP-associated MHC class I molecules and d) the dissociation rates of HLA-A2/HA-1^H and HLA-A2/HA-1^R complexes.

MATERIALS AND METHODS

Cell lines and clones

The HLA-A2 restricted HA-1-specific CD8⁺ CTL clones 3HA15 and 5W38 were both isolated from patients suffering from GvHD after HLA-identical SCT^{4,6}. The HA-1^H specific CTLs were maintained and used in cytotoxicity and epitope reconstitution assays as described previously⁷.

Synthetic peptides

Peptides were synthesized on an AMS 1400 multiple peptide synthesizer (Gilson Medical Electronics) using solid-phase Fmoc chemistry and Wang resins. Peptides were HPLC purified to > 98 % on a C-8 column. Purity and identity of all synthetic peptides were confirmed using ESI with an LCQ MS.

Fluorescent HA-1^H and HA-1^R peptides

Fluorescent analogs of HA-1^H and HA-1^R peptides were synthesized by replacing the aa L at position 7 or E at position 8 with a fluorescein (Fl)-labeled Cys-derivative. These replacements did not affect binding of the HA-1^{H/R} peptides to HLA-A2 in competition based binding assays (data not shown). Fluorescent labeling was performed with 4-(iodoacetamido) fluorescein (Fluka Chemie AG, Buchs, Switzerland) at pH 7.5 (Na-phosphate in water/acetonitrile 1:1). The fluorescent peptides were desalted over Sephadex G-10, purified by C18 RP-HPLC and analyzed by MALDI-MS (Lasermat, Finnigan, UK).

Prediction of proteasomal digestion

Proteasomal cleavage prediction of the HA-1^H and HA-1^R alleles was performed using the NetChop (<http://www.cbs.dtu.dk/services/NetChop/>)⁸ and PAMPro (<http://www.paproc.de/>) programs. NetChop analyses were performed with the C-term 2.0 network and a threshold of 0.5. PAMPro analyses were run with the human type III algorithm.

Proteasomal digestion assays

20S proteasomes from HeLa, and EBV-LCL ROF were prepared and purified (purity > 95%) as described before⁹. To determine the proteasome-mediated cleavage, 10 µg of 28 or 32 aa long, > 98% pure HA-1^H and HA-1^R synthetic peptides were incubated with 1 µg of purified 20S proteasomes in 100 µl assay buffer (20 mM HEPES/KOH (pH 7.8), 2 mM MgAc₂, 5 mM DTT) at 37°C for different time periods ranging from 0.25 to 24 hours. The proteolysis products were analyzed by electrospray ionization mass spectrometry on a hybrid quadruple time-of-flight mass spectrometer, (Q-TOF; Micromass, Manchester, UK), equipped with an on-line nano-electrospray interface as described elsewhere¹⁰.

Streptolysin-O-mediated peptide transport assay

In vitro assays of TAP-mediated peptide transport were performed as previously described¹¹. In short, serial dilutions of peptides of interest were tested for their ability to compete for TAP-dependent translocation of a ¹²⁵I-iodinated model peptide in streptolysin-O- permeabilized EBV-LCLs, and the results are expressed as the IC₅₀ values (the concentration of the test peptide that inhibits the translocation of the radiolabeled peptide with 50%).

Peptide binding to TAP-associated MHC class I molecules

Peptide loading on MHC class I from the class I-loading complex was performed essentially as described¹². Briefly, TAP and associating proteins were isolated from cell lysates of 5.10⁷ 35S-methionine/cystein labeled HLA-A2⁺ JY EBV-LCLs, using protein G-beads coated with rabbit anti-human TAP1 and TAP2 sera. Ten percent of the beads was loaded on 12.5% SDS-PAGE without further treatment. The remainder was split into equal portions and incubated with serial concentrations of VLHDDLLEA or VLRDDLLEA peptides that were dissolved in 10 µl DMSO. TAP molecules were also incubated with DMSO alone to serve as negative control. The TAP precipitates were thus incubated for 16 h at 4°C in digitonin lysis mixture. Subsequently these precipitates were incubated in NP40 lysis mixture for 2 h at 37°C. The intact MHC class I/peptide complexes were then immunoprecipitated from the supernatant with monoclonal antibody (moab) W6/32 and analyzed by 12.5% SDS-PAGE.

Class I MHC-peptide dissociation assay

The dissociation rates of the recombinant-HLA-A2 molecules complexed with fluorescent peptides VLHDDLC*EA, VLRDDLC*EA, VLHDDLCA and VLRDDLCA were determined using HPLC. The fluorescent peptide FLPSPDC*FPSV, known to display high affinity for HLA-A2 was tested as a positive control. Fluorescent peptides (1nM) were added to a freshly made mixture of recombinant HLA-A2 (20 nM), β₂M (150 nM; Sigma, Zwijndrecht, The Netherlands), and protease inhibitors (Complete; Roche, Almere, The Netherlands) in binding buffer (75 mM NaCl, 100 mM Phosphate pH 7,

1 mM CHAPS). The HLA- peptide mixture was kept in the dark and shaken gently for 48 hours at room temperature. To determine the level of binding at $t=0$, 75 μ l of each mix was injected on HPLC (Column GCP100 Synchopack 250 x 4.0 mm ID) furnished with fluorescence detector. Immediately thereafter, the HLA binding of fluorescent peptides was inhibited by adding 10,000 fold non-fluorescent peptides. The level of fluorescent peptides bound to HLA was then determined at $t= 0.5, 1, 2, 4, 6, 8$ and 24 h. The dissociation rates were distracted from the percentages heavy chain/FL-peptide complexes still intact at the different time points.

RESULTS

Proteasome-mediated generation of the HA-1^H CTL epitope

The major pathway for the generation of antigenic peptides in the cytosol is degradation by proteasomes¹⁵. The proteasome dependent generation of the HA-1 peptide, was analyzed with the proteasome-specific inhibitor lactacystin. Figure 1 shows that lactacystin had no effect on the CTL lysis of target cells pulsed with the synthetic HA-1 peptide. However, lactacystin inhibited CTL recognition of the natural HA-1^H ligand in a dose dependent manner. These results indicate that the generation of the HA-1^H CTL epitope is dependent on intracellular proteasomal activity.

To subsequently test the generation of the HA-1^H CTL epitope by proteasomes, a 29 aa-long HA-1^H peptide was incubated with 20S proteasomes derived from EBV-LCLs for 1 and 3 h (figure 2). Degradation products generated by proteasomal activity were fractionated by HPLC. Each HPLC fraction was tested for the capacity to reconstitute recognition of HLA-A2⁺, HA-1^H negative target cells by HA-1^H specific CTLs. In both 1h. and 3h. proteasomally digested samples, a single fraction (#35) reconstituted the HA-1^H specific CTL activity. Fraction 35 from the sample digested for 3h triggered significantly enhanced CTL responses, indicating the active generation of HA-1^H nonameric peptides in time. The presence of the nonameric HA-1^H peptide in fraction #35 was confirmed by mass spectrometric analysis (data not shown).

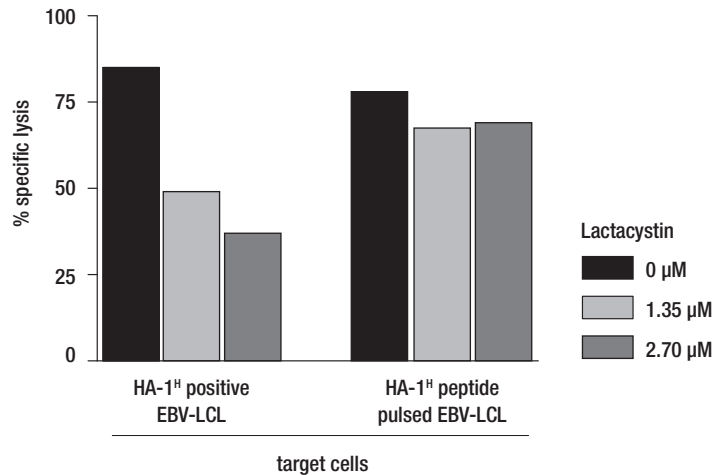


Figure 1.

Inhibition of HA-1 specific lysis by proteasome inhibitor lactacystine.

HA-1^H and HA-1^{RR} EBV-LCL were treated overnight with the indicated concentrations of lactacystine prior to use as target cells for the HA-1^H specific CTL clone 3HA15. The lactacystine treated HA-1^{RR} EBV-LCL were pulsed with 1 μg/ml of the HA-1^H peptide for 1 h during the ⁵¹Cr labeling. Results shown are at an effector to target (E:T) ratio of 10:1. Similar inhibition levels were observed at E:T ratios of 5:1.

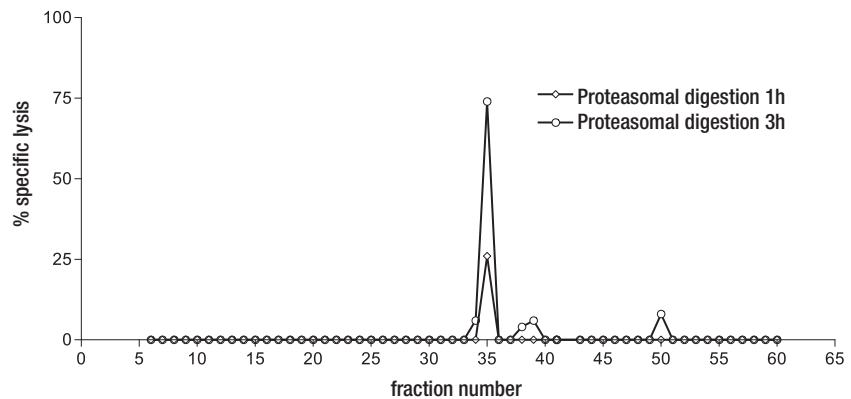


Figure 2.

Reconstitution of the minor H antigen HA-1 with HPLC-fractionated peptides.

Peptide fractions were generated by proteasomal digestion of a 29 aa long HA-1^H peptide for 1 h and for 3 h. Aliquots of each fraction were pre-incubated with ⁵¹Cr-labeled HLA-A2⁺, HA-1^{RR} cells and tested for their ability to reconstitute epitope activity of the HA-1^H specific CTL clone 5W38 at an E:T ratio of 10:1.

Generation of HA-1^H and HA-1^R nonameric peptides by proteasome-mediated digestion

The allelic counterpart of mHag HA-3 appears to be destroyed by proteasomes¹⁴. To assess whether the HA-1^{H/R} polymorphism may affect the proteasomal cleavage of the HA-1 peptides, we first subjected the aa sequences of HA-1^H and HA-1^R to a computational analysis with the proteasomal cleavage prediction programs NetChop and PAMPro. Whereas the PAMPro program predicted the destruction of the HA-1^R putative epitope (table I), the NetChop program predicted no differences between HA-1^H and HA-1^R peptides (data not shown). Subsequently, we subjected 28 aa-long HA-1^H and HA-1^R peptides to *in vitro* digestion by 20S immunoproteasomes purified from EBV-LCL ROF and by constitutive proteasomes from HeLa cells. The degradation products were analyzed by mass spectrometry (tables IIa and IIb). Digestion of HA-1^H and HA-1^R peptides with immuno- or constitutive proteasomes revealed some cleavage sites within the epitope for both HA-1^H and HA-1^R peptides. The cleavage site at the N terminus of the H/R polymorphic aa was more predominant in the HA-1^R peptide as predicted by the PAMPro program. However, this cleavage site did not destroy the HA-1^R peptide because the digested products of both 28-meric HA-1^H and HA-1^R peptides contained significant and similar amounts of material corresponding exactly with the mass of the nonameric HA-1^H and HA-1^R peptides. Subsequent MS/MS sequencing analysis confirmed the presence of the nonameric sequences HA-1^H (VLHDDLLEA) and HA-1^R (VLRDDLLEA) in the relevant fractions, demonstrating that the HA-1^{H/R} polymorphism did not affect the proteasome mediated generation of the right sizes of the HA-1^{H/R} peptides.

Table I.

Predicted cleavage sites in the HA-1^{H/R} polymorphic region by PAMPro human proteasome type II algorithm

HA-1 ^H	ADVARFA ↓ EGLEK ↓ L ↓ KEC ↓ VLHDDLLEA ↓ RRPRAHEC ↓ LGEALRV
HA-1 ^R	ADVARFA ↓ EGLEK ↓ L ↓ KEC ↓ VL ↓ RDDLLEA ↓ RRPRAHEC ↓ LGEALRV

The predicted cleavage sites in 40 aa long stretches of HA-1^H and HA-1^R polypeptides are indicated. Algorithms based on human proteasome type I or type III, and NetChop software revealed no differences between HA-1^H and HA-1^R peptides.

Table IIa.

In vitro digestion of a 28 aa long HA-1^H peptide by 20s proteasomes

Substrate: 28 aa HA-1 ^H polypeptide	% fragment generated by 20s Proteasomes derived from	
GLEKLKEC VLHDDLLEARRPRAHE CLGE	HeLa	EBV
Fragments		
GLEKLKEC VLHDDLLEARRPRAHE CLGE	1.68	2.11
GLEKLKEC VLHDDLLEARRPRAHE CLG	2.63	2.39
GLEKLKEC VLHD	0.83	1.26
GLEKLKEC VL	0	1.65
LEKLKEC VLHDDL	1.25	1.05
LEKLKEC VLHDDLLEARRPRAHE CLG	0	1.61
EKLKEC VLHDDLLEARRPRAHE CLGE	3.80	4.93
KLKEC VLHDDLLEARRPRAHE CLGE	78.12	78.24
KEC VLHDDLLEARRPRAHE CL	1.86	1.19
CVLHDDLLEARR	0.98	0.13
VLHDDLLEARRPRAHE	1.23	0.43
VLHDDLLEARRPRAH	1.01	0.52
VLHDDLLEARRPRA	0.47	0.56
VLHDDLLEA	3.24	1.02
DLLEARRPRAHE	1.27	1.36
DLLEARRPRA	1.20	0.58

The aa sequences corresponding to the HLA-A2 restricted HA-1^H CTL epitope and its HA-1^R allelic counterpart are indicated in bold. The rows shaded in gray indicate the proteasomal digestion products that correspond with the nonameric HA-1^H CTL epitope and its allelic counterpart. Proteasomal digestions was carried out for 3 h at 37°C. The amounts of the generated fragments are indicated as the percentage of all fragments found in the digested substrate. The indicated peptides were identified by their molecular masses calculated from the m/z peaks of the single or multiple charged ions and were confirmed by mass spectrometric sequencing analyses. Similar results were obtained using 32meric HA-1^{H/R} peptides FAEGLEKLKECVL H/R DDLEARRPRAHECLGEA as substrates.

Table IIb.

In vitro digestion of a 28 aa long HA-1^R peptide by 20s proteasomes

Substrate: 28 aa HA-1 ^R polypeptide	% fragment generated by 20s Proteasomes derived from	
	HeLa	EBV
GLEKLKEC VLRDDLLEARRPRAHE CLGE		
Fragments		
GLEKLKEC VLRDDLLEARRPRAHE CLG	3.36	3.28
GLEKLKEC VL	1.11	1.73
GLEKLKEC VLRD	2.00	1.65
EKLKEC VLRDDLLEARRPRAHE CLGE	60.34	67.90
KLKEC VLRDDLLE	1.55	1.81
LKEC VLRDDLLEARRPRAHE CLGE	0.98	2.15
KEC VLRDDLLEARRPRAHE CLGE	4.03	5.69
CVLRDDLLEARR	1.86	1.94
VLRDDLLEARRPRAHE	0.56	0.30
VLRDDLLEARRPRAH	1.10	0.72
VLRDDLLEARRPRA	0.45	0.89
VLRDDLLEA	2.58	1.05
RDDLLEARRPRAHE	0.82	0.53
RDDLLEARRPRAH	0.48	0.34
RDDLLEARRPRA	0.43	0.54
DLLEARRPRAHE	1.53	0.28
DLLEARRPRAH	0.48	0.34
DLLEARRPRA	1.71	0.71
DLLEARRPR	0.25	0.22
LEARRPRAHE	0.52	0
LEARRPRAH	0.54	0

Efficient TAP-translocation of both HA-1^H and HA-1^R peptides

It has been shown that defective translocation of antigenic peptides into the ER by TAP molecules can be also a mechanism why the mHag peptides are not available for cell surface expression¹⁵. We therefore compared the TAP-dependent transport of various synthetic length-variants of HA-1^H and HA-1^R peptides. Both nonameric HA-1^H and HA-1^R peptides were transported equally well by the TAP transporter (table III). Although a 12-meric HA-1^H peptide was transported significantly better than the corresponding HA-1^R peptide, this difference may not be relevant because these 12-meric HA-1^{H/R} peptides were not generated by proteasomes (table II). These results indicate that TAP translocation could not explain the differential expression of the HA-1^H and HA-1^R peptides on the cell surface.

Table III.

In vitro TAP translocation of HA-1^H and HA-1^R peptides

Sequence	No. of aa	IC ₅₀ (μM)
KECVLHDDLLEARRP	15	>100
KECVLRDDLLEARRP	15	>100
KECVLHDDLLEA	12	8
KECVLRDDLLEA	12	53
ECVLHDDLLEA	11	>100
ECVLRDDLLEA	11	45
CVLHDDLLEA	10	92
CVLRDDLLEA	10	87
VLHDDLLEA	9	17
VLRDDLLEA	9	10

Different lengths of HA-1^H and HA-1^R peptides were tested for their ability to compete for translocation of the radioiodinated model peptide TVNKTERAY in streptolysin O-permeabilized EBV-LCLs. The IC₅₀ values represent the concentration of the test peptide that inhibits the transport of the model peptide with 50%. The HLA-A2 restricted nonameric HA-1^H CTL epitope is indicated in bold.

HA-1^{H/R} peptide binding to TAP-associated HLA-A2 molecules

In vitro competition based peptide-binding assays have shown that the HA-1^R peptide can compete with reference peptides for binding to HLA-A2 molecules^{3,5}.

However, peptide binding to recombinant HLA molecules or to HLA molecules already present on the cell surface may not be representative for intracellular peptide binding to TAP-associated HLA molecules in the ER, which is necessary for cell surface expression of the MHC-peptide complex. Therefore, we measured HA-1^H and HA-1^R peptide binding to TAP-associated HLA-A2 molecules in a cell free system. The HA-1^R peptide, like the HA-1^H peptide, bound to TAP-associated HLA-A2 molecules, but only at 10 fold higher concentrations (figure 3). These results are similar to the MHC-peptide binding results described previously^{3,5}.

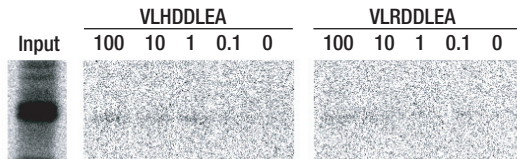


Figure 3.

Binding of HA-1^H and HA-1^R peptides to TAP-associated HLA-A2.

JY EBV-LCL cells, positive for HLA-A2, were biosynthetically labeled with 250 μ Ci 35S-methionine/cysteine and then lysed. TAP and associating proteins were isolated using protein G-beads coated with rabbit anti-human TAP1 and TAP2 sera. Ten percent of the beads were loaded on 12.5% SDS-PAGE without further treatment (left column), while the remainder was split into equal portions and incubated over night at 4°C with 100, 10, 1, 0.1 or 0 μ M peptide VLHDDLLEA or VLRDDLLEA. Subsequently NP40 lysis mixture was added and the lysate was incubated for 2 hr at 37°C. MHC class I/peptide complexes were then isolated from the supernatant with moab W6/32 and analyzed by 12.5 SDS-PAGE.

Dissociation rates of HLA-A2/HA-1^{H/R} peptide complexes

Competition based peptide-binding assays mainly reflect the rate of formation of the HLA-peptide complexes. However, it has been established that, MHC class I peptide binding affinity is mainly controlled by the rate of dissociation of the HLA-peptide complexes, rather than the rate of formation of the complex¹⁶. Thus, instability of the MHC-peptide complex could be a reason why the HA-1^R peptides are not present in cell surface HLA-A2 molecules. We therefore tested whether the H/R substitution in the HA-1 peptides influences the stability of the HLA binding. The dissociation rates of the HLA-A2/HA-1^H and HLA-A2/HA-1^R complexes were compared in a cell free HLA-binding assay using recombinant HLA molecules and fluorescent analogs of the HA-1^H and HA-1^R peptides (figure 4). As expected, the dissociation rates of HLA-A2/HA-1^H complexes were slow. After 24h still 65% of the HLA-A2/VLHDDLC*EA and 40% of the HLA-A2/VLHDDLLC*A peptide complexes were intact. In contrast, the HLA-A2/HA-1^R complexes dissociated rapidly. No intact HLA-A2/HA-1^R complexes were found

after 2 hours, indicating that the rapid dissociation rates contribute significantly to the absence of HLA-A2/HA-1^R peptides from the cell surface.

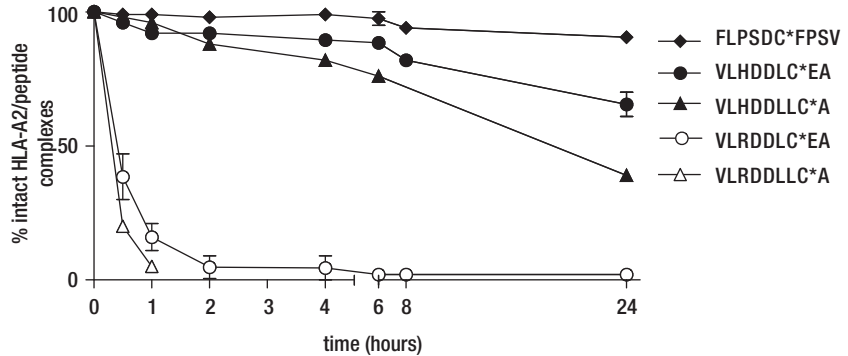


Figure 4. Dissociation rates of HA-1^H and HA-1^R peptides.

Recombinant HLA-A2 molecules were incubated with the indicated fluorescent peptides for 48 hours. At t=0 fluorescent peptides were diluted out by the addition of 10,000 fold non fluorescent peptides. The amount of bound fluorescent peptide was determined at the indicated time points. The intact HLA-peptide complexes were expressed as percentage of the intact complexes at t=0. The peptide FLPSDC*FPSV was used as a positive control.

DISCUSSION

Generation of class I restricted mHags from polymorphic intracellular proteins is dependent on their proper handling by several intracellular processes including ubiquitination, degradation by proteasomes into peptides and translocation of the peptides into the ER, as well as the capacity of the peptides to bind HLA molecules. Previous studies on viral CTL epitopes have revealed that even a single amino acid exchange in a viral protein can interfere with intracellular processing and can result in the absence of cell surface expression of the MHC/peptide complexes¹⁷⁻¹⁹. With the biochemical identification of a number of autosomally encoded human mHags it became also evident that single amino acid substitutions can have a significant impact on the cell surface expression of the allelic counterparts. To date, such a “functional null allelism” has been demonstrated for four mHags: the HA-1, HA-2, HA-3 and HA-8^{5,14,15,20}. It appears that distinct mechanisms can account for the loss of cell surface expression of the allelic peptides. For instance, in case of HA-8 the allelic peptide is poorly translocated into the ER, whereas in case of HA-3 the allelic counterpart appears to be destroyed by proteasomes. The allelic counterpart of the BCL2A1 antigen might not be produced endogenously as a result of the fact that the COOH terminus is not properly formed by proteasomes²¹. In contrast, cell surface expression of the UGT2B17 antigen is lost as a consequence of homozygous gene

deletion in UGT2B17 negative individuals²². Furthermore, the novel HLA-A33 restricted mHag TMSB4Y is not derived from a functional polypeptide, but is probably encoded outside a conventional open reading frame (ORF) of a nonmutated gene²⁵. The results in this study reveal another mechanism and show that absence of HA-1^R cell surface expression in the context of HLA-A2 molecules is due to the low association and extremely rapid dissociation rates of HLA-A2/HA-1^R complexes. Previously, we have shown that the HA-1^R peptide binds with ten fold lower affinity to recombinant HLA-A2 molecules as compared to the HA-1^H peptide⁵. Although the peptide binding to recombinant HLA molecules may not be representative for intracellular peptide binding to TAP-associated HLA molecules in the ER, we show in this study that the HA-1^R peptide also binds with ten fold lower affinity to intracellular TAP-associated HLA-A2. However, this ten fold affinity difference is unlikely to account for the complete absence of HLA-A2/HA-1^R complexes on the cell surface as the HA-1^H peptide is expressed at 80 copies per cell⁵. Moreover, it was previously shown for a set of naturally processed peptides that the MHC binding affinities were not directly related to their cell surface density (reviewed in²⁴). Furthermore low affinity peptides can represent important T cell epitopes^{25,26}. However, a common feature of these CTL epitopes with low MHC binding affinity is that they slowly dissociate from MHC, which of course has a major impact on the cell surface expression of the MHC/peptide complexes.

Our results revealed that while the HA-1^H peptide dissociates slowly from the HLA-A2 molecules, the dissociation rate of HLA-A2/HA-1^R peptide complexes was high. No detectable HLA-A2/HA-1^R complexes were left within two hours. This extremely rapid dissociation rate may be explained by a different configuration of the HA-1^R peptide compared with the HA-1^H peptide resulting in a less stable HLA-A2/HA-1^R complex²⁷, and can result in the loss of HLA-A2/HA-1^R cell surface expression.

Furthermore, our results rule out other important possibilities. First, we could not demonstrate any significant differential effect of the HA-1^{H/R} polymorphism on proteasome-mediated digestion. Although the PAMPro software predicted a major cleavage site in the HA-1^R peptide by human type II proteasomes, this cleavage site appeared not to destroy the generation of relevant peptides. Both immuno- and constitutive-proteasomes generated the HLA-A2 binding nonameric peptides of HA-1^H and HA-1^R in significant and equal amounts. We detected a second cleavage site within the HA-1^{H/R} polymorphic region. This cleavage site between two D residues, which was also predicted by the NetChop program (data not shown) is present in both HA-1^H and HA-1^R peptides but did not prevent the generation of the HA-1^H CTL epitope and of its allelic counterpart. Thus, our results are in line with previous findings that once the relevant peptides are generated by proteasomal cleavage, other cleavage sites found within the epitope weigh less heavily for the cell surface expression of the peptides¹⁰.

Our results also demonstrated that the TAP translocation of HA-1 peptides is not affected by the HA-1^{H/R} polymorphism. As TAP can transport different lengths of peptides up to 15-mers²⁸, we tested the TAP translocation of 9-15-meric HA-1^{H/R} peptides. The nonameric HA-1^{H/R} peptides, as well as several longer variants of the HA-1^{H/R}, were equally transported by TAP. Only the 12-meric HA-1^R peptide (KECVRDDLLEA) was transported less efficiently as compared to the corresponding HA-1^H peptide. However, this difference may be irrelevant since these 12-meric peptides were not generated by *in vitro* proteasome-mediated-digestion of 28 or 32-meric HA-1^H and HA-1^R peptides. Taken together, our data indicate that the loss of surface expression of HA-1^R peptide is not caused by differential antigen processing or TAP translocation, neither is it caused by gene deletion or an ORF shift. As the exact lengths of HLA-A2 binding nonameric peptides were generated by proteasomes, we did not study the possibility whether the HA-1^{H/R} polymorphism may have an impact on N terminal trimming by aminopeptidases in the ER²⁹⁻⁵².

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CHAPTER 3

Competition-based cellular peptide binding assays for 13 prevalent HLA class I alleles using fluorescein-labeled synthetic peptides

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ABSTRACT

We report the development, validation and application of competition based peptide binding assays for 13 prevalent HLA class I alleles. The assays are based on peptide binding to HLA molecules on living cells carrying the particular allele. Competition for binding between the test peptide of interest and a fluorescein-labeled known HLA class I binding peptide is used as read out. The use of cell membrane bound HLA class I molecules circumvents the need for laborious biochemical purification of these molecules in soluble form. Previously, we have applied this principle for HLA-A2 and HLA-A3. We now describe the assays for HLA-A1, HLA-A11, HLA-A24, HLA-A68, HLA-B7, HLA-B8, HLA-B14, HLA-B35, HLA-B60, HLA-B61 and HLA-B62. Together with HLA-A2 and HLA-A3, these alleles cover over 95% of the Caucasian population. Several allele-specific parameters were determined for each assay. Using these assays we identified novel HLA class I high affinity binding peptides from HIVpol, p53, PRAME and minor histocompatibility antigen (mHag) HA-1. Thus, these convenient and accurate peptide binding assays will be useful for the identification of putative CTL epitopes presented on a diverse array of HLA class I molecules.

INTRODUCTION

The identification of HLA restricted cytotoxic T lymphocyte (CTL) epitopes is crucial for our understanding of immunity in bacterial or viral infections, autoimmune diseases and cancer as well as for the development of defined vaccines that induce CTL and the monitoring of such immunotherapies. The peptide-binding based prediction of CTL epitopes in protein sequences has led to the identification of CTL epitopes in viral proteins^{1,2} bacterial proteins³ and tumor antigens⁴. Often these CTL epitopes are presented in HLA-A2, being the class I allele that predominates in the Caucasian population. However, an ongoing search for new CTL epitopes restricted by other prevalent HLA class I molecules is necessary for the development of immunotherapies covering all class I haplotypes and multi-epitope vaccines (table I).

The peptide binding groove of HLA molecules contains highly polymorphic allele-specific pockets that accommodate side chains of the so-called anchor residues of the bound peptide^{5,6}. The peptide binding groove of HLA class I molecules is closed at both sides⁶ and thus HLA class I accommodates peptides with a length of 8 - 11 amino acids (aa). Allele-specific peptide binding motifs were defined by the analysis of naturally presented peptide pools eluted from class I molecules^{7,8}. Each HLA class I molecule displays a preference for certain aa at the major (primary) peptide anchor positions (relative position 2 and the C-terminus for most HLA class I molecules) that bind in the binding pockets. Amino acids at other positions in the peptide can significantly contribute to binding by their engagement in secondary pockets⁹⁻¹⁶. The knowledge of allele specific peptide binding motifs has led to the development of peptide binding prediction algorithms by several groups¹⁷⁻¹⁹. Although these algorithms

are extremely helpful to select potential HLA class I binding peptides, experimental determination of the HLA class I binding capacity is still considered necessary due to the partly undefined contributions to binding of each possible aa in every position of the peptide.

Table I.

Phenotype frequency distribution of HLA class I antigens for which assays were developed expressed as percentages among major populations^a

HLA class I	Population			
	Black	Caucasoid	Oriental	Amerindian
A1	9	26	7	11
A2	29	44	47	43
A3	13	22	6	8
A11	3	13	30	4
A24	6	20	42	52
A68	18	8	3	12
B7	15	17	7	5
B8	9	14	3	2
B14	7	6	1	3
B35	11	20	10	32
B60	1	6	17	5
B61	0	6	9	23
B62	2	8	16	21

^aPhenotype frequencies for the HLA antigens have been deduced using the gene frequencies as given by Marsh et al³⁸.

Peptide-HLA class I binding assays employ either cell-bound class I molecules²⁰⁻²⁸ or solubilized class I molecules²⁹⁻³⁴. Assays using cell-bound HLA class I molecules are either based on upregulation of class I molecules in processing defective cell lines^{22,25,26} or on reconstitution of HLA class I molecules^{24,25,27,28}. Cell-free assays are quantitative and are based on competition for binding between a labeled reference peptide and a test peptide³². We previously applied the competition principle in easy to perform cell-bound HLA class I binding assays for HLA-A2 and HLA-A3²⁷. In these assays, EBV transformed B cell lines (B-LCL) expressing the class I allele of interest are used from which naturally bound class I peptides are eluted to obtain free class I molecules. Subsequently, B-LCL are incubated with a mixture of a fluorescein (Fl)-labeled reference peptide, known to bind efficiently to the allele of interest, and titrated amounts of a competing test peptide. Cell-bound fluorescein is determined by flowcytometry and the inhibition of binding of the Fl-reference peptide is calcu-

lated as readout. We now report the adoption of this principle for an additional set of highly prevalent HLA class I alleles (HLA-A1, -A11, -A24, -A68, -B7, -B14, -B8, -B35, -B60, -B61, -B62). Together with HLA-A2 and HLA-A5, these alleles cover over 95% of the Caucasian populations. For each assay, the allele-specific parameters were established: a) a suitable reference peptide with known binding capacity for the allele, b) the optimal position of the FI-label in the reference peptide, c) the required concentration of the labeled peptide, d) the pH of the elution buffer used for acid stripping of class I molecules, e) a B-LCL expressing the HLA class I molecule of interest and f) exclusion of binding of FI-reference peptide to co-expressed class I molecules on the used B-LCL. The assays were used to identify several HLA class I binding peptides derived from HIV-1_{pol}, p53, PRAME and mHag HA-1. Finally, we analyzed the predictive power of a commonly used peptide binding prediction algorithm for a set of HLA-A2 binding peptides to assess the need to actually assay the peptide binding affinity after prediction of binding.

MATERIALS AND METHODS

Cell lines

The Epstein-Barr virus transformed B-lymphoblastoid cell lines (B-LCL) used for the binding assays were either obtained from the international histocompatibility workshop cell line repository or newly generated from PBMC of healthy blood donors. All B-LCL were cultured in complete culture medium consisting of IMDM (Biowithaker, Verviers, Belgium) supplemented with 8% fetal calf serum (FCS) (Gibco BRL, Breda, The Netherlands), 100 IU/ml penicillin and 2 mM L-glutamine.

Peptides

Peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Syro II, MultiSyntech, Witten, Germany) using Fmoc-chemistry. Peptides were analyzed by reversed-phase HPLC and mass spectrometry, dissolved in 50 μ l dimethyl sulfoxide, diluted in 0.9% NaCl to a peptide concentration of 1 mM and stored at -20°C until usage. Fluorescein-labeled reference peptides were synthesized as Cys-derivative. Labeling was performed with 5-(iodoacetamido)fluorescein (Fluka Chemie AG, Buchs, Switzerland) at pH 7.5 (Na-phosphate in water/acetonitrile 1:1 vol/vol). The labeled peptides were desalted over Sephadex G-10 and further purified by C18 RP-HPLC. Labeled peptides were analyzed by mass spectrometry.

Selection of test peptides for binding assays

Peptides derived from HIV-1_{pol}, p53, PRAME and mHag HA-1 that contain HLA class I peptide binding motifs were selected using either the BIMAS peptide binding algorithm available via the internet (http://bimas.cit.nih.gov/molbio/hla_bind/)¹⁸ or an algorithm that was developed in our department¹⁷.

Mild acid elution of HLA class I bound peptides on B-LCL

Mild acid treatment of B-LCL to remove naturally HLA class I bound peptides was performed with minor modifications according to the principle first described by Sugawara et al.³⁵ and elaborated by our group²⁷. B-LCL were harvested and washed in phosphate buffered saline (PBS) and the pellet (2 – 15x10⁶ cells) was put on ice for 5 min. The elution was performed by incubating the cells for exactly 90 s in ice-cold citric-acid buffer (1:1 mixture of 0.263 M citric acid and 0.123 M Na₂HPO₄, adjusted to the pH listed in table II). Immediately thereafter, cells were buffered with ice-cold IMDM containing 2% FCS, washed once more in the same medium and resuspended at a concentration of 4x10⁵ cells/ml in IMDM medium containing 2% FCS and 2 µg/ml human β₂-microglobulin (β₂M) (Sigma, St. Louis, MO, USA).

Table II.

Allele specific characteristics of HLA class I binding assays.

HLA class I allele ^a	Reference peptides used in the assays			Ref. ^c	Assay Cell line		pH ^e
	FL-labeled seq. ^b	[FL-pep.]	Original seq.		Name	HLA class I type	
A1 (A*0101)	YLEPAC(F)AKY	150 nM	YLEPAIAKY	32	CAA	A*0101, B*0801, Cw*0701	3.1
A2 (A*0201) ^d	FLPSDC(F)FPSV	150 nM	FLPSDFFPSV	39	JY	A*0201, B*0702, Cw*0702	3.2
A3 (A*0301) ^d	KVFPC(F)ALINK	150 nM	KVFPYALINK	32	EKR	A*0301, B*0702, Cw*0702	2.9
A11 (A*1101)	KVFPC(F)ALINK	150 nM	KVFPYALINK	32	BVR	A*1101, B*3501, Cw*0401	3.1
A24 (A*2402)	RYLKC(F)IQLL	150 nM	RYLKDQQLL	40	Vijf	A*2402; B*0702, Cw*0702	3.1
A68 (A*6801)	KTGGPIC(F)IKR	150 nM	KTGGPIYKR	41	A68HI	A*6801, B*4402, Cw*0704	3.1
B7 (B*0702)	APAPAPC(F)WPL	150 nM	APAPAPSWPL	N.P.	JY	A*0201, B*0702, Cw*0702	3.1
B8 (B*0801)	FLRGRAC(F)GL	50 nM	FLRGRAYGL	42	Vavy	A*0101, B*0801, Cw*0701	3.1
B14 (B*1402)	DRYIHAC(F)ILL	150 nM	DRYIHAVLL	43	CHE	A*2402, A*3301, B*1402, Cw*0201	4.0
B35 (B*3501)	NPDIVC(F)YQY	150 nM	NPDIVIYQY	44	BVR	A*1101, B*3501, Cw*0401	2.9
B60 (B*4001)	KESTC(F)HLVL	125 nM	KESTLHLVL	36	DKB	A*2402, B*4001, Cw*0304	3.1
B61 (B*4002)	GEFGGC(F)GSV	50 nM	GEFGFGSV	36	Swei007	A*2902, B*4002, Cw*0202	3.1
B62 (B*1501)	YLGEFSC(F)TY	150 nM	YLGEFSITY	36	BSM	A*0201, B*1501, Cw*0304	2.9

^a HLA class I allele of binding assay. Mostly, B-LCL were used expressing the most common subtype of the allele (see HLA class I type).

^b A non-anchor residue was substituted with a cysteine derivatized by a fluorescein group, denoted as C(F).

^c Most reference peptides were derived from the SYFPHEITI database¹⁹; here the original reference is cited. For HLA-B7, APAPAPSWPL (human p53 84-93) was found as high affinity binder in a molecular binding assay (N.P. = not published).

^d Characteristics of HLA-A2 and HLA-A3 binding assays have been published before²⁷.

^e Optimal pH of the elution buffer used for stripping naturally bound peptides.

HLA class I competition binding assays

Eight serial twofold dilutions of each competitor test peptide in PBS/BSA 0.5% were made (highest concentration 600 μM , 6-fold assay concentration). In the assay, test peptides were tested from 100 μM to 0.8 μM . The FI-labeled reference peptide was dissolved in PBS/BSA 0.5% at 6-fold final assay concentration (see table II). In a well of a 96-well V-bottom plate 25 μl of competitor peptide was mixed with 25 μl FI-labeled reference peptide. Subsequently, the stripped B-LCL were added at 4×10^4 /well in 100 μl /well. After incubation for 24 h at 4°C, cells were washed three times in PBS containing 1% BSA, fixed with 0.5% paraformaldehyde, and analyzed with FACScan flowcytometry (Becton Dickinson) to measure the mean fluorescence (MF). The percentage inhibition of FI-labeled reference peptide binding was calculated using the following formula:

$$(1 - (\text{MF}_{\text{reference + competitor peptide}} - \text{MF}_{\text{background}}) / (\text{MF}_{\text{reference peptide}} - \text{MF}_{\text{background}})) \times 100\%$$

The binding affinity of competitor peptide is expressed as the concentration that inhibits 50% binding of the FI-labeled reference peptide (IC_{50}). IC_{50} was calculated applying non-linear regression analysis (with software CurveExpert 1.3, SPSS Science Software, Erkrath, Germany). An $\text{IC}_{50} \leq 5 \mu\text{M}$ was considered high affinity binding, $5 \mu\text{M} < \text{IC}_{50} \leq 15 \mu\text{M}$ was considered intermediate affinity binding, $15 \mu\text{M} < \text{IC}_{50} \leq 100 \mu\text{M}$ was judged low affinity binding and $\text{IC}_{50} > 100 \mu\text{M}$ was regarded as no binding. These IC_{50} cutoff values are based on our experience with HLA-A2 and HLA-A3 binding ligands and CTL epitopes²⁷.

RESULTS

Selection of HLA class I alleles

The HLA-A2 and HLA-A3 alleles cover around 70% of the Caucasian population²⁷. To enlarge the haplotype coverage we chose to develop binding assays for 11 additional alleles (HLA-A1, -A11, -A24, -A68, -B7, -B8, -B14, -B35, -B60, -B61 and -B62) with high prevalence among most populations (table I). Together with HLA-A2 and HLA-A3, these alleles cover over 95% of the Caucasian population, as calculated from a group of 1000 HLA-typed Dutch blood donors.

Selection of optimal fluorescein labeled reference peptides

For each allele one or two peptides to be used as fluorescein (FI)-labeled reference were derived from aa sequences shown to bind strongly to the particular allele. These peptides were originally identified as naturally presented class I ligand, CTL epitope, or consensus sequence, with the exception of the peptide for HLA-B7 (table II). For each peptide several labeled variants were made by substituting at various positions a non-anchor residue for a fluorescein conjugated cysteine. FI-labeled reference peptides were titrated on B-LCL homozygously expressing the class I molecule of interest to identify for each allele the one which best retained their high binding capacity as well as to determine an

optimal concentration of the FI-labeled peptide to be used in the competition assay. As exemplified for HLA-B61 two peptides were selected, GEFGGFGSV (histone acetyltransferase 127-135³⁶), of which the phenylalanine at position 6 was substituted rendering GEFGGC(FI)GSV and GEFVDLYV (ribosomal protein S21 6-13³⁶), of which both GEFVC(FI)LYV and GEFVDC(FI)YV were tested (figure 1A). Differences in binding capacity occurred depending on which original sequence was used and the particular residue that was substituted. The difference in binding capacity between the two variants of GEFVDLYV can be explained by altered contribution to overall binding affinity of the fluorescein conjugated cysteine as compared to the original residue depending on (position of) the residue that is substituted. The FI-labeled reference peptide GEFGGC(FI)GSV, displaying highest binding capacity, was chosen as the labeled reference peptide for the assay (figure 1A and table II). Optimal FI-labeled reference peptides for the other alleles were likewise determined (table II). For each FI-labeled peptide sub-optimal saturating concentrations were used in the assay to optimally enable competition by the test peptides (table II). The maximal binding of FI-labeled reference peptides at the chosen concentration after 24 h incubation at 4°C resulted in a mean fluorescence (MF) of at least 5 times the background staining with PBS, as shown for HLA-B61 in figures 1A and 1B.

Selection of HLA class I expressing cell lines

B-LCL homozygously expressing the allele of interest were used for the assays (table II). Control B-LCL were tested to exclude binding of the FI-labeled reference peptide to co-expressed class I molecules. As exemplified for the HLA-B61 binding assay, the FI-labeled reference peptide GEFGGC(FI)GSV did efficiently bind to B-LCL Sweig007 (HLA-A29, -B61, -Cw2) whereas binding to control B-LCL Mann and 4B5 expressing HLA-A29 and HLA-Cw2 respectively was absent (figure 1B). This indicates that binding of the FI-reference peptide to HLA-A29 and HLA-Cw2 can be excluded and binding on Sweig007 was exclusively accomplished via binding to HLA-B61. B-LCL functioning optimally in assays for binding to HLA-A1, -A11, -A24, -A68, -B7, -B8, -B14, -B35, -B60 and -B62 were likewise found as listed in table II.

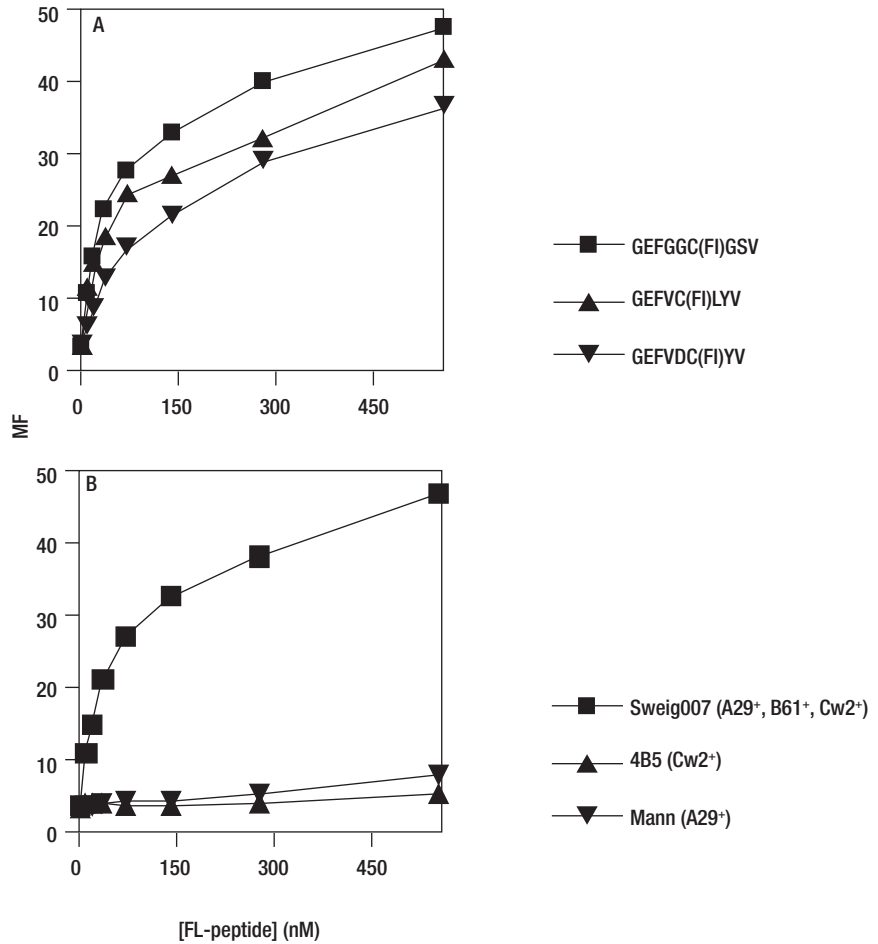


Figure 1

Determination of the optimal FI-labeled HLA-B61 binding reference peptide and exclusion of binding of the FI-labeled peptide to alleles other than HLA-B61 that are expressed on B-LCL Swei007 (HLA I type: HLA-A29, -B61, -Cw2).

(A) Binding affinities of 3 FI-labeled HLA-B61-binding reference peptides. The peptides were titrated at the indicated concentrations on B-LCL Swei007. After incubation for 24 h at 4°C fluorescence was measured with flowcytometry. (B) Exclusion of binding of the FI-labeled reference peptide GEFGGC(FI)GSV for HLA-B61 to co-expressed alleles on Swei007. The FI-reference peptide was incubated for 24 h at 4°C with B-LCLs Swei007, Man (expressing HLA-A29) and 4B5 (expressing HLA-Cw2) and fluorescence was measured with flowcytometry at a FACScan. Results of one representative experiment of at least three performed are shown.

Determination of the optimal elution - pH for each allele

We previously observed differences in the pH required for optimal elution of naturally bound peptides from HLA-A2 and HLA-A3^{27,28}. Therefore, several pHs were tested for each new allele in order to find optimal conditions for removal of endogenous peptides, enabling efficient reconstitution of HLA class I – peptide complexes. For 8 of the 11 alleles for which novel assays were developed elution at pH 3.1 produced the best results. However, for HLA-B14, -B35 and -B62 a different pH was chosen. For instance, elution of naturally presented peptides in HLA-B35 at pH 2.0 resulted in a considerable higher level of FI-labeled reference peptide binding than application of pH 2.4 – 4.0 (figure 2). However, at pH lower than 2.8 cell viability decreased dramatically. We therefore chose pH 2.9 as an optimal compromise between these two phenomena. The optimal pH for every allele is listed in table II.

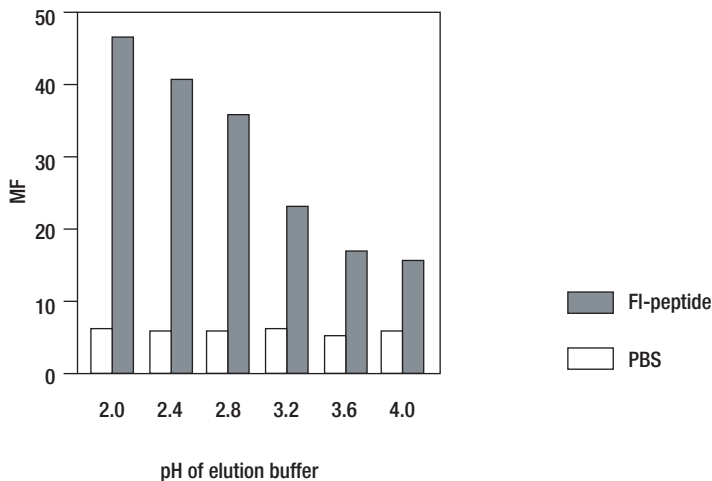


Figure 2.

Determination of optimal pH of the elution buffer for HLA-B35.

The elution buffer was adjusted to the various pHs and elution of naturally presented peptides from the surface of B-LCL BVR was performed at the indicated pHs as described in Materials and Methods. Subsequently, B-LCL BVR was incubated with the HLA-B35 binding FI-labeled reference peptide NPDIVC(FI)YQY for 24 h at 4°C and was measured with flowcytometry at a FACScan. Results of one representative experiment of at least three performed are shown.

Optimization and validation of the competition assays

A general improvement of the assay protocol was realized, compared to the published protocol²⁷, by adding FCS during incubation. Addition of 2% FCS improved binding of the FI-labeled reference peptide (figure 3) and increased cell viability from 30% to 90% after 24 h (data not shown), which greatly enhanced cell recovery for FACS sample preparation. To validate each assay the non-labeled reference peptide and/or another positive control peptide, known from literature to be either a naturally presented ligand or CTL epitope, were tested in 8 serial twofold dilutions (100 μ M – 0.8 μ M) for competition with the FI-labeled peptide. The competition of binding of the HLA-B60 FI-labeled reference peptide by the non-labeled reference peptide KESTLHLVL is shown in figure 4 as an example. Unmodified reference peptides and other positive control peptides were able to inhibit at least 50% of binding of the FI-labeled reference peptide at concentrations lower than 5 μ M ($IC_{50} < 5 \mu$ M) (table III). These results are in line with those obtained with high affinity binding positive control peptides in the published binding assays for HLA-A2 and HLA-A3²⁷.

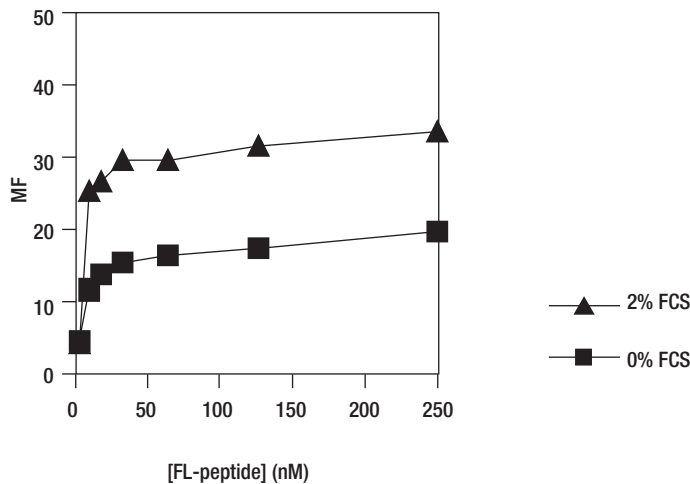


Figure 3.

Influence of incubation without or with addition of 2% FCS on binding of the FI-labeled reference peptide.

B-LCL JY expressing HLA-A2 was incubated with titrated amounts of the HLA-A2 – binding FI-labeled reference peptide FLPSDC(FI)FPSV for 24 h at 4°C without or with addition of 2% FCS in the medium. Subsequently, fluorescence was measured with flowcytometry at a FACScan. Results of one representative experiment of at least three performed are shown.

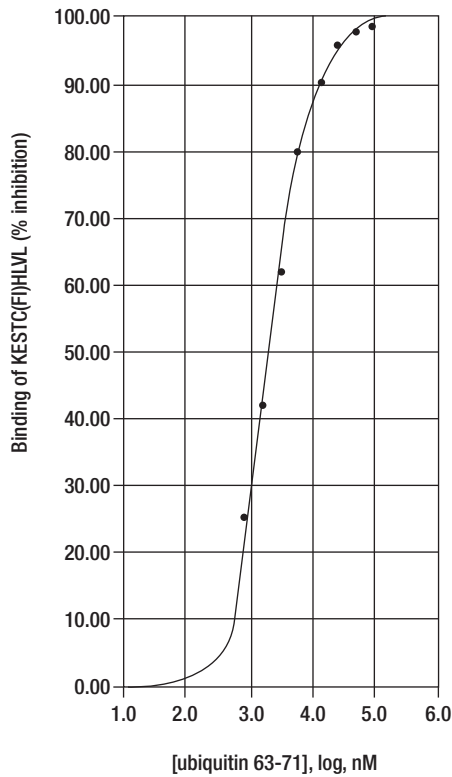


Figure 4.

Competition of binding of the HLA-B60 FI-labeled reference peptide KESTC(F)HLVL by the non-labeled original aa sequence KESTLHLVL to validate the HLA-B60 assay.

The unlabeled peptide was titrated in 8 serial twofold dilutions (100 μ M – 0.8 μ M) on B-LCL DKB (HLA-B60⁺) together with the FI-labeled peptide (125 nM) and was incubated for 24 h at 4°C. Fluorescence was measured with flowcytometry at a FACScan and the data were analyzed by regression analysis using software program CurveExpert 1.3 to determine the precise IC_{50} value expressed at a logarithmic scale. Results of one representative experiment of at least three performed are shown.

Table III.

Positive control peptides used to validate binding assays

HLA class I allele	Positive control peptides ^a		Ref.	IC ₅₀ (μM)
	Sequence	Source		
HLA-A1	YLEPAIAKY	Consensus sequence	32	
HLA-A2	FLPSDFFPSV	HBV cAg 18-27	39	0.5
	YIGEVLVSV	mHag HA-2	45	3.5
HLA-A3	KVFPICALINK	Consensus sequence	32	0.7
	QVPLRPMTYK	HIV-1nef 73-82	46	0.2
HLA-A11	QVPLRPMTYK	HIV-1nef 73-82	46	2.0
	KQSSKALQR	BCR-ABL b3a2	47	5.7
HLA-A24	RYLKDQQLL	HIV-1env gp41 583-591	40	1.8
	AYIDNYNKF	Consensus sequence	48	0.6
HLA-A68	KTGGPIYKR	Influenza A NP 91-99	41	1.3
HLA-B7	APAPAPSWPL	Human p53 84-93	N.P.	0.5
	SPSVDKARAEEL	Human SMCY 950-960 (mHag HY)	49	0.7
HLA-B8	FLRGRAYGL	EBNA-3 339-347	42	0.2
	GFKQSSKAL	BCR-ABL b3a2 fusion region	47	1.5
HLA-B14	ERYLKDQQL	HIV-1env gp41 584-592	50	7.5
HLA-B35	NPDIVYQY	HIV-1 RT 330-338	44	1.2
HLA-B60	KESTLHLVL	Ubiquitin 63-71	36	1.9
HLA-B61	GEFGGFGSV	Histone acetyltransferase 127-135	36	0.2
	GEFVDLYV	40S ribosomal protein S21 6-13	36	0.3
HLA-B62	YLGEFSITY	40S ribosomal protein S15 114-122	36	0.6

^a The unlabeled reference peptides were used as positive control peptide for all alleles except for HLA-A11 and -B14. For several alleles additional positive control peptides were tested. (N.P. = not published)

Identification of novel HLA class I binding peptides

The binding assays described herein were used for the identification of novel HLA class I binding peptides derived from various protein sequences (HIV-1pol; p53; PRAME; mHag HA-1). For several alleles, candidate class I binding peptides were selected complying with the different HLA class I binding motifs of interest and their binding capacity was assessed. For these alleles we successfully identified peptides binding with high or intermediate affinity (table IV). Four peptides of HIV-1pol were found to bind with high affinity in HLA-A11 ($IC_{50} \leq 5 \mu M$), whereas one peptide displayed intermediate affinity ($5 \mu M < IC_{50} \leq 15 \mu M$). In HLA-A24, 3 peptides from HIV-1pol bound with high affinity, one peptide with intermediate affinity and 2 with low affinity ($15 \mu M < IC_{50} \leq 100 \mu M$). Six peptides of p53 displayed high affinity binding to HLA-B7. Seven out of eight peptides derived from PRAME, predicted to bind in HLA-B35, displayed

Table IV.

Identified HLA class I binding peptides

Allele	Sequence	Source	IC ₅₀ (μM) ^a
		HIV-1pol	
HLA-A11	AIKKKDSTK	221-229	4
	GIPHPAGLK	252-260	1
	QLDCTHLEGK	781-790	9
	AVFIHNFKR	898-906	2
	KIQNFRVYY	938-946	4
		HIV-1pol	
HLA-A24	FWEVQLGI	242-249	20
	RYQYNVLPQGW	298-309	1.4
	QYNVLPQGW	300-308	1
	PFLWMGYEL	381-389	1.2
	GYELHPDKW	386-394	20
	LWKGEGAVVI	957-966	6.5
		human p53	
HLA-B7	LPENNVLSPL	26-35	1.2
	SPALNKMFCQL	127-137	0.9
	RPILTIITL	249-257	0.2
	LPPGSTKRAL	299-308	0.2
	SPQPKKKPL	315-323	0.6
		PRAME	
HLA-B35	LPRELFPPPL	48-56	0.7
	LPRRLFPPPLF	48-57	1.6
	FPPLFMAAF	53-61	0.8
	RPRRWKLQV	113-121	>100
	IPVEVLVDLF	173-182	0.1
	LPTLAKFSPY	246-255	0.1
	CPHCGDRTFY	487-497	1.5
	EPILPCCFM	499-507	0.3
		mHag HA-1	
HLA-B60	KECVLHDDL	134-142	5.3
	KECVLRDDL	134-142	3.9
	KECVLHDDL	134-143	1
	KECVLRDDL	134-143	1.6

^a Bindings affinity can be classified according to the following cutoffs. High affinity: IC₅₀ ≤ 5 μM; Intermediate affinity: 5 μM < IC₅₀ ≤ 15 μM; Low affinity: 15 μM < IC₅₀ ≤ 100 μM; No binding IC₅₀ > 100 μM.

high binding affinity for HLA-B35. Furthermore, we found four peptides of minor histocompatibility antigen (mHag) HA-1 that bound with high affinity in HLA-B60³⁷. For the other alleles as well, several high affinity binding peptides (derived from PRAME and BCR-ABL) were successfully identified by applying the present binding assays (manuscript in preparation). In summary, in all assays peptides could be classified in the range from high affinity binding to no observable binding affinity.

Correlation between peptide binding prediction and peptide binding capacity

While peptide binding prediction algorithms are extremely useful to select potential HLA class I binding peptides, the currently prevailing view is that these predictions are not accurate enough to bypass binding measurements. We chose to analyze the binding prediction for HLA-A2 (-A*0201), because a refined binding motif is known for this extensively studied allele^{10,12,18}. Previously, we identified 19 high and 27 intermediate affinity HLA-A2 binding peptides of tumor antigen PRAME (length 509 aa) out of 65 nona- and 63 decamers selected⁴ by using the BIMAS peptide binding prediction algorithm¹⁸. Analysis of the data revealed that a relatively low prediction score did not necessarily exclude high affinity binding. Examples of this group of peptides were decamers SLYSFPEPEA (PRAME 142-151) and FLKEGACDEL (PRAME 182-191) that ranked 35th and 46th in binding-prediction for HLA-A2 (BIMAS algorithm) respectively (data not shown). Despite these low scores, SLYSFPEPEA bound second best (IC₅₀ 1.9 μM) and FLKEGACDEL bound with high affinity as well (IC₅₀ 3 μM, ranking 5th for binding)⁴. Low prediction scores in these cases were caused by the lack of a canonical C-terminal anchor in SLYSFPEPEA and residues with a predicted deleterious effect on binding (E at P7 for SLYSFPEPEA and K at P3 for FLKEGACDEL). Conversely, a high prediction score for HLA-A2 did not necessarily correlate with high affinity binding. Fifty percent of the predicted 16 best binding 9-mers and 18.7% of the 10-mers from the analogous group displayed only low or no binding affinity at all (table V). For instance, nonamer KMILKMVQL (PRAME 224-232) that ranked 5th in binding-prediction for HLA-A2 actually failed to bind (IC₅₀ > 100 μM)⁴. A possible explanation is that the strong deleterious effect on binding of glutamine at position 9 of the peptide¹⁸ may also results from this aa in position 8¹², but is not incorporated in the binding prediction score¹⁸. Taken together we conclude that binding prediction for this particular set of peptides did not accurately correlate with binding affinities, confirming the need for actual peptide binding assays.

Table V.

Accuracy of binding prediction in HLA-A*0201 of 128 peptides in PRAME

Binding prediction ^a		Binding affinity measured by HLA-A2 assay ^c			
Length	Ranking ^b	High	Intermediate	Low	No binding
9-mers	ranked 1-16	6 (37.5%)	2 (12.5%)	6 (37.5%)	2 (12.5%)
	ranked 17-32	1 (6.2%)	4 (25%)	10 (62.5%)	1 (6.3%)
	ranked 33-48	1 (6.2%)	4 (25%)	6 (37.5%)	5 (31.3%)
	ranked 49-65	0 (0.0%)	0 (0.0%)	7 (41.0%)	10 (59.0%)
	total (ranked 1-65)	8 (12.3%)	10 (15.4%)	29 (44.6%)	18 (27.7%)
10-mers	ranked 1-16	6 (37.5%)	7 (43.7%)	3 (18.7%)	0 (0.0%)
	ranked 17-32	1 (6.2%)	6 (37.5%)	7 (43.8%)	2 (12.5%)
	ranked 33-48	4 (25%)	4 (25.0%)	5 (31.2%)	3 (18.7%)
	ranked 49-63	0 (0.0%)	0 (0.0%)	6 (40.0%)	9 (60.0%)
	total (ranked 1-63)	11 (17.5%)	17 (27.0%)	21 (33.3%)	14 (22.2%)

^aPrediction by BIMAS algorithm, accessible via http://bimas.cit.nih.gov/molbio/hla_bind/¹⁸.

^bRanking: no. 1 is peptide with highest prediction score, which is predicted to bind best.

^cBinding affinity classified according to the following cutoffs. High affinity: $IC_{50} \leq 6 \mu M$; Intermediate affinity: $6 \mu M < IC_{50} \leq 15 \mu M$; Low affinity: $15 \mu M < IC_{50} \leq 100 \mu M$; no binding $IC_{50} > 100 \mu M$.

DISCUSSION

Measurement of peptide HLA class I binding affinity can be exploited for the identification of HLA class I presented epitopes as is needed for e.g. vaccine development and insight in autoimmunity and graft versus host reactions. For these purposes especially HLA class I molecules with a prevalent distribution among different human populations are of interest (table I). The current report presents a concise summary of binding assays that were developed for 13 highly prevalent HLA class I molecules according to a competition based strategy which utilizes a fluorescein labeled class I binding reference peptide and cell surface expressed HLA class I molecules.

This type of binding assay has several advantages over molecular HLA class I binding assays. First, the assays are rapid and convenient, because there is no need for time-consuming production and purification of soluble HLA class I molecules. Furthermore, the readout is not dependent on either radioactive peptide labeling or conformation specific antibodies, of which the latter are not available for every allele, but instead on fluorescein labeled reference peptides the synthesis of which is straightforward. Finally, as equipment a flow-cytometer suffices.

We show that the concept of the assay can be adapted for basically every HLA class I allele of interest. Therefore the present report can also be read as an instruction for the development of class I binding assays that are still lacking. Several important allele-specific features need to be determined for each allele. Differences in binding capacity of the FI-labeled reference peptides were observed depending on which residue was substituted for a FI-labeled cysteine (figure 1A). However, when a proper non-anchor residue was chosen for substitution (figure 1A), the substitution did not appear detrimental for binding. Exceptionally, we met problems in finding a suitable FI-labeled reference peptide. For example, we did not succeed thusfar in obtaining a sufficiently binding FI-labeled peptide for HLA-B44 (data not shown). We used B-LCL homozygously expressing HLA class I molecules of interest, as B-LCL are broadly available in the scientific community and can easily be generated from PBMC. The required exclusion of binding of the FI-labeled reference peptide to co-expressed alleles was accomplished with the use of properly selected negative control B-LCL (figure 1B). Like in other competition based assays, in our assays, the measured binding affinity of the test peptides is relative to the binding capacity of the FI-labeled reference peptide. Therefore, we used well defined HLA class I ligands or CTL epitopes as reference peptides (table II). As we have shown before for the HLA-A2 and HLA-A3 binding assays²⁷, the kinetics of peptide binding in our assays at 4°C with an incubation time of 24 h followed the same pattern as those in assays applying soluble HLA molecules. Also, the ranking of peptides according to their IC₅₀ was comparable to the ranking found in cell free binding assays²⁷. Validation of the newly developed assays with either the unlabeled reference peptide or other defined class I binding peptides, showed IC₅₀ values below or around 5 μM (figure 4 and table III), which is in line with previously published results²⁷.

We were able to use the assays described for the identification of novel HLA class I binding peptides as exemplified for HIVpol derived peptides binding in HLA-A11 and HLA-A24, peptides of p53 with high affinity for HLA-B7, PRAME derived peptides binding in HLA-B35 and peptides from mHag HA-1 with high affinity for HLA-B60 (table IV). These peptides have been used for CTL inductions to identify new class I presented epitopes⁵⁷.

An analysis of the motif-based peptide binding prediction in HLA-A2 revealed that rankings of the peptide binding prediction and binding capacity (IC₅₀) did not accurately correlate (table V). This is caused by the incomplete knowledge of the contribution of each aa in every position of a peptide to HLA class I binding and therefore we consider actual peptide binding assays compulsory for precise assessment of peptide binding capacity to all HLA class I molecules. The currently presented peptide binding assays will be conveniently applicable for this purpose.

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CHAPTER 4

Identification of a novel HLA-B60
restricted T cell epitope of the minor
histocompatibility antigen HA-1 locus

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ABSTRACT

The polymorphic minor histocompatibility antigen HA-1 locus encodes two peptides HA-1^H and HA-1^R with a single amino acid difference. Whereas the immunogenicity of the HA-1^R allele has not yet been shown, the nonameric HA-1^H peptide induces HLA-A2 restricted cytotoxic T cells *in vivo* and *in vitro*. It is not known whether the mHag HA-1^H or HA-1^R associates with other HLA class I molecules. Therefore, the polymorphic regions of both HA-1 alleles were analyzed to identify HLA class I binding peptides that are properly processed by proteasomal degradation. Peptide binding analyses were performed for all nonameric HA-1^{H/R} peptides for binding to nine HLA class I molecules that have more than 10% prevalence in the Caucasian population and for seven nonameric/decameric HA-1^{H/R} peptides that were predicted to bind to HLA-A3, -B14 and -B60. Only the nonameric KECVL^{H/R}DDL and decameric KECVL^{H/R}DDL peptides showed strong and stable binding to HLA-B60. *In vitro* digestion of 29 amino acid long HA-1 peptides by purified 20S proteasomes revealed proper cleavage at the COOH termini of both HLA-B60 binding HA-1^H and HA-1^R peptides. In subsequent analyses, dendritic cells pulsed with the nonameric HA-1^R peptide did not induce CTLs that recognize the natural HLA-B60/HA-1^R ligand. In contrast, dendritic cells pulsed with the nonameric HA-1^H peptide induced IFN- γ secreting T cells specific for the natural HLA-B60/HA-1^H ligand in three HLA-B60⁺ HA-1^{RR} individuals, demonstrating the immunogenicity of the HLA-B60/HA-1^H ligand. In conclusion, this study shows a novel HLA-B60 restricted T cell epitope of the minor histocompatibility antigen HA-1 locus.

INTRODUCTION

Differences in minor histocompatibility antigens (mHags) between HLA identical stem cell (SC) donor and recipient may lead to alloimmune T cell responses post SC transplantation (SCT). The mHag specific T cells are key players in both the Graft versus Host Disease and the Graft versus Leukemia reaction¹. It is important to understand the basis of the mHag induced alloimmune responses for both latter Graft versus Host reactivities. Now that mHags can be characterized, information on issues such as mHag processing and presentation will give insight into their potential clinical relevance. With the molecular identification of the autosomally encoded mHag HA-1, it was shown for the first time that a human mHag is encoded by a locus with two alleles with a single amino acid difference². The difference between the HA-1 alleles comprises a histidine (H) to an arginine (R) substitution. Although nonameric peptides of both the HA-1^H and the HA-1^R alleles bind to HLA-A2, the HA-1^R allele fails to be expressed at the cell surface. In contrary, the HA-1^H peptide is presented at the cell surface and is known to induce HLA-A2 restricted CTLs both *in vivo* and *in vitro*^{3,4}. Because of its hematopoietic system restricted expression, the mHag HA-1 can be used for the treatment of relapsed leukemia after HLA identical HA-1 mismatched SCT^{4,5}.

Since the HA-1 specific immunotherapy is currently restricted to HLA-A2 positive patients, we have investigated whether the HA-1^{H/R} polymorphic region contains peptides that can be presented by other HLA molecules. We analyzed the binding capacities of HA-1 polymorphic peptides to nine HLA-A and -B molecules that have a frequency of > 10% in the Caucasian population. Since cognate T cell epitopes of mHags do not always confirm the predicted MHC class I binding motifs^{6,7}, all nonameric HA-1^{H/R} peptides (n=18) were tested for binding to these frequent HLA alleles. The peptide binding analyses were extended with two decameric HA-1^{H/R} peptides that contained binding motives for HLA-A3 and with five nonameric/decameric peptides that were predicted to bind to HLA-B14 or to -B60. After the binding studies, cellular processing was executed by *in vitro* proteasome digestion of 29 amino acid long HA-1^H and HA-1^R peptides. To enlarge the patient population for HA-1 specific immunotherapy, the HLA-B60 binding peptides were analyzed for their *in vitro* immunizing potential. Peptide loaded dendritic cells (DCs) were used to induce T cell responses from healthy individuals.

MATERIALS AND METHODS

HA-1 peptides

HA-1^H and HA-1^R peptides were synthesized using an automated multiple peptide synthesizer (Syrto II, MultisynTech, Witten, Germany) according to the known HA-1 amino acid sequence². The purity of the peptides was > 90%. The peptides were dissolved in dimethyl sulfoxide (DMSO), diluted in 0.9% NaCl and stored at -20°C until use.

Prediction of HLA peptide binding

The polymorphic HA-1^H and HA-1^R regions were screened with the HLA-peptide binding prediction software of BIMAS (BioInformatics & Molecular Analysis Section, NIH, Bethesda, MD; url: <http://bimas.dcrt.nih.gov/>) for octameric, nonameric or decameric HA-1 peptides capable to bind to HLA class I molecules. The selection of peptide candidates was made by comparison of the computed scores with that of the HLA-A2 restricted HA-1^H CTL epitope with amino acid (aa) sequence VLHDDLLEA (score of 79.6). This score corresponds to the estimated half-time of dissociation of complexes containing the peptide at 37°C at pH 6.5. Five HA-1^{H/R} peptides with scores ranging from 32 (intermediate binding score) to 176 (strong binding score) were selected to assay for binding to the relevant HLA class I molecules. The predicted HLA class I /HA-1^{H/R} peptide associations and their computed binding scores are presented in table I. In addition, we selected two decameric HA-1^{H/R} peptides that contained anchor residues for binding to HLA-A3 but were not predicted by the BIMAS software.

Table I.

Peptides of the HA-1 polymorphic region tested for binding to different HLA class I molecules^a

Peptide no.	HA-1 ^{H/R} polymorphic region sequence											Binding predicted to (BIMAS score) ^b										
	E	K	L	K	E	C	V	L	H/R	D	D		L	L	E	A	R	R				
1	E	K	L	K	E	C	V	L	H													
2	E	K	L	K	E	C	V	L	R													
3		K	L	K	E	C	V	L	H	D												
4		K	L	K	E	C	V	L	R	D												
5			L	K	E	C	V	L	H	D	D											
6			L	K	E	C	V	L	R	D	D											
7				K	E	C	V	L	H	D	D	L				HLA-B60 (176)						
8				K	E	C	V	L	R	D	D	L				HLA-B60 (176)						
9				K	E	C	V	L	H	D	D	L	L			HLA-B60 (160)						
10				K	E	C	V	L	R	D	D	L	L			HLA-B60 (160)						
11					E	C	V	L	H	D	D	L	L									
12					E	C	V	L	R	D	D	L	L			HLA-B8 (32), -B14 (90)						
13						C	V	L	H	D	D	L	L	E								
14						C	V	L	R	D	D	L	L	E								
15							V	L	H	D	D	L	L	E	A	HLA-A2 (79.6)						
16								V	L	R	D	D	L	L	E	A						
17									V	L	H	D	D	L	L	E	A	R				
18										V	L	R	D	D	L	L	E	A	R			
19											L	H	D	D	L	L	E	A	R			
20												L	R	D	D	L	L	E	A	R		
21													R	D	D	L	L	E	A	R	R	
22														R	D	D	L	L	E	A	R	R

^a Peptides 1-8, 11-16 and 19-22 were assayed for binding to HLA-A1, -A2, -A3, -A11, -A24, -B7, -B8, -B35, -B62 regardless of prediction. Polymorphic amino acids are indicated in bold.

^b Prediction of HLA/peptide associations was executed using BIMAS software except for peptides 17 and 18, which were not predicted by BIMAS but contain HLA-A3 anchor amino acids at position 2 and 10.

HLA peptide binding assays

We used the competition-based HLA peptide binding assay as described previously, with some modifications⁸. Briefly, HLA typed EBV-LCLs were washed with PBS, kept on ice for 5 min. and treated with an ice-cold 0.132 M citric acid/0.062 M Na₂HPO₄·2H₂O elution buffer for 90 sec⁸. The pH of the elution buffer was optimized for each HLA molecule to enable maximal elution of HLA

bound peptides (chapter 3). Immediately after mild acidic treatment, the cells were washed with 12 ml Iscove's modified Dulbecco's medium (IMDM, Bio Whittaker, Belgium) containing 2% FCS and resuspended in IMDM containing 2% FCS, 1.5 µg/ml β_2 microglobulin (Sigma, St. Louis, MO, USA). 4×10^4 acid treated EBV-LCLs were then incubated in 96-well-V-bottom plates (Costar, Cambridge, MA, USA) with fluorescent-labeled reference peptide (25 µl/well, final concentration: 150 nM) mixed with serial dilutions of competitor (test) peptides (25 µl/well; final concentrations: 100 to 0.78 µM) in a total volume of 150 µl. All reference peptides were deduced from previously reported peptides that show strong binding to the respective HLA class I molecules⁹. After incubation for 24 h at 4°C, the cells were washed twice with 100 µl/well PBS/1% FCS and fixed with 0.5% paraformaldehyde in PBS. The mean fluorescence expressed by the cells was determined by a FACScalibur flow cytometer (Becton-Dickinson, St. Louis CA, USA). Percentage inhibition of the HLA binding of the fluorescent reference peptide is calculated with the formula:

$$\left(\frac{1 - (MF_{\text{reference + competitor peptide}} - MF_{\text{background}})}{(MF_{\text{reference peptide}} - MF_{\text{background}})} \right) \times 100\%$$
The relative binding affinity of the peptides is expressed as the peptide concentration that inhibits 50% of the binding of the reference peptide (IC_{50}).

Proteasomal cleavage of the HA-1 polymorphic region

Twenty-nine amino acid long HA-1^H and HA-1^R peptides were purified to > 95% by reverse phase HPLC. 10 µg/ml of the peptides were incubated with 20S proteasomes isolated from EBV-LCLs for 15 min, 30 min and 45 min as described elsewhere¹⁰⁻¹². The proteolysis products were analyzed by tandem mass spectrometry as previously described¹³.

Dendritic cell culture

Monocyte derived DCs (MoDCs) were generated from healthy individuals by culturing peripheral blood derived CD14⁺ monocytes with 1000 U/ml IL-4 (Genzyme, Cambridge, MA, USA) and 800 U/ml GM-CSF (donated by Dr. S. Osanto, LUMC, Leiden, The Netherlands) for 6 days as described elsewhere¹⁴. On day 6, the DCs were matured by culturing on irradiated (750 Gy) CD40 ligand-transfected mouse fibroblasts at a DC to fibroblast ratio of 2:1 or by adding 50% of monocyte-conditioned medium¹⁴. Mature DCs were pulsed with HA-1 peptides for 2 hours at 37°C in Aim-V medium (Invitrogen, Breda, The Netherlands) before their use as stimulator cells.

In vitro induction of HLA-B60/HA-1 specific T cell responses

Peptide pulsed DCs were cocultured with autologous PBMC at a DC to PBMC ratio of 1:10 in IMDM, 10% human serum supplemented with 1 U/ml IL-2 (Cetus, Emeryville, CA, USA) and 1 U/ml IL-12 (R&D systems, Minneapolis, MN, USA). On day 5, 20 U/ml IL-2 was added. On day 7, the T cell lines (TCL) were

depleted of CD4⁺ cells using immunomagnetic beads (Dynal AS, Oslo, Norway) and were restimulated with irradiated (150 Gy) peptide pulsed mature DCs (DC:T cell ratio, 1:10) or with irradiated (150 Gy) peptide pulsed monocytes (monocyte:T cell ratio, 1:5). Twenty-four hours and 96 hours after restimulation, medium containing 20 U/ml IL-2 was added. TCL were subsequently restimulated every 7 days and were tested for HA-1 specific activity in Interferon- γ (IFN- γ) ELISPOT assays¹⁵ before each restimulation.

RESULTS

Effective binding of nonameric and decameric HA-1^H and HA-1^R peptides to HLA-B60

Three categories of HLA molecules were selected for the peptide binding assays: those molecules with a frequency of more than 10 % in the Caucasian population, those with binding motifs and those that were predicted to bind nonameric / decameric HA-1^{H/R} peptides. All nonameric HA-1^H and HA-1^R peptides (n=18) were tested for binding to the so called frequent HLA class I molecules HLA-A1, -A2, -A3, -A11, -A24, -B7, -B8, -B35, -B62. The peptide analysis was extended with two decameric HA-1^{H/R} peptides with a binding motif for HLA-A3 and with five nonameric/decameric peptides predicted to bind either to HLA-B14 or -B60 (table I). The HLA-A1, A11, -A24, -B7, -B8, -B14, -B35 and -B62 molecules did not bind nonameric HA-1^{H/R} peptides, despite the predictions of BIMAS software for intermediate to strong binding of peptide ECVLRDDL to HLA-B8 or to -B14 (table I). The decameric HA1^{H/R} peptides VL^{H/R}DDLLEAR showed weak to intermediate binding to HLA-A3 molecules with IC₅₀ values of 15.6 μ M and 37.5 μ M respectively (figure 1). In agreement with the prediction of the BIMAS software, the nonameric and decameric HA-1^{H/R} peptides KECVLHDDL, KECVLRDDL, KECVLHDDL and KECVLRDDL showed strong binding to HLA-B60 molecules with very low IC₅₀ values of 5.3 μ M, 3.9 μ M, 1.0 μ M and 1.6 μ M respectively (figure 2). As expected, the original HLA-A2/HA-1^H CTL epitope, also predicted by the BIMAS software, displayed binding to HLA-A2 with an IC₅₀ value of 6.4 μ M (data not shown).

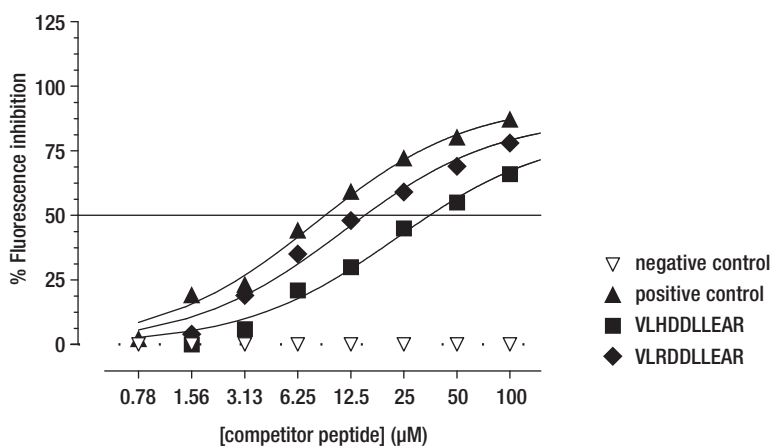


Figure 1.

Binding of HA-1^{H/R} peptides to HLA-A3.

The results are expressed as the percentage inhibition of the HLA binding of the 150 nM fluorescent reference peptide by the indicated peptides added at serial dilutions (see Materials and Methods). Curves were fitted by nonlinear regression and one site binding equation. The IC₅₀ value of the HLA-A3 binding positive control peptide KQSSKALQR⁹ was 9.4 μM.

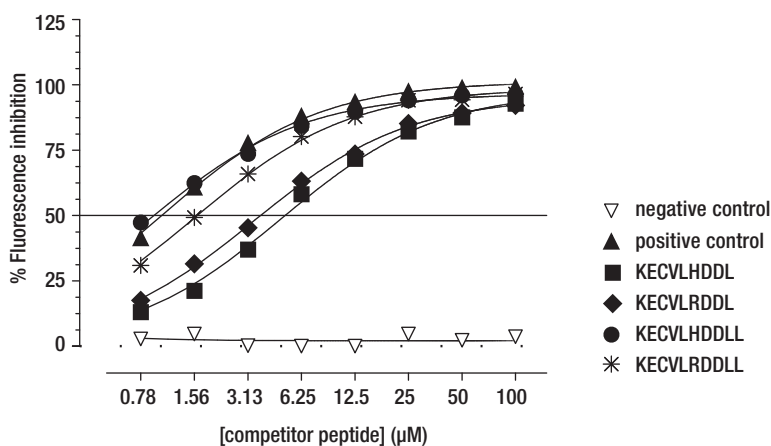


Figure 2.

Efficient binding of HA-1^{H/R} peptides to HLA-B60.

The results are expressed as the percentage inhibition of the HLA binding of the 150 nM reference peptide by the indicated peptides added at serial dilutions (see Materials and Methods). Curves were fitted by nonlinear regression and one site binding equation. The IC₅₀ value of the HLA-B60 binding positive control peptide KESTLHLVL⁹ was 1.1 μM.

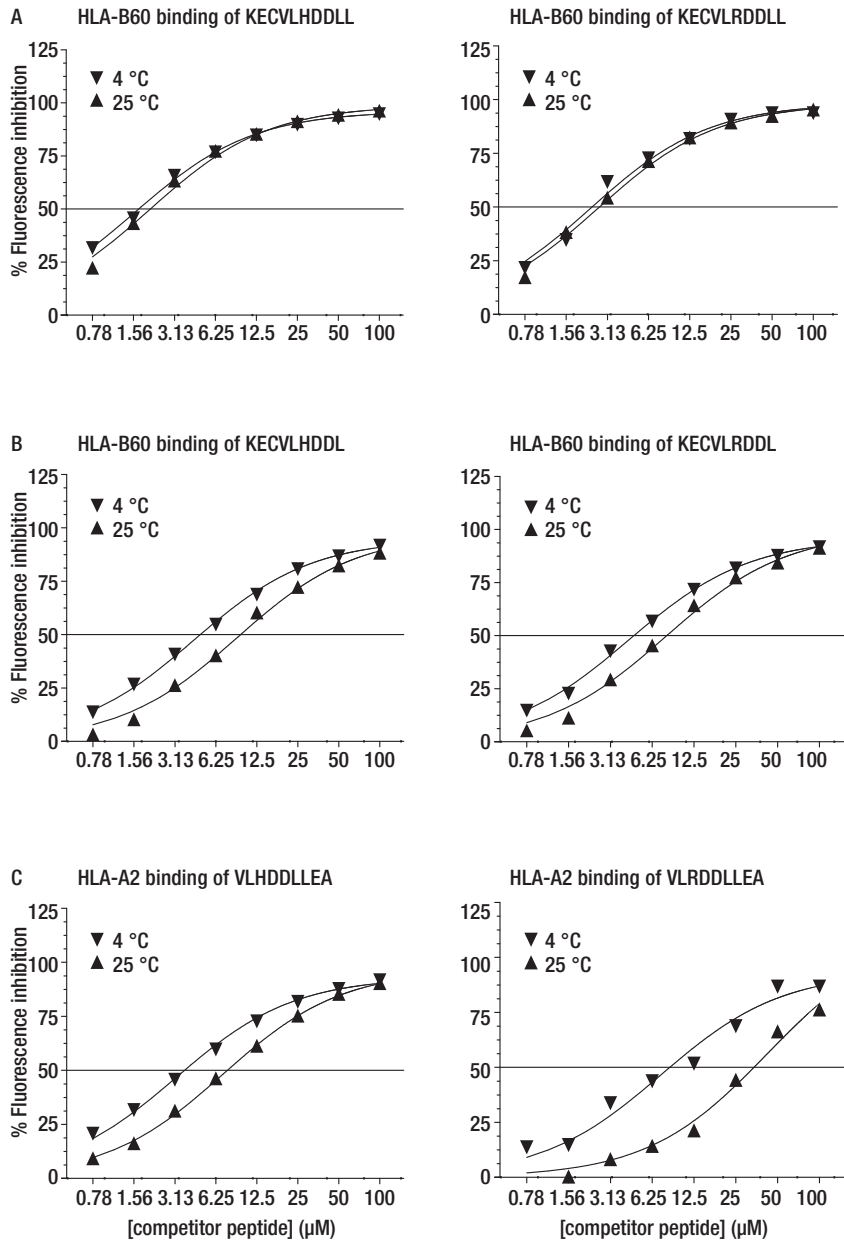


Figure 3.

Stable binding of nonameric and decameric HA-1^{H/R} peptides to HLA-B60.

The nonameric and decameric HA-1^{H/R} peptides were tested for binding to HLA-B60 (A, B) and to HLA-A2 (C) at the indicated temperatures. The results are expressed as the percentage inhibition of HLA binding of the reference peptide. Curves were fitted by nonlinear regression and one site binding equation.

Stable binding of nonameric and decameric HA-1^H and HA-1^R peptides to HLA-B60

The stability of the HLA-B60/HA-1^{H/R} peptide binding was addressed by testing for the HLA peptide binding capacities at 4°C and 25°C. HLA-A2/HA-1^{H/R} peptide binding stability was analyzed in parallel as comparison. Increasing the temperature from 4°C to 25°C did not affect the strong binding of decameric HA-1^{H/R} peptides to HLA-B60 (figure 3A). Less binding was observed with the nonameric HA-1^{H/R} peptides to HLA-B60 (figure 3B), which was comparable to the nonameric HA-1^H peptide to HLA-A2 (figure 3C). Increasing the temperature from 4°C to 25°C further decreased the intermediate binding of the nonameric HA-1^R peptide to HLA-A2 (figure 3C). Thus, the binding of both HA-1^H and HA-1^R peptides to HLA-B60 were stable and not temperature sensitive.

Proper proteasomal cleavage of the HLA-B60 binding HA-1^{H/R} peptides

Twenty-nine amino acid long HA-1^{H/R} peptides were subjected to *in vitro* digestion with EBV-LCL derived 20S immuno-proteasomes. Within a time frame of 15 minutes, major peptide fragments were cleaved at the COOH-termini of both nonameric and decameric HLA-B60 binding HA-1^{H/R} peptides. The latter cleavage products contained the intact HLA-B60 binding sequences with 3-5 additional amino acid residues at the N termini for the HA-1^H and HA-1^R peptides as demonstrated in tables II and III, respectively. Thus, both the HA-1^H and the HA-1^R products can be effectively cleaved by proteasomes to generate the precursors of the peptides that bind to HLA-B60.

Table II.

In vitro proteasomal cleavage of a 29-amino acid long HA-1^H peptide^a

			GL EKL <u>KECVLHDDL</u> LEARRPRAHECLGEA
% fragment digested in			
15 min	30 min	45 min	
17.2	22.4	0	GLEKLKECVLHDDL
14.7	11.8	14.7	GLEKLKECVLHDDLLEARRPRAHECLG
13.9	16.4	21.7	HDDLLEARRPRAHECLGEA
13.0	10.3	13.9	GLEKLKECVLHDDLLEARRPRA
10.5	8.3	12.1	EKLKECVLHDDL
9.6	8.8	12.3	GLEKLKECVLHDDLLEARRPRAHEC
8.5	9.2	13.8	GLEKLKECVLHD
7.8	7.4	11.5	GLEKLKECVLHDDLLEA
4.8	5.5	0	GLEKLKECVL

^a The peptide sequences that bind to HLA-B60 are underlined. The proteolytic fragments cleaved at the COOH termini of the HLA-B60 binding peptides are indicated in bold. The amounts of the generated fragments after cleavage with 20S immuno proteasomes for 15, 30 and 45 min are expressed as the percentage of all fragments found in the digested substrate.

Table III.

In vitro proteasomal cleavage of a 29-amino acid long HA-1^R peptide^a

			G L E K L <u>K E C V L R D D L</u> L E A R R P R A H E C L G E A									
% fragment digested in												
15 min	30 min	45 min										
26.2	28.0	23.8	G L E K L K E C V L R D D L L E A R R P R A H E C L G									
14.0	16.0	13.5	G L E K L K E C V L R D D L L E A R R P R A H E C L G E									
11.1	14.3	12.7	G L E K L K E C V L R D D L									
7.9	9.6	7.9	G L E K L K E C V L R D D L L E A R R P R A									
6.6	8.6	8.3	E K L K E C V L R D D L L									
6.2	7.5	7.3	C V L R D D L L E A R R									
5.3	7.0	5.7	G L E K L K E C V L R D D L L E A R R P R A H E C L									
4.9	7.1	6.4	G L E K L K E C V L R D D L L E A R R P R A H E C									
4.2	6.1	5.6	G L E K L K E C V L R D									
3.9	4.2	3.9	G L E K L K E C V L R D D L L E A									
3.6	4.4	4.4	G L E K L K E C V L R D D L L E A R R									
3.4	4.2	3.6	G L E K L K E C V L R D D L L E A R R P R									
2.6	4.2	4.0	G L E K L K E C V L R D D L L E A R R P R A H									

^a The peptide sequences that bind to HLA-B60 are underlined. The proteolytic fragments cleaved at the COOH termini of the HLA-B60 binding peptides are indicated in bold. The amounts of the generated fragments after cleavage with 20S immuno proteasomes for 15, 30 and 45 min are expressed as the percentage of all fragments found in the digested substrate.

In vitro induction of HLA-B60 restricted T cells against the nonameric HA-1^H peptide

To test the immunogenicity of both the HA-1^H and the HA-1^R peptides in the context of HLA-B60, PBMCs from three HLA-B60⁺ HA-1^{RR} and from two HLA-B60⁺ HA-1^{HH} healthy individuals were stimulated with autologous DCs pulsed with the nonameric HA-1^H or HA-1^R peptide respectively. After two or three rounds of stimulation, the two T cell lines (TCL) induced with the HA-1^R peptide contained significant numbers of IFN- γ producing T cells that recognized HA-1^R peptide pulsed HLA-B60-transfected T2 cells. Nevertheless, neither TCL induced with HA-1^R peptide produced IFN- γ upon stimulation with EBV-LCLs that express the natural ligand HLA-B60/HA-1^R (data not shown). On the contrary, all three TCL induced with the HA-1^H peptide contained besides HA-1 non-specific T cells, a significant number of T cells that produced IFN- γ not only upon stimulation with HA1^H peptide pulsed HLA-B60 transfected T2 cells but also upon stimulation with EBV-LCLs that express the natural HLA-B60/HA-1^H ligand (figure 4).

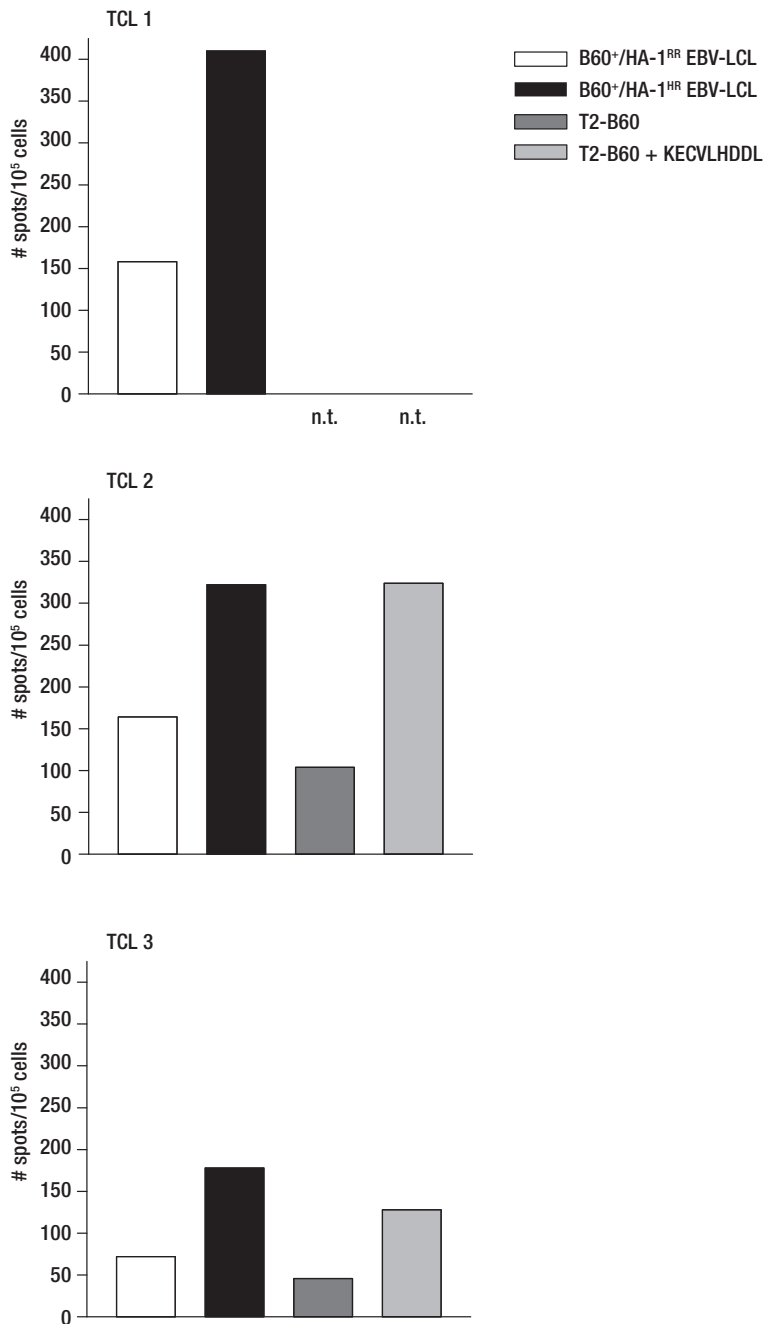


Figure 4.

T cell recognition of HLA-B60/HA-1^H ligand.

T cell lines (TCL) secreting IFN- γ in response to the target cells are indicated. The EBV-LCLs (HLA-B60/HA-1^{RR} and HLA-B60/HA-1^{HR}) are derived from HLA-identical but HA-1-non-identical siblings. The number of IFN- γ spots per 10⁵ cells is expressed on the Y-axis. The SEM was < 5%.

DISCUSSION

Earlier studies demonstrated the immunogenicity of one allele of the HA-1 locus i.e.: the nonameric HA-1^H peptide VLHDDLLEA presented by the HLA-A2 molecule². Here, we show for the first time that the mHag HA-1 locus comprises, beside the known HLA-A2/HA-1^H T cell epitope, an HLA-B60/HA-1^H T cell epitope.

In search for novel T cell epitopes in the HA-1^{H/R} polymorphic region, we studied the binding of polymorphic HA-1 peptides to 11 HLA class I molecules and analyzed the proteasomal cleavage sites in the HA-1^{H/R} polypeptides. These analyses suggested novel interactions of both alleles of the mHag HA-1 locus with HLA-B60 molecules. Both nonameric and decameric HA-1^{H/R} peptides effectively bind to HLA-B60. *In vitro* proteasomal analysis showed cleavage at the COOH termini of HLA-B60 binding peptides, indicating proper intracellular processing. Functional analyses revealed the immunogenicity of the HLA-B60/HA-1^H ligand but as yet not of the HLA-B60/HA-1^R ligand.

Both nonameric and decameric HA-1^{H/R} peptides show strong binding to HLA-B60, with IC₅₀ values between 1.6-5.3 μM. These HLA binding levels are similar to or higher than the HLA binding of the immunogenic HLA-A2/HA-1^H CTL epitope and of other reported T cell epitopes measured in similar assays⁸⁻¹⁶. Furthermore, we compared the stability of the HLA-B60/HA-1^{H/R} with HLA-A2/HA-1^{H/R} peptide interactions by increasing the temperature of the binding assays. These assays reveal that unlike the HLA-A2/HA-1^R peptide interaction, the HLA-B60/HA-1^{H/R} and HLA-A2/HA-1^H interactions are stable. The stability of HLA-B60/HA-1^{H/R} interactions were confirmed in separate experiments using fluorescent HA-1^{H/R} peptides (data not shown). Thus, both HA-1^H and HA-1^R peptides can efficiently interact with HLA-B60, which is an important biochemical feature of strongly immunogenic T cell epitopes¹⁶. This actually predicts immunogenicity of both HA-1^H and HA-1^R locus products in association with HLA-B60.

The HLA peptide binding is preceded by intracellular processing of cellular proteins. In the endoplasmatic reticulum (ER), proteasomally cleaved peptides can undergo NH₂-terminal trimming by aminopeptidases¹⁷. COOH-terminal trimming in de ER has not been demonstrated. The proper generation of the correct COOH-terminus by an early major cleavage site by proteasomes is thus a key event for efficient epitope generation as demonstrated by recent studies¹⁸⁻²¹. In our *in vitro* cleavage studies, the correct COOH termini of HLA-B60 binding sequences of both the HA-1^H and the HA-1^R allele were generated within 15 minutes. These peptide fragments contained the intact HLA-B60 binding sequences. The exact sequences of the HLA-B60 binding peptides were not present as proteasomal degradation products. Some additional cleavage sites within the putative T cell epitopes were also observed. Nonetheless, the successful generation of HLA-B60/HA-1^H-specific T cells demonstrates the proper cleavage of the HLA-B60 binding HA-1^H peptides by cellular antigen processing machinery.

Our results confirm the recent reports on the importance of the generation of the exact COOH terminus for the intracellular generation of T cell epitopes. Once the relevant peptides are generated by proteasomal cleavage, other cleavage sites found within the epitope do not prevent the generation of immunogenic T cell epitopes expressed on the cell surface¹⁵. Based on these results, one may expect that the HA-1^R peptides are also expressed on the cell surface in association with HLA-B60. However, the TCL generated against the HA-1^R peptide loaded HLA-B60⁺ stimulator cells recognized only peptide-pulsed stimulator cells, not the stimulator cells that naturally express HLA-B60/HA-1^R ligand. It is possible that these TCL may have low affinity to the natural HLA-B60/HA-1^R ligand. Alternatively, the naturally expressed HLA-B60/HA-1^H complex may act as an altered peptide ligand to antagonize the high avidity HLA-B60/HA-1^R-specific T cells. In murine models, it was shown that *in vivo* expression of altered peptide ligand as a natural MHC/peptide complex can antagonize mature T cells specific for the agonistic ligand²². The antagonistic complex may also cause negative selection of the high avidity T cells in the thymus^{25,24}. Thus both mechanisms may result in the failure of the detection of HLA-B60/HA-1^R specific T cells in the periphery. However, it may also be possible that similar to the HLA-A2/HA-1^R ligand, the HA1^R peptide is not expressed on the cell surface. HLA-B60 HA-1^R peptide elution experiments are currently underway, but to date have not shown the presence of the HLA-B60/HA-1^R peptide. Moreover, we will apply a more efficient CTL induction protocol and use HA-1-ransduced DCs instead of peptide-pulsed DCs as antigen presenting cells. cDNA-transduced DCs result in natural processing and constitutive expression of the mHag HA-1, which is more effective than the peptide pulsed DCs in the generation of HA-1 specific CTLs²⁵.

In conclusion, our analyses reveal a novel HLA-B60-restricted HA-1^H specific T cell epitope. Herewith, the HA-1-specific immunotherapy for relapsed leukemia can be extended to patients who receive HLA-B60-matched HA-1^H-mismatched SCT.

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CHAPTER 5

Cord blood comprises antigen-experienced T cells specific for maternal minor histocompatibility antigen HA-1

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ABSTRACT

Umbilical cord blood transplantation is applied as treatment for mainly pediatric patients with hematological malignancies. The clinical results show a relatively low incidence of Graft-versus-Host-Disease and leukemia relapse. Since maternal cells traffic into the fetus during pregnancy, we questioned whether cord blood has the potential to generate cytotoxic T cells specific for the hematopoietic minor Histocompatibility (H) antigen HA-1 that would support the Graft-versus-Leukemia effect. Here we demonstrate the feasibility of *ex vivo* generation of minor H antigen HA-1 specific T cells from cord blood cells. Moreover, we observed already pre-existing HA-1 specific T cells in cord blood samples. Both the circulating and the *ex vivo* generated HA-1 specific T cells show specific and hematopoietic restricted lysis of HLA-A2⁺/HA-1⁺ target cells, including leukemic cells. The cord blood derived HA-1 specific cytotoxic T cells are from child origin. Thus, the so-called naïve cord blood can comprise cytotoxic T cells directed at the maternal minor H antigen HA-1. The apparent immunization status of cord blood may well contribute to the *in vivo* Graft-versus-Leukemia activity after transplantation. Moreover, since the fetus cannot be primed against Y chromosome encoded minor H antigens, cord blood is an attractive stem cell source for male patients.

INTRODUCTION

In the last decade, umbilical cord blood transplantation (CBT) is available as alternative to HLA matched sibling or unrelated donor stem cell transplantation (SCT) for the treatment of hematological malignancies¹⁻⁷. The clinical outcome of transplanted CB grafts with one or two HLA antigen mismatches demonstrates a similar risk of developing Graft versus Host Disease (GvHD) as compared to HLA-matched unrelated SCT^{5,6}. A significant lower incidence of acute and chronic GvHD was reported after HLA identical CBT when compared to sibling SCT⁴. Collectively, these clinical results point to a decreased incidence of GvHD after CBT.

GvHD is often associated with a curative Graft versus Leukemia (GvL) response. Minor H antigen disparities between donor and recipient play important roles in both the GvH and GvL reactivity after HLA-matched SCT as reviewed⁸. One of the well-described minor H antigens is HA-1. The immunodominant minor H antigen HA-1 is encoded by a diallelic gene with a single amino acid polymorphism⁹. The HA-1 'positive' allele (HA-1⁺) contains a histidine at position 3 (HA-1^H), whereas the HA-1 'negative' allele (HA-1⁻) contains an arginine (HA-1^R). The HA-1^H peptide is recognized by HLA-A2 restricted CD8⁺ cytotoxic T cells from HA-1⁻ donors¹⁰⁻¹². The expression of HA-1 is restricted to the hematopoietic lineage and to epithelial carcinomas^{13,14}. This restricted expression makes HA-1 an attractive target antigen for GvL and Graft-versus-Tumour responses¹⁵.

Despite the lower incidence of GvHD after CBT, there is no indication of increased leukemia relapse rates when compared with sibling or unrelated donor SCT⁵⁻⁵. Comparable survival rates point to an as yet unexplored GvL potential of cord blood. Relatively little is known about the development of antigen-specific T cell responses around birth¹⁶. Mature monocyte-derived neonatal dendritic cells (DC) are able to efficiently prime antigen-specific cytotoxic T cells *in vitro*¹⁷. In bulk cultures, cord blood T cells proliferate in response to alloantigen¹⁸. Yet, the development of functional alloreactive cytotoxic T cells is impaired¹⁸⁻²⁰. Limiting dilution studies have however reported normal precursor frequencies of cytotoxic T cells specific for allo-HLA class I and class II in cord blood²⁰⁻²². Thus, the capacity to develop allogeneic cytotoxic T cells is intact at birth, despite overall diminished magnitude of responses²⁵.

It is known that fetomaternal hemorrhage occurs during pregnancy. Fetal cells expressing paternal minor H antigens can prime maternal T cells^{12,24}. Since cells of the mother also traffic into the fetus during pregnancy, we tested the hypothesis that maternal minor H antigens can prime fetal T cells. Fifteen HLA-A2⁺/HA-1⁻ CB samples derived from HLA-A2⁺/HA-1⁻ or HLA-A2⁺/HA-1⁺ mothers were analyzed for their feasibility to generate HA-1 specific cytotoxic T cells *ex vivo* as well as for the presence of pre-existing HA-1 specific T cells.

METHODS

Cord Blood

After informed consent of the mother, cord blood was collected from the umbilical cord vein with the placenta still *in utero*. HLA-A2⁺/HA-1⁻ CB samples derived from HLA-A2⁺/HA-1⁻ and HLA-A2⁺/HA-1⁺ mothers were selected after high resolution HLA class I typing and HA-1 genomic typing as described previously²⁵. Cord blood mononuclear cells (CB-MNC) were isolated by Ficoll-Isopaque density gradient centrifugation and stored in liquid nitrogen.

HLA class I/minor H antigen peptide tetrameric complexes

Expression of the T cell receptor specific for HLA-A2/HA-1^{H1} peptide (VLHDDLLEA) complexes was analyzed by staining T cells with phycoerythrin (PE)-conjugated HLA-A2/HA-1 tetrameric complexes (HA-1^{A2}) in combination with allophycocyanin (APC)-conjugated anti-CD8 monoclonal antibody (BD Biosciences, Amsterdam, The Netherlands). Tetramers were generated as previously described²⁶. Specificity analysis of the HA-1^{A2} tetramer was performed in parallel experiments using HA-1 specific and HA-1 non-specific CTL clones (data not shown).

Culture, retroviral transduction and maturation of CD34⁺-derived dendritic cells

CD34⁺ cells were isolated via positive selection using CD34 magnetic-activated cell sorting (MACS) beads (Miltenyi GmbH, Bergisch Gladbach, Germany). CD34⁺ cells were cultured in complete IMDM containing 10% pooled human serum (HS), 250 U/ml GM-CSF (Leucomax, Novartis, Arnhem, The Netherlands), 25 µg/ml SCF (Peprotech, London, UK), 100 U/ml TNF-α (Peprotech) and 50 µg/ml FLT3 ligand (R&D Systems, Mineapolis, MN). Dendritic cells were transduced with retroviral supernatants containing HA-1^H encoding cDNA, as previously described²⁷, and further cultured in complete medium supplemented with 300 U/ml IL-4 (Peprotech). Maturation of HA-1^H transduced dendritic cells was induced by co culturing immature dendritic cells for 3 days with irradiated CD40 ligand-transfected fibroblasts.

Generation of HA-1 specific cytotoxic T cells from cord blood

A slightly modified protocol, originally designed for the induction of minor H antigen specific cytotoxic T cells from adult PBMC, was applied¹¹. In short, CD8⁺ T cells were isolated via positive selection using CD8 MACS beads (Miltenyi) and cultured with irradiated autologous HA-1^H transduced dendritic cells (generated as described above) at a CD8 to dendritic cell ratio of 5:1 or 10:1 in IMDM supplemented with 10% HS, 0.5 U/ml IL-2 (Cetus, Emeryville, CA) and 1 U/ml IL-12 (R&D Systems). After 3 days, irradiated autologous T helper cells were added to the culture at a CD8 to T helper ratio of 10:1. T helper cells were generated by stimulating CD34/CD8 depleted CB-MNC with anti-CD3/CD28 beads according to supplier's protocol (DynaL Biotech, Smestad, Norway). This mode of stimulation results in ≥ 80% activated CD4⁺ T cells that produce IFN-γ, TNF-α and IL-2 (data not shown). From day 7 onwards, oligoclonal T cell lines were restimulated weekly using irradiated dendritic cells and T helper cells at a CD8:T helper:dendritic cell ratio of 10:1:1. Fresh medium containing IL-2 was added every 3–4 days.

Bulk T cell lines were tested for the presence of HA-1^{A2} tetramer positive CD8⁺ T cells after 2–4 rounds of stimulation with T helper cells and dendritic cells. HA-1^{A2} tetramer staining cells were subsequently sorted on a FACS Vantage (Becton Dickinson, San Jose, CA) and cloned by limiting dilution. Cloned T cells were expanded in the presence of 5x10⁴ irradiated allogeneic PBMC and 5x10⁵ HLA-A2⁺/HA-1⁺ EBV transformed B cells (EBV-LCL) and 30 U/ml IL-2.

Direct isolation and culture of HA-1 specific cytotoxic T cells from cord blood

The protocol for detection of circulating minor H antigen specific cytotoxic T cells in PBMC from healthy multiparous female blood donors was applied¹². In short, CB-MNC were depleted for various cell subsets using CD4, CD14, CD19, CD16 and glycophorin-A MACS beads (Milteny). The depleted fraction was subsequently stained with CD8-APC, CD45RA-FITC (BD Biosciences) and PE-conjugated HA-1^{A2} tetrameric complexes. Further enrichment of HA-1^{A2} tetramer positive CD8⁺ cells was performed on a FACS Vantage cell sorter using the "enrich mode", whereby cells are sorted at 20.000 events/s. The enriched fraction was reanalyzed and resorted immediately at 10.000 events/s using the more stringent "normal-R" mode. Double FACS-sorted cells were expanded in the presence of irradiated autologous HA-1⁻ CB-MNC cells, 1% phytohemagglutinin and 30 U/ml IL-2. Fresh IL-2 containing medium was added every 3-4 days.

Cell-mediated lympholysis assay

Standard 4-hour ⁵¹Cr-release assays were performed as previously described¹⁵.

RESULTS

Ex vivo generation of HA-1-specific cytotoxic T cells lines from HLA-A2⁺/HA-1⁻ cord blood

Six HLA-A2⁺ CB samples, four HA-1⁻ and two HA-1⁺, were used for the *ex vivo* generation of HA-1 specific cytotoxic T cell lines. CD8⁺ T cells were isolated and cultured with autologous HA-1^H transduced dendritic cells and T helper cells. The generation of HA-1 specific cytotoxic T cell lines was successful in three out of four HLA-A2⁺/HA-1⁻ CB samples, whereas no growth at all was observed in the two HLA-A2⁺/HA-1⁺ CB samples. Results of two HLA-A2⁺/HA-1⁻ CB samples (I and II) are shown in figure 1. The percentages of HA-1^{A2} tetramer and CD8 double positive T cells are shown after 14 days of culture (figure 1A and B). HA-1^{A2} tetramer staining CD8⁺ T cells were further enriched after additional rounds of stimulation (figure 1C and D). The latter populations were FACS sorted, expanded for 14 days, and functionally analyzed. Strong HA-1 specific lytic activity is demonstrated for both CB-derived T cell cultures (figure 1E and F).

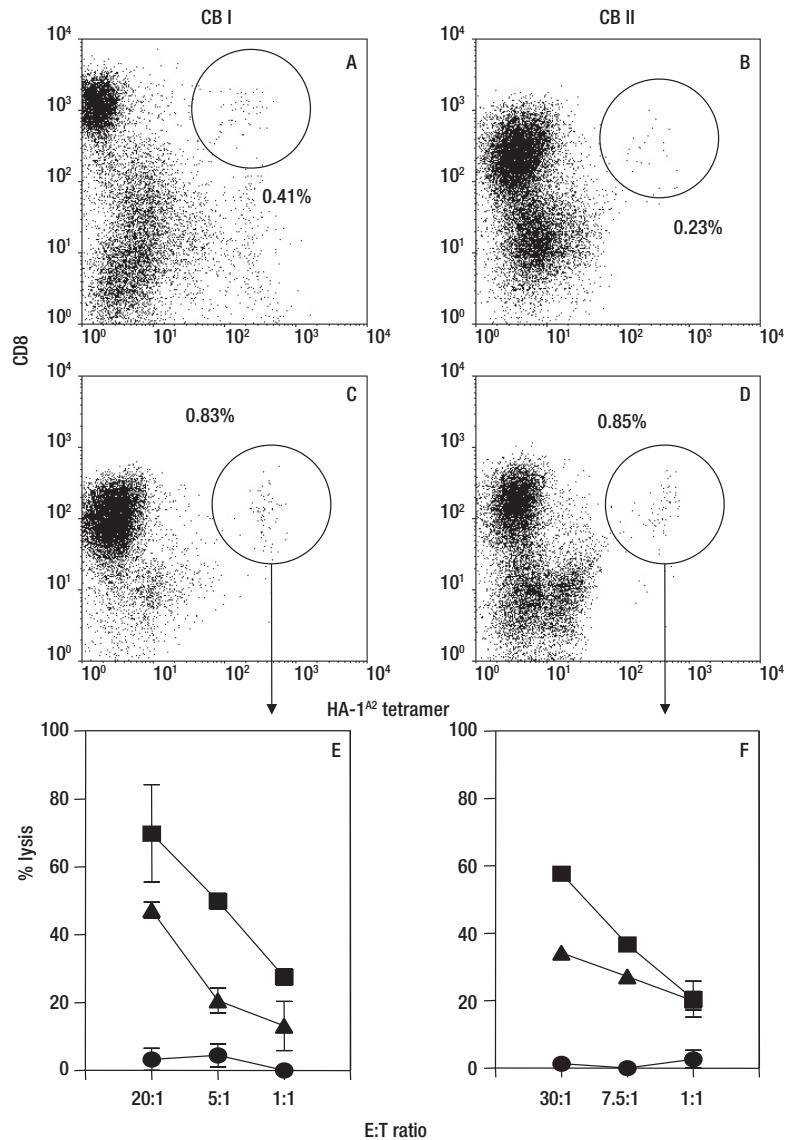


Figure 1.

Ex vivo generation of HA-1 specific cytotoxic T cells from HLA-A2⁺/HA-1⁻ cord blood

Purified CB CD8⁺ T cells were cultured in the presence of autologous HA-1^H expressing dendritic cells and T helper cells. Results of two different CB cultures are shown (I and II). Samples were stained with HA-1^{A2} tetramers (x-axis) and CD8 antibodies (y-axis). The percentages of HA-1^{A2} tetramer positive CD8⁺ T cells are shown after 14 (A, B), 28 (C) or 21 days of culture (D). HA-1^{A2} tetramer positive CD8⁺ T cells were FACS sorted (indicated by arrow), expanded and tested for cytotoxic activity (E, F). Target cells: HLA-A2⁺/HA-1⁻ EBV-LCL (dots), HLA-A2⁺/HA-1⁻ EBV-LCL pulsed with HA-1^H peptide (squares) and HLA-A2⁺/HA-1⁺ EBV-LCL (triangles). Data are presented as mean percentage of lysis ± SD.

Origin of cord blood-derived HA-1 specific cytotoxic T cells

Since cord blood may contain maternal cells, we determined whether the HA-1 specific T cells were child or mother derived. DNA typing of the HA-1 alleles showed unequivocally that *ex vivo* generated HA-1 specific T cells are from child origin (data not shown).

Hematopoietic-restricted cytolytic activity of HA-1 specific cord blood-derived T cells

T cell clones were generated from CB I (n=5), CB II (n=29) and CB III (n=8) and analyzed for HA-1 hematopoietic-restricted specificity and leukemic cell lysis. Results of three representative T cell clones (clones 1, 2, 3) are shown in figure 2. Clone 1 lysed HLA-A2⁺/HA-1⁺ PHA blasts, but not fibroblasts, while fibroblasts pulsed with HA-1^H peptide were lysed (figure 2A). Clones 1, 2 and 3 were analyzed against a panel of HA-1⁺ and HA-1⁻ leukemic cells. Each clone recognized the three HLA-A2⁺/HA-1⁺ leukemic targets, whereas HA-1⁻ leukemic cells and autologous HA-1⁻ PHA blasts, tested in parallel, were not recognized (figure 2B-D). Thus, HA-1 specific cytotoxic T cells can be generated *ex vivo* from HLA-A2/HA-1⁻ CB samples. These T cells display specific and hematopoietic-restricted recognition.

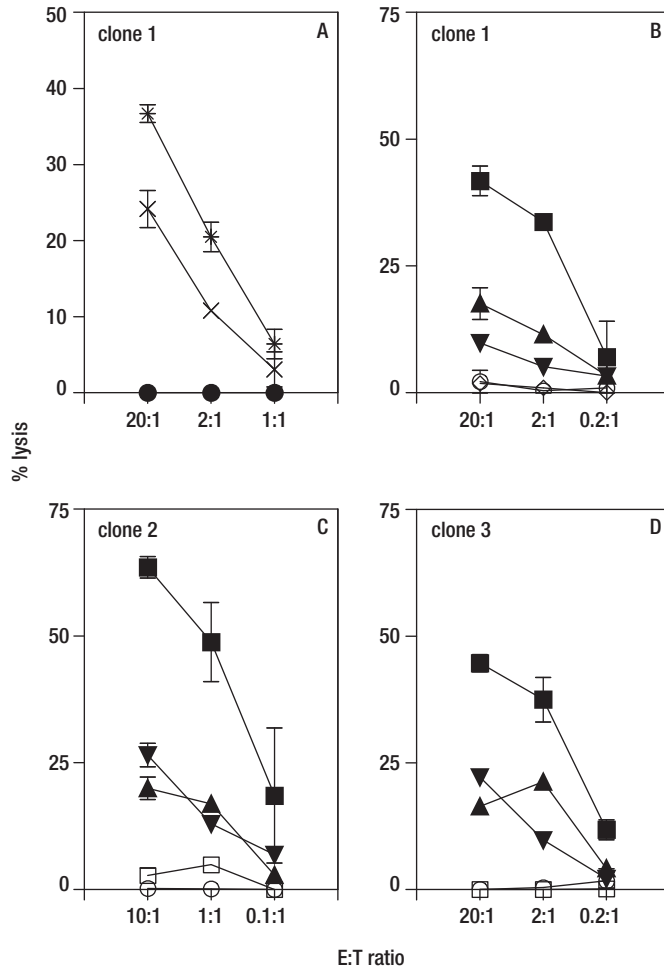
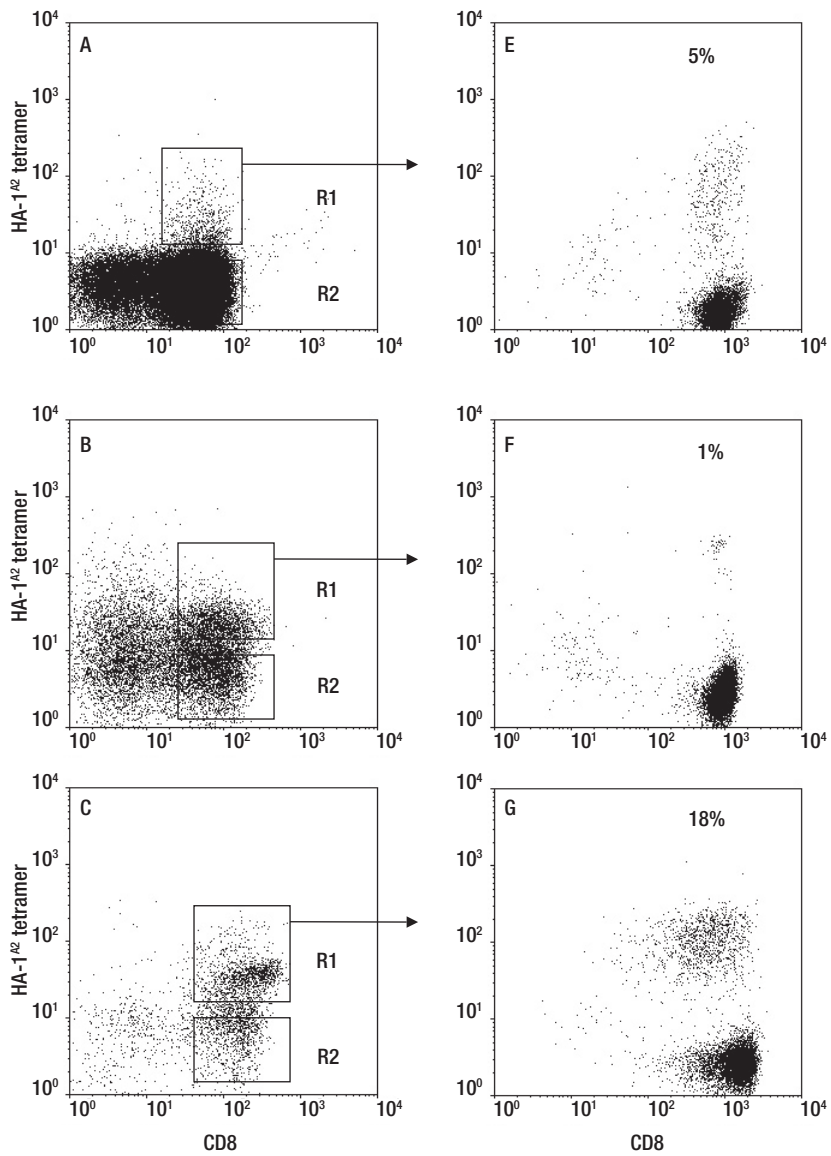


Figure 2.

Hematopoietic-restricted lysis of CB-derived cytotoxic T cell clones

(A) The cytotoxic activity of one representative HA-1 specific T cell clone (1) is shown against fibroblasts (closed circles); fibroblasts pulsed with HA-1^H peptide (asterisks) and PHA blasts (crosses). The fibroblasts and PHA blasts are derived from the same HLA-A2⁺/HA-1⁺ donor. (B-D) The cytotoxic activity of three HA-1 specific cytotoxic T cell clones (clones 1, 2 and 3) is shown against three different HLA-A2⁺/HA-1⁺ leukemic cells (closed squares, triangles and reverse triangles; acute lymphocytic lymphoma cells); HLA-A2⁺/HA-1⁻ leukemic cells (open circles) and autologous HLA-A2⁺/HA-1⁻ CB PHA blasts (open diamonds or squares).



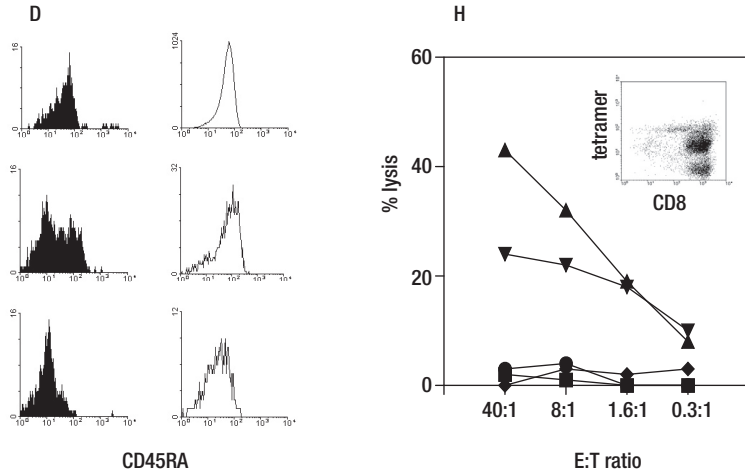


Figure 3.

Direct isolation of HA-1 specific cytotoxic T cells from HLA-A2⁺/HA-1⁻ cord blood

Results from three (A, B and C) HLA-A2⁺/HA-1⁻ CB samples obtained from HLA-A2⁺/HA-1⁺ mothers are shown. (A-C) Analysis of cells collected after the first enrichment sort for HA-1^{A2} tetramer and CD8⁺ cells. (D) CD45RA expression on HA-1^{A2} tetramer positive CD8⁺ cells (gate R1, filled histograms) and tetramer negative CD8⁺ cells (gate R2, open histograms) from CB samples A, B and C respectively. (E-G) CD8 and HA-1^{A2} tetramer staining of polyclonal cultures expanded after the enrichment sort followed by R1 sort of tetramer positive CD8⁺ cells from CB samples A, B and C respectively. (H) Cytotoxic activity of culture G, after a second expansion phase, against various target cells: HLA-A2⁺/HA-1⁻ EBV-LCL (squares); HLA-A2⁺/HA-1⁻ EBV-LCL pulsed with HA-1^H peptide (triangles); HLA-A2⁺/HA-1⁺ leukemic cells (reverse triangles); autologous HLA-A2⁺/HA-1⁻ CB PHA blasts (diamonds) and K562 cells (circles). Insert in H shows the corresponding HA-1^{A2} tetramer staining.

Isolation of circulating HA-1-specific T cells from HLA-A2⁺/HA-1⁻ cord blood

The presence of circulating HA-1 specific T cells was analyzed in eleven HLA-A2⁺/HA-1⁻ CB samples derived from seven HA-1⁺ mothers and from four HA-1⁻ mothers. All eleven CB samples were stained with HA-1^{A2} tetramers and CD8 antibodies, FACS sorted and non-specifically expanded, omitting any *in vitro* HA-1 specific stimulation. Subsequently, HA-1^{A2} tetramer analysis was performed after 21 days of expansion. Cells isolated from all four HA-1⁻ CB samples derived from HA-1⁻ mothers failed to grow *in vitro*, despite a few detectable tetramer-staining CD8⁺ cells. The latter observed events are most likely due to background staining. Cord blood samples contain variable percentages of CD3⁻CD8⁺ NK cells and erythroblasts that display non-specific staining in our hands. The level of background tetramer staining depends on the degree of depletion of these cells by magnetic bead separation. Yet, a substantial number of cells isolated from three out of seven HA-1⁻ CB samples derived from HA-1⁺ mothers stained with the HA-1^{A2} tetrameric complexes (figure 3A-C, gate R1) and expanded to sufficient numbers for tetramer analysis. A variable percentage (1–18%) of HA-1^{A2} tetramer staining CD8⁺ cells was observed at day 21 of non-specific expansion (figure 3E-G). Culture G was expanded non-specifically for another 14 days, which resulted in a further enrichment of tetramer positive CD8⁺ cells (40%, insert figure 3H). Functional analysis of the latter culture showed lysis of HLA-A2⁺/HA-1⁺ leukemic cells and HLA-A2⁺/HA-1⁻ EBV-LCL pulsed with HA-1^H peptide, while autologous HLA-A2⁺/HA-1⁻ PHA blasts, HLA-A2⁺/HA-1⁻ EBV-LCL and K562 cells were not lysed (figure 3H).

CD45RA expression on CB CD8⁺ cells was determined directly after the first enrichment sort prior to *in vitro* culture (figure 3D). The majority of tetramer negative CD8⁺ cells (figure 3A-C, gate R2) expressed CD45RA (open histograms). In contrast, tetramer positive CD8⁺ cells (figure 3A-C, gate R1) from two of the three CB samples clearly expressed lower levels of CD45RA (filled histograms), suggesting a primed phenotype at the time of isolation. Thus, antigen-experienced circulating T cells specific for maternal minor H antigen HA-1 can be detected in cord blood.

DISCUSSION

Our study demonstrates the presence of circulating HA-1 specific T cells in HLA-A2⁺/HA-1⁻ CB samples derived from children delivered by HLA-A2 matched but HA-1 mismatched mothers. We also show that HA-1 specific T cells can be generated *ex vivo* from HLA-A2⁺/HA-1⁻ CB samples. CB-derived HA-1 specific T cells show the expected cytotoxic activity that includes lysis of HA-1⁺ leukemic cells.

The majority of unrelated CBT is performed with one or two HLA mismatched grafts as reviewed²⁸. An inverse correlation between the number or type of HLA disparities and relapse risk was recently found²⁹, suggesting that alloreactivity to mismatched HLA antigens contributes to GvL responses. The fact that minor H antigen specific cytotoxic T cells are generated across HLA haplo barriers and observed in fetal blood underlines the immunogenicity of the hematopoietic-specific minor H antigen HA-1. We speculate that pre-existing HA-1 specific T cells may contribute to GvL reactivity upon CBT in HLA-A2⁺/HA-1⁺ recipients. Alternatively, HA-1 specific cytotoxic T cells can be generated *ex vivo* and stored for adoptive transfer in case of leukemic relapse.

The alloreactive potential of cord blood is shaped during pregnancy by fetal-maternal interactions. Both fetal and maternal HLA alloreactive T cells are however subjected to immune regulatory mechanisms to prevent fetal reactivity towards maternal tissues or fetal rejection⁵⁰⁻⁵². Despite the apparent immunological tolerance, normal precursor frequencies of cytotoxic and helper T cells specific for non-inherited maternal HLA antigens (NIMA) can be detected in cord blood^{21,53}. Similarly, non-inherited maternal minor H antigens can prime fetal cytotoxic T cells, as shown in this study. Whether these T cells have any implications for the immunology of the maternal-fetal interface is subject of further studies.

The tetramer positive CD8⁺ T cells directly sorted from two different CB samples clearly expressed lower levels of CD45RA than tetramer negative CD8⁺ T cells. Low CD45RA expression is indicative of recent antigen exposure, suggesting that HA-1 specific T cell priming has occurred *in utero*. Similar fetal CD8 T cell responses have been observed in case of maternal infections with *T. cruzi* or cytomegalovirus^{16,54}, as well as allergen- or Epstein-Barr Virus-specific CD4⁺ T helper cells⁵⁵⁻⁵⁷. In line with these observations, our results demonstrate that also T cell priming for minor H antigens occurs *in utero*. This is a remarkable finding since allelic variants of minor H antigens can be considered as “modified self” in contrast to foreign viral antigens. The broadness of the autosomal encoded minor H antigen repertoire in CB samples needs to be investigated.

Maternal microchimerism is frequently found in CB samples³⁸ and in newborn tissue³⁹. Nucleated maternal cells have been found in the fetal circulation as early as the second trimester of pregnancy⁴⁰. Whether the presence of HA-1 cytotoxic T cells is associated with the presence of maternal HA-1 microchimerism in the CB samples is as yet unknown. If so, we will analyze whether HA-1 is expressed by professional antigen-presenting cells, as we observed in another study⁴¹. If maternal cells persist, they could provide a continuous antigen source of HA-1 that may maintain HA-1 specific cytotoxic T cells into adult life. This would explain the low but significant precursor frequencies of HA-1 specific cytotoxic T cells that we observe in some healthy blood or stem cell donors (personal communication). Pre-existing cytotoxic T cell precursor frequencies may facilitate the *ex vivo* generation of HA-1 specific T cells for adoptive immunotherapy.

Recipients of HLA-identical SCT have a poorer transplant outcome if the donor is female rather than male^{42,45}. The explanation of this clinical observation is that pregnancy can lead to alloimmune responses. Over decades, several types of alloimmune responses, varying from immunization against erythrocyte- and HLA-specific antibodies⁴⁴ to alloimmune responses against fetal paternal minor H antigens have been reported⁴⁵. With regard to the latter, both autosomal and Y chromosome encoded minor H antigen responses have been observed^{12,24}. Evidently, priming of fetal cells restricts itself to the autosomal minor H repertoire, since maternal cells lack the Y-chromosome encoded H-Y antigens. The absence of fetal anti H-Y priming, disadvantageous for female to male SCT, makes cord blood an attractive stem cell source for male patients.

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CHAPTER 6

Adult and cord blood T cells can acquire HA-1 specificity through HA-1 T cell receptor gene transfer

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ABSTRACT

Background and Objectives: Minor Histocompatibility antigen (mHag) specific graft-versus-leukemia reactivities are observed following unselected donor lymphocyte infusion for the treatment of relapse after HLA matched mHag mismatched stem cell transplantation (SCT). Adoptive transfer of donor-derived *ex vivo* generated HA-1 specific oligoclonal T cells or HA-1 peptide patient vaccination are currently explored as curative tools for stem cell (SC) based immunotherapy of hematological malignancies. Another treatment modality to eradicate residual leukemic cells after SCT is the transfer of the HA-1 hematopoietic-specific T cell receptor (TCR) into cells of the SC donor. This strategy would be particularly useful in case of relapse after cord blood transplantation (CBT) and is explored in the underlying study.

Design and Methods: HLA-A2⁻ adult and cord blood PBMC were transduced with the genes encoding the HA-1 α - and β TCR chains derived from established HA-1 specific CTL clones.

Results: The HA-1 TCR $\alpha\beta$ transduced T cells showed consistent marker gene expression, but low staining with HLA-A2/HA-1 tetrameric complexes. The HA-1 TCR $\alpha\beta$ transduced T cells showed hematopoietic-restricted cytolytic activity against HLA-A2⁺/HA-1⁺ target cells, including leukemic cells.

Interpretation and Conclusions: Low level of HA-1 specific tetramer staining of HA-1 TCR $\alpha\beta$ transduced T cells may be caused by hybrid TCR formation of the transferred TCR α and β chains with endogenous TCR α and β chains. This may cause unwanted alloreactivity and requires attention. The HA-1 TCR $\alpha\beta$ transduced T cells show that the HA-1 TCR can be functionally transferred into donor PBMC, which can be exploited in immunotherapeutic settings of SCT and CBT for hematological malignancies.

INTRODUCTION

The minor Histocompatibility antigen (mHag) HA-1 is a polymorphic antigen that is presented in the context of HLA-A2¹. The tissue distribution of HA-1 is restricted to hematopoietic cells and carcinomas^{2,3}. It therefore can function as a tumor target antigen for stem cell (SC) based immunotherapy of malignancies. In an HLA matched HA-1 mismatched SCT setting for hematological malignancies, T cells from the HA-1⁻ SC donor can recognize HA-1 expressed by the patient's leukemic cells⁴. *In vivo* and *in vitro* generation of HLA-A2 restricted HA-1 specific CTLs have previously been reported^{5,6}. T cells expressing the HA-1 specific TCR can be monitored by staining PBMC with HLA-A2/HA-1 tetramers⁷ and by TCRBV spectratyping⁸. Both *in vitro* and *in vivo* generated HA-1 specific T cell clones analyzed so far exclusively use the TCR BV7S9 variable domain in combination with different TCR BD, BJ, TCR AV and AJ regions^{8,9}. The CDR1 region of the TCR BV does however not seem to play a major role in the interaction with the HLA-A2/HA-1 ligand¹⁰.

Donor lymphocyte infusion (DLI) with HA-1 specific CTLs generated from adult and cord blood donor cells provides a feasible treatment for relapsed HLA-A2⁺/HA-1⁺ leukemia patients^{6,11}. *Ex vivo* CTL induction and expansion for adoptive immunotherapy is however time-consuming and not successful in all SC donors. Gene transfer of the HLA-A2 restricted HA-1 specific TCR into donor T cells may provide an alternative treatment strategy. Several studies have described the transmission of various antigenic specificities by TCR transfer¹²⁻¹⁵. We earlier reported on the successful gene transduction of the TCR specific for the mHag HA-2 into peripheral T lymphocytes^{16,17}. Since the hematopoietic specific HA-1 antigen is additionally expressed on a series of epithelial carcinomas^{5,18}, we studied the feasibility of HA-1 TCR gene transfer into peripheral blood cells derived from adult or CB donors. We chose to transfer the HA-1 TCR specificity into HLA-A2⁻ donor T cells which enables usage of the HA-1 specific immunotherapy in HLA mismatched SCT settings. The HA-1 TCR α and β genes used for transduction were derived from two established HA-1 specific HLA-A2 restricted T cell clones 3HA15 and 5W38, previously isolated from different patients after HLA-identical SCT⁵. Both clones expressed the same TCR BV7S9, but different TCR AV chains. The individual genes encoding the α - and β TCR chains of both CTL clones were cloned into retroviral vectors and specificity and functional studies on TCR transduced adult and cord blood CD8⁺ T cells were performed.

MATERIALS AND METHODS

Construction of retroviral vectors and generation of retroviral supernatant

Total RNA from the mHag HA-1 specific HLA-A2 restricted T cell clones 3HA15 and 5W38 was extracted using Trizol (Gibco, Carlsbad, CA). The mRNA was reverse transcribed into single-strand cDNA by reverse transcriptase using oligo dT primers (Pharmacia, Uppsala, Sweden). Using primers that cover the complete repertoire of known TCR chains, TCR α and β usage of the two clones was determined. Both T cell clones expressed the TCR BV7S9, as previously described^{8,9}. T cell clone 5W38 expressed the TCR AV10S1; clone 3HA15 expressed two in frame TCR α chains, TCR AV32 and TCR AV3S1 (data not shown). Pilot experiments revealed that TCR AV3S1, in combination with TCR BV7S9, forms the functional HA-1 specific HLA-A2 restricted TCR of CTL clone 3HA15. Two bicistronic retroviral vectors based on the pLZRS backbone¹⁹ were used, containing an internal ribosome entry site (IRES) and the marker gene enhanced green fluorescent protein (pLZRS-eGFP)²⁰ or a truncated form of the nerve growth factor receptor (pLZRS-dNGF-R)²¹. The individual genes encoding the α - and β TCR chains were PCR amplified using primers containing relevant restriction sites and cloned into the pLZRS-vectors. The 5' forward primer sequences used were ATTGAATTCAGAAGAATGGAACTCTC contain-

ing the EcoRI restriction-site for the TCR AV3S1 chain, CGCGGATCCACCAT-GTCTGAAATTCTCCG containing the BamHI restriction-site for the TCR AV10S1 chain, TATGGATCCCTGCCATGGGCACCAG containing the BamHI restriction-site for the 3HA15 TCR BV7S9 chain and TAGAGAATTCACCAT-GGGCACCAGTCTCCTATGC containing the EcoRI restriction-site for the 5W38 TCR BV7S9 chain. The 3' reverse primer sequences used were TATCTCGAGA-TAAATTCGGGTAGGATC containing the XhoI restriction-site for both TCR AV chains, GGTGTGCGACTGGGATGGTTTTGGAG containing the Sall restriction-site for the 3HA15 TCR BV7S9 chain and CCGGAATTCAGAAATCCTTTCTCTGACC containing the EcoRI restriction-site for the 5W38 TCR BV7S9 chain (Eurogentec, Seraing, Belgium). The TCR AV3S1 chain of 3HA15 was cloned into the pLZRS-dNGF-R vector, while the 3HA15 TCR BV7S9 chain was cloned into the pLZRS-eGFP vector. The TCR AV10S1 chain of clone 5W38 was cloned into the pLZRS-eGFP vector, whereas its TCR BV7S9 chain was cloned into the pLZRS-dNGF-R vector. Retroviral vectors encoding eGFP or dNGF-R without additional insert were used as control (mock) vectors in the experiments. Control cycle sequencing was performed after which the constructs were transfected (0.66 µg/ml) into the amphotropic phoenix packaging cell line (kindly provided by G. Nolan, Stanford University School of Medicine, Stanford, CA) using the calcium phosphate transfection kit (Life Technologies, Gaithersburg, MD). The phoenix cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS. Two days following transfection, 2 µg/ml puromycin (Clonotech Laboratories, Palo Alto, CA) was added and 10 to 14 days later 20 x 10⁶ cells were plated per 150 cm² culture flask (Beckton Dickinson, San Jose, CA) in fresh medium without puromycin. The following day the medium was refreshed and 24 hours thereafter retroviral supernatant was harvested, centrifuged, and frozen in aliquots at -70°C.

Retroviral transduction of TCR $\alpha\beta$ deficient Jurkat cells

Prior to transduction of donor T cells, Jurkat clones deficient for TCR α ($\alpha^{-/-}$) or TCR β ($\beta^{-/-}$) or for both chains ($\alpha\beta^{-/-}$), clone 3, clone 4, and clone 76 respectively¹⁶, were transduced using the various viral supernatants according to the procedure described below. At day three, correct expression of transduced TCR α and/or β chains was confirmed by measuring the TCR $\alpha\beta$ expression by FACS analysis, using PECy5 conjugated anti-TCR $\alpha\beta$ moab (Pharmingen, San Diego, CA).

Isolation and retroviral transduction of T cells derived from adult- or cord blood

PBMC isolated from HLA-A2⁻ adult or cord blood donors were stimulated with 800 ng/ml phytohemagglutinin (PHA) and 25 U/ml IL-2 (Cetus, Emeryville, CA) in IMDM containing 10% human serum (HS) at a concentration of 1 x 10⁶/ml. After 2 days of culture, T cells were transduced with retroviral supernatant using recombinant CH-296 human fibronectin fragments²² (Retronectin, Takara,

Otsu, Japan). Briefly, 0.5×10^6 T cells per well were cultured overnight at 37°C together with 0.25 ml TCR α - and 0.25 ml TCR β retroviral supernatant and 0.5 ml of fresh IMDM containing 10% fetal calf serum (FCS) and 25 U/ml IL-2 in non tissue culture treated CH-296-coated 24-well plates¹⁶. Next, the cells were washed and transferred to tissue culture treated 24-wells plates at a concentration of 0.5×10^6 cells per well in IMDM containing 10% HS and 25 U/ml IL-2.

Flow cytometric analysis and fluorescence-activated cell sorting

Transduction efficiencies were measured 3-5 days after transduction by the expression of the markers eGFP and dNGF-R. T cells positive for both markers and negative for CD4 were sorted at 1 or 25 cells/well by fluorescence-activated cell sorting using a FACSVantage (Becton Dickinson). PE-conjugated and PECy5-conjugated antibodies (Pharmingen) were used to detect dNGF-R expression and CD4 expression respectively. FACS sorted cells were restimulated with 30 Gy irradiated randomly selected PBMC, 50 Gy irradiated HLA-A2⁺/HA-1⁺ EBV-LCLs, 25 U/ml IL-2 and 800 ng/ml PHA.

Tetramer staining and cytotoxicity assay

Expression of the TCR specific for HLA-A2/HA-1 complexes was measured by staining the cells with PE-conjugated HLA-A2/HA-1 tetrameric complexes (HA-1^{A2}), either or not in combination with APC-conjugated CD8 antibodies (BD Biosciences, Amsterdam, The Netherlands). Tetramers were generated and validated as previously described⁷.

CTL activity was measured in a chromium release assay. HLA-A2⁺ EBV-LCLs either positive or negative for HA-1 were used as target cells. Peripheral blood or bone marrow containing more than 95% morphologically recognizable malignant cells derived from HLA-A2⁺ chronic myeloid leukemia (CML) patients were used as leukemic target cells. HLA-A2⁺ fibroblasts derived from a HA-1⁺ donor were used to test hematopoietic-restricted specificity of HA-1 TCR transduced T cells. Fibroblasts were treated for 24 hours with 250 U/ml IFN γ and TNF α before incubation with effector cells. Target cells were pulsed with 1 μ M HA-1^H peptide (VLHDDLLEA) where indicated. Chromium labeled target cells were added to various numbers of effector cells and were cultured for 4 or 18 hours at 37°C. Supernatant was harvested and measured in a luminescence counter (Topcount-NXT; Packard, Meriden, CT). The mean percentage specific lysis of triplicate wells was calculated using the following formula:

(Experimental release-Spontaneous release) / (Maximal release- Spontaneous release) x 100%.

RESULTS

TCR $\alpha\beta$ cell surface expression following retroviral transduction into Jurkat cells

Retroviral vectors encoding the different TCR α or β chains derived from HA-1 specific CTL clones 3HA15 or 5W38 were transduced into various Jurkat cells. The cell surface expression of the transduced TCR $\alpha\beta$ gene products was analyzed. The TCR α deficient Jurkat cells (see Materials and Methods) transduced with the HA-1 TCR α chain from either HA-1 specific CTL clone 3HA15 (figure 1A) or 5W38 (data not shown) expressed TCR $\alpha\beta$ complexes at the cell surface. Similarly, the TCR β deficient Jurkat cells (see Materials and Methods) transduced with the HA-1 TCR β chain from either HA-1 CTL clone 3HA15 or 5W38 expressed TCR $\alpha\beta$ complexes at the cell surface. These data demonstrate that the retrovirally transduced HA-1 TCR α chains as well as the HA-1 TCR β chains can pair with the endogenous Jurkat TCR chains respectively. However, some HA-1 TCR β chain transduced Jurkat cells did not express the HA-1 TCRs or expressed TCRs at a low level. Transduction of both the HA-1 TCR α and β chain of the two different HA-1 CTL clones into TCR $\alpha\beta$ deficient Jurkat cells resulted into intact HA-1 TCR $\alpha\beta$ cell surface expression (data not shown). Thus, the individual HA-1 TCR chains pair with each other and are able to form stable TCR complexes at the cell surface.

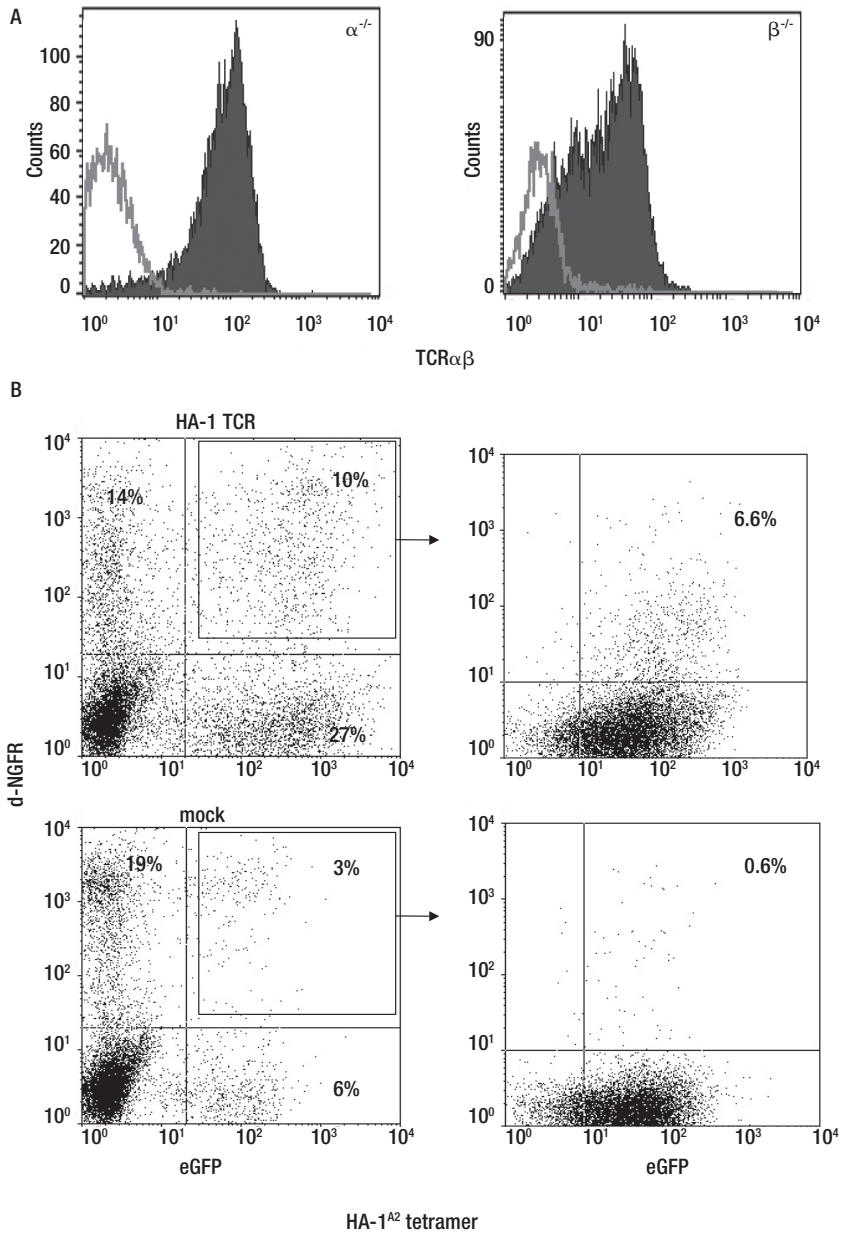


Figure 1.

TCR cell surface expression upon transfer of HA-1 TCR α and TCR β chain encoding genes into Jurkat cells and adult T cells

TCR α or β deficient Jurkat cells (A) or peripheral blood-derived adult T cells (B) were transduced with HA-1 TCR α and β chains from CTL clone 3HA15. Intact TCR $\alpha\beta$ expression is shown as filled histogram plots. Open histograms represent marker gene positive mock-transduced Jurkat cells. HA-1 TCR $\alpha\beta$ or mock-transduced adult T cells double positive for eGFP and dNGF-R expression were FACS sorted (indicated by arrow), expanded and stained with HA-1^{A2} tetrameric complexes.

Transduction of the HA-1 TCR $\alpha\beta$ chains into adult peripheral blood-derived T cells

The TCR α and β chain derived from the HA-1 specific CTL clones 3HA15 and 5W38 were transduced into PBMC isolated from various HLA-A2⁻ adult donors. The transduction efficiency of the HA-1 TCR α chains varied between 14–23%, whereas the transduction efficiency of the HA-1 TCR β chains varied between 27–35%. T cells isolated from adult PBMCs that were negative for CD4 and expressed both marker genes (6–10%) were FACS sorted and expanded (see Materials and Methods). Despite stable expression of the marker genes eGFP and dNGFR (data not shown), only low numbers of HA-1 TCR $\alpha\beta$ transduced adult T cells stained specifically with HA-1^{A2} tetramers (figure 1B).

Functional analysis of HA-1 TCR $\alpha\beta$ transduced adult peripheral blood-derived T cells

The HA-1 TCR $\alpha\beta$ transduced T cells were subsequently analyzed for their functional activity and specificity. The cytolytic activities of 3HA15 TCR $\alpha\beta$ transduced T cells from one representative donor are depicted in figure 2. The 3HA15 or 5W38 TCR $\alpha\beta$ transduced T cells specifically lysed HLA-A2⁺/HA-1⁻ EBV-LCL target pulsed with HA-1 peptide and importantly, the natural ligand expressing HLA-A2⁺/HA-1⁺ EBV-LCL target cells. Compared with the original CTL clone 3HA15 analyzed in parallel, the HA-1 TCR $\alpha\beta$ transduced T cells required a prolonged incubation time to lyse HA-1 expressing target cells. After 4 hours of effector and target cell incubation, HA-1 TCR $\alpha\beta$ transduced T cells showed specific lysis of target cells pulsed with HA-1 peptide, but no lysis on the natural ligand. After 18 hours of incubation, HA-1 TCR $\alpha\beta$ transduced T cells displayed strong and specific lytic capacities on both the peptide loaded and the natural ligand target cells comparable to the original CTL clones. The mock-transduced bulk population did not lyse any of the target cells.

Next, we tested the lysis of leukemic targets by 3HA15 or 5W38 TCR $\alpha\beta$ transduced T cells (figure 3). Short term expanded HA-1 TCR $\alpha\beta$ transduced T cells lysed HA-1 expressing leukemic cells, but not HA-1⁻ leukemic cells, after 4 hours of incubation. Specific and much stronger lysis was observed after 18 hours of incubation. Similar results were obtained when HA-1 expressing EBV-LCL target cells were used (data not shown). In line with the results on bulk HA-1 TCR $\alpha\beta$ transduced T cells, short term expanded HA-1 TCR $\alpha\beta$ transduced T cells stained low but specifically with HA-1^{A2} tetramers.

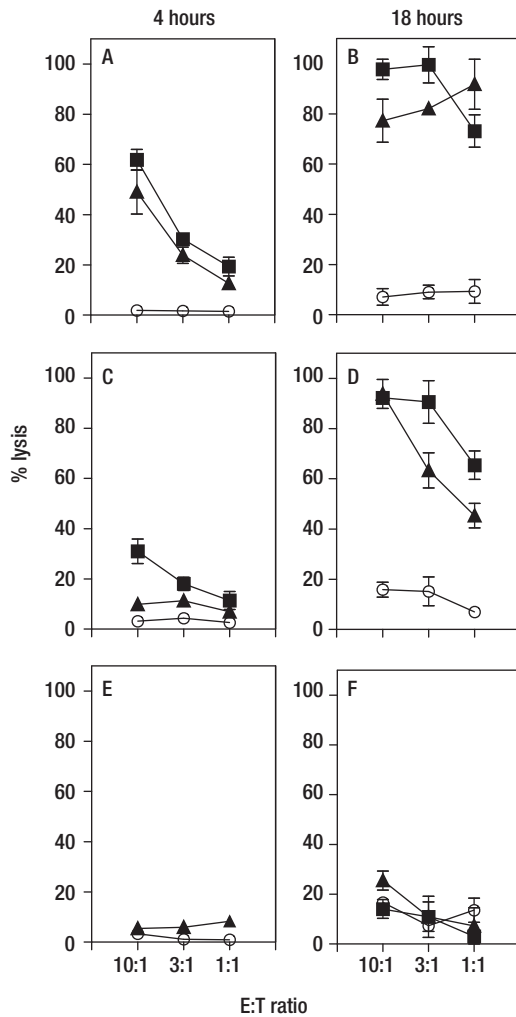


Figure 2.

Functional analysis of HA-1 TCR $\alpha\beta$ transduced adult T cells

The HA-1 specific cytotoxic activity of HA-1 TCR $\alpha\beta$ transduced T cells (C,D) and mock-transduced T cells (E,F) is shown after 4 and 18 hours of incubation. The original CTL clone 3HA15 was tested in parallel (A,B). Target cells: HA-1⁻ EBV-LCL (open dots), HA-1⁻ EBV-LCL pulsed with HA-1 peptide (filled squares) and HA-1⁺ EBV-LCL (filled triangles).

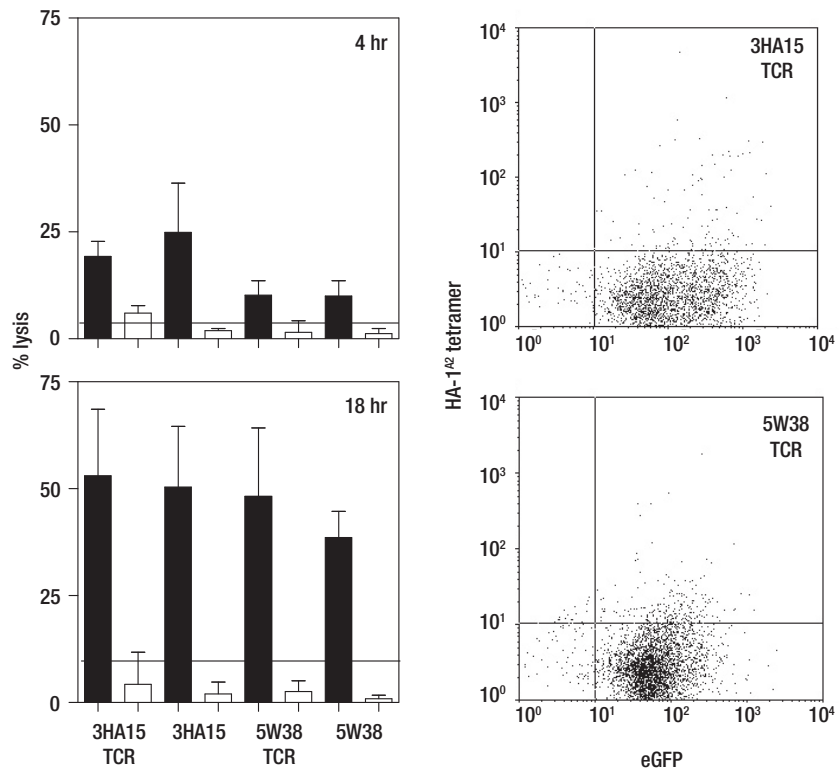


Figure 3.

Recognition patterns of HA-1 TCR $\alpha\beta$ transduced adult T cells against leukemic cells

HA-1 specific lysis of two representative HA-1 TCR $\alpha\beta$ transduced T cell clones was measured after 4 or 18 hours of effector/target cell incubation at an E:T ratio of 10:1. The original HA-1 specific CTL clones 3HA15 and 5W38 were analyzed in parallel. The horizontal lines in the left figures represent the background lysis by mock-transduced T cells tested in parallel. Target cells: HA-1⁺ chronic myeloid leukemia cells (CML, filled bars), HA-1⁻ CML cells (open bars). Corresponding HA-1^{A2} tetramer staining of the 3HA15 and 5W38 HA-1 TCR $\alpha\beta$ transduced T cell clones is shown.

Transduction of the HA-1 TCR $\alpha\beta$ chains into cord blood-derived T cells

The TCR α and β chains derived from the HA-1 specific CTL clone 3HA15 were transduced into PBMC isolated from various HLA-A2⁻ CB donors. The transduction efficiencies of both the TCR α and the TCR β chains were in the same range as observed with adult derived PBMC (15–40%). HA-1 TCR $\alpha\beta$ transduced CB derived T cells (4–20%) were FACS sorted (depleted for CD4⁺ T cells) and expanded. HA-1 TCR $\alpha\beta$ transduced CB T cells displayed low HA-1^{A2} tetramer staining comparable to the HA-1 TCR $\alpha\beta$ transduced adult T cells described above (data not shown).

Functional analysis of HA-1 TCR $\alpha\beta$ transduced cord blood-derived T cells

The HA-1 TCR $\alpha\beta$ transduced CB T cells were subsequently analyzed for their hematopoietic-specific lytic capacities (figure 4). HA-1 TCR $\alpha\beta$ transduced CB T cells lysed HLA-A2⁺/HA-1⁺ leukemic cells and EBV-LCL target cells that were either pulsed with HA-1 peptide or naturally expressed HA-1. Similar to the results obtained with the HA-1 TCR $\alpha\beta$ transduced adult T cells, specific and strong lysis of HA-1 expressing target cells required prolonged incubation of effector and target cells. The original HA-1 specific CTL clone 3HA15 and the mock-transduced T cells were analyzed in parallel. Mock-transduced T cells did not lyse any of the target cells.

Besides the recognition of the relevant EBV-LCL and leukemia cells, the HA-1 TCR $\alpha\beta$ transduced CB T cells were analyzed for their hematopoietic-restricted specificity (figure 4B). HA-1 TCR $\alpha\beta$ transduced CB T cells did not lyse fibroblasts derived from an HLA-A2⁺/HA-1⁺ donor, whereas these target cells were recognized by an allo HLA-A2 specific T cell clone tested in parallel. The same fibroblasts pulsed with HA-1 peptide were efficiently lysed by HA-1 TCR $\alpha\beta$ transduced CB T cells. Herewith, the recognition pattern of the HA-1 TCR $\alpha\beta$ transduced CB T cells is indicated to be restricted to hematopoietic system specific cells.

Collectively, these results show that HA-1 TCR $\alpha\beta$ transfer into HLA-A2⁻ adult or into HLA-A2⁻ CB T cells results in functional cytotoxic T cells that display specific reactivity against HLA-A2⁺ HA-1 expressing target cells including leukemic cells.

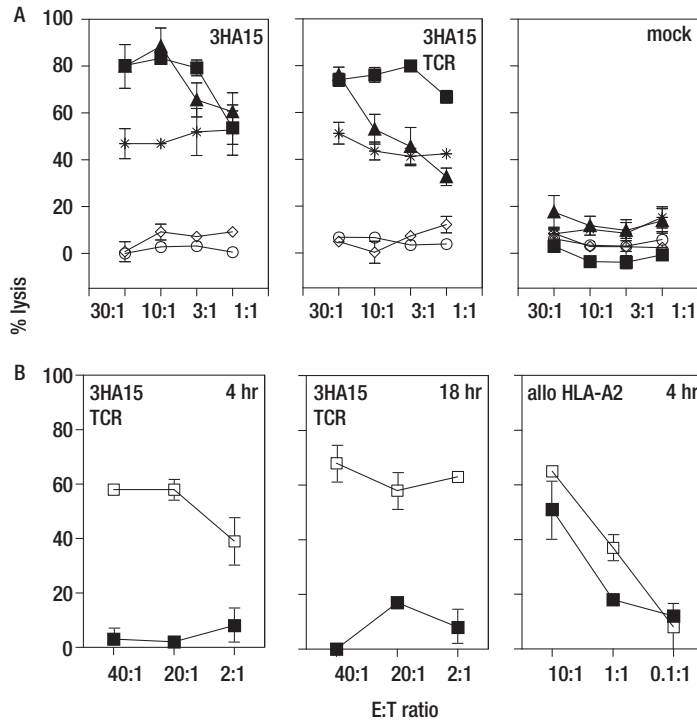


Figure 4.

Functional analysis of HA-1 TCR $\alpha\beta$ transduced cord blood T cells

(A) HA-1 specific lysis by HA-1 TCR $\alpha\beta$ transduced CB T cells and by mock-transduced CB T cells are shown after 18 hours of incubation. The original HA-1 specific CTL clone 3HA15 was tested in parallel. Target cells: HA-1⁻ EBV-LCL (open dots), HA-1⁻ EBV-LCL pulsed with HA-1 peptide (filled squares), HA-1⁺ EBV-LCL (filled triangles), HA-1⁻ CML cells (open diamonds) and HA-1⁺ CML cells (asterisks). (B) Hematoipoietic restricted lysis by HA-1 TCR $\alpha\beta$ transduced CB T cells is shown after 4 and 18 hours of effector/target cell incubation. A control allo HLA-A2 specific CTL clone was tested in parallel. Target cells: fibroblasts (filled squares) and fibroblasts pulsed with HA-1 peptide (open squares). The fibroblasts are derived from an HLA-A2⁺/HA-1⁺ donor.

DISCUSSION

We studied the feasibility of transferring the HA-1 TCR α and β chain encoding genes into HLA-A2⁻ adult and into HLA-A2⁻ CB T cells and analyzed the HA-1 TCR $\alpha\beta$ transduced T cells for their antigen-specific lytic potential. We here show that these HA-1 TCR $\alpha\beta$ transduced HLA-A2⁻ adult and CB T cells can indeed acquire HA-1 specific and lytic activity. The feasibility of the functional HA-1 TCR $\alpha\beta$ transfer into HA-1 TCR negative cells lays the basis for a potential broad spectrum of applications in SC based immunotherapy of hematological malignancies and solid tumors. It is worthwhile mentioning that besides the hematoipoietic-restricted specificity, HA-1 is also expressed on epithelial cancer

cells. Moreover, our results set the stage for broadening the use of the immunodominant and hematopoietic specific mHag HA-1 to the HLA mismatched SCT setting. It should be noted however that although the specific functional activity of the HA-1 TCR can indeed be transferred, significant improvements in transduction efficiency, HA-1 TCR avidity for its ligand and relevant expansion of HA-1 TCR $\alpha\beta$ transduced T cells need to be established before HA-1 TCR transduced T cells can be therapeutically applied.

Endogenous TCR, transduced TCR and hybrid TCR possibly compete for CD3 association and therewith for functional cell surface expression²⁵. This feature may explain the lack of correlation between the intensity of double marker expression and the cell surface expression, as measured by HLA-A2/HA-1 specific tetramers, of stable HA-1 specific TCR complexes following transduction. The presence of other TCRs on the cell surface may also hamper HA-1 TCR clustering, lipid raft formation^{24,25} and rapid activation upon antigen encounter. Moreover, the granzyme depot and thus the intrinsic cytolytic capacity of the TCR transduced T cells may be inferior compared with the non-transduced CTL clones. We also noticed that HA-1 TCR $\alpha\beta$ transduced T cells generally require more time than the original non-transduced HA-1 specific CTL clones to lyse their target cells. We encountered the same phenomenon of low tetramer staining and 'slow' but antigen-specific lysis in our earlier study using HA-2 specific TCR transfer¹⁶. A single chain construct containing both the TCR α and β chains combined with strategies that can prevent the formation of hybrid TCR or suppress endogenous TCR expression²⁵ is necessary to improve functional HA-1 TCR transfer. Improved gene expression may be obtained with more effective retroviral vector systems²⁶ or a lentiviral-based transduction procedure. Hybrid pairing of the different TCR α and β chains following retroviral transduction may also result in the formation of new TCRs of unknown specificities²⁵. It is clear that the above mentioned, as yet unsolved, processes need extensive additional analyses before *in vivo* transfer with TCR transduced T cells can be executed. Serious attention should be focused on the potential risks of Graft-versus-Host-Disease (GvHD) as well as undesired autoimmune reactions that may occur upon adoptive transfer of TCR modified T cells. Suicide gene control of the *ex vivo* HA-1 TCR $\alpha\beta$ transduced T cells may be included to potentially control undesired alloreactivity^{27,28}.

Foremost, HA-1 TCR $\alpha\beta$ transfer may be of special use in the setting of CBT. Currently, a treatment for relapsed hematological malignancies after CBT is lacking. Cord blood is obtained anonymously and the number of donor lymphocytes for the purpose of DLI is far too little. CBT is usually performed with 1-2 HLA mismatched grafts²⁹. HLA disparity is however not significantly associated with a higher risk of GvHD in this transplantation modality. HA-1 specific TCR $\alpha\beta$ transfer into cryopreserved HLA-A2⁻ CB T cells may be a strategy requiring low numbers of donor cells for immunotherapeutical purposes for HLA-A2 positive patients. A universal option would be to generate 'prefab'

HA-1 TCR $\alpha\beta$ transduced T cells derived from HLA-A2⁻ CB donors who have frequent HLA-homozygous haplotypes. HLA-A2⁺ patients who match the remaining HLA typing of the CB donor can be treated with these 'off the shelf' HA-1 TCR $\alpha\beta$ transduced T cells.

In summary, our results provide the proof of principle that transfer of the HA-1 specificity into HA-1 TCR negative cells is feasible. Current studies focus on the generation of sufficient numbers of HA-1 TCR $\alpha\beta$ transduced CB T cells with high *ex vivo* expansion potential and lytic capacity.

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CHAPTER 7
General discussion



Contents

Future of minor histocompatibility antigen HA-1 directed immunotherapy

- 7.1 HA-1 peptides potentially suitable for HA-1 directed immunotherapy
 - 7.1.1 Immunogenic potential of the HLA-A2/HA-1^R counterpart
 - 7.1.2 Expansion of the MHC class I associated HA-1 epitope pool
- 7.2 HA-1 directed immunotherapy following UCB SCT
- 7.3 HA-1 specific TCR transfer

Concluding remarks

Future of minor histocompatibility antigen HA-1 directed immunotherapy

The minor histocompatibility (mH) antigen HA-1 is an important target antigen for the induction of a graft versus leukemia (GvL) response with a low risk of graft versus host disease (GvHD). Applying HLA-A2/HA1^H specific cytotoxic T cells (CTLs) as cellular immunotherapy for relapsed leukemia is an option for HLA-A2 HA-1^H patients transplanted with HLA-A2 HA-1^{RR} donor stem cells.^{1,2} Currently this adoptive immunotherapy is however not available for patients not expressing HLA-A2 and further depends on the presence of recipient-donor disparity for HA-1. The studies in this thesis were designed to extend the patient population that can benefit from HA-1 specific immunotherapy in the future. A number of options have been studied.

First, we examined several possible causes for the absence of cell surface HLA-A2/HA-1^R expression in order to evaluate the option of HA-1^R directed immunotherapy. Secondly, we investigated whether the HA-1^{H/R} polymorphic region contains peptides that can be presented by MHC class I molecules other than HLA-A2. Furthermore, we explored the feasibility of generation of HA-1^H specific HLA-A2 restricted CTLs from umbilical cord blood (UCB). Finally, we investigated whether HA-1 specific TCR transfer may be a useful alternative strategy to generate HA-1^H specific HLA-A2 restricted adult- or UCB CTLs. Here I will discuss the results of this thesis and the possible future of HA-1 directed immunotherapy.

7.1 HA-1 peptides potentially suitable for HA-1 directed immunotherapy

7.1.1 Immunogenic potential of the HLA-A2/HA-1^R counterpart

Thus far, HA-1^H specific adoptive immunotherapy has been suggested as a feasible option for HLA-A2 HA-1^H positive patients who relapsed following a stem cell transplantation (SCT) from an HLA-A2 HA-1^H negative donor¹⁻³. In theory, the reciprocal immunogenicity of the HA-1^R allele would also provide an immunotherapy option for HLA-A2 HA-1^R positive patients who relapsed following a SCT from an HLA-A2 HA-1^R negative donor. Currently, HLA-A2 HA-1^R homozygous patients are no candidates for HA-1 specific immunotherapy. Of the HLA-A2 positive population 30% is HA-1^R homozygous⁴. The additional option of HA-1^R specific adoptive immunotherapy would thus mean a considerable expansion of the patient population that can benefit from HA-1 specific immunotherapy, providing that an HLA matched HA-1^R negative donor is available. CTLs directed against HA-1^R in the context of HLA-A2 have been generated⁵. However, these CTLs were only responsive to HLA-A2 positive target cells pulsed with the HA-1^R peptide. Target cells positive for HA-1^R were not recog-

nized. This may be explained by the fact that the HA-1^R peptide is not naturally expressed on the cell-surface since peptide elution studies did not detect HA-1^R cell-surface expression in the context of HLA-A2⁶. It has been demonstrated that the HA-1^R peptide binds with ten-fold lower HLA-A2 binding affinity as compared with the HA-1^H peptide⁶. However this ten-fold difference is unlikely to solely account for the absence of HA-1^R on the cell-surface. Furthermore, the low binding affinity of the HA-1^R peptide does not per se result in low HA-1^R cell-surface expression since MHC-peptide binding affinities are not directly related to the cell-surface density of the peptide⁷. In addition, low affinity peptides can still represent important T cell epitopes^{8,9}. Therefore, as described in chapter 2, we studied mechanisms involved in HLA-A2/HA-1^R expression in order to thoroughly evaluate the option of HA-1^R directed immunotherapy. We showed that the intracellular processing mechanisms that we have tested did not interfere with the generation or expression of the HA-1^R peptide, and were thus not responsible for the absence of this peptide on the cell-surface. We found however, that the HLA-A2/HA-1^R complex was extremely instable, which can explain the complete absence of HLA-A2/HA-1^R on the cell-surface. Stability of MHC-peptide complexes has been demonstrated as an important parameter for their immunogenicity¹⁰. Therefore, even if this peptide was shortly displayed on the cell surface (which could not be detected by biochemical methods), immunotherapy directed against HLA-A2/HA-1^R is not a feasible option.

However, since the HA-1^R antigen is processed correctly it could possibly be naturally expressed on the cell surface in the context of other MHC class I molecules. In that case HA-1^R could still be capable of triggering HA-1^R specific CTLs in vivo, necessary for HA-1^R directed immunotherapy.

7.1.2 Expansion of the MHC class I associated HA-1 epitope pool

Approximately 45% of the Caucasian population expresses HLA-A2¹¹ and is thus a potential candidate for HA-1 directed immunotherapy. Of the HLA-A2 positive population 16% is HA-1^H homozygous and 54% is HA-1 heterozygous, whereas 30% is HA-1^R homozygous⁴. This suggests that approximately 5% of the Caucasian patient population could be candidates for HA-1^H specific immunotherapy, since 32% of the Caucasian population is both HLA-A2 and HA-1^H positive and 14% of the population can serve as an HLA-A2 matched HA-1^{RR} stem cell donor. The percentage potential candidates for HA-1 specific therapy could be increased if the option of HA-1 specific immunotherapy also becomes available for patients not expressing HLA-A2. To this end HA-1 epitopes, either from the HA-1^H or from the HA-1^R allele, presented by other MHC class I molecules need to be identified. The option of HA-1 specific immunotherapy may be extended to a large population if HA-1 epitopes are identified that are presented by highly prevalent MHC class I molecules¹¹. Several cellular processes are involved in the formation of an immunogenic epitope. First, the antigenic

peptide needs to be correctly processed. Secondly, this peptide should be translocated into the endoplasmatic reticulum (ER) where it binds an MHC molecule. Subsequently, the MHC-peptide complex stability is an important factor for epitope immunogenicity.

In order to predict which HA-1 peptides containing the polymorphism are naturally cleaved and presented, several peptide processing prediction programs¹² may be used. Some investigators solely rely on the predictions by these programs¹⁵, however, in chapters 2 and 4 we show that this approach is not sufficient. The HA-1^R epitope VLRDDLLEA and several other putative HA-1 epitopes containing the R polymorphism, were properly cleaved by proteasomes although the PAProc program predicted these epitopes to be destroyed by an intra-peptide cleavage site. This can be explained by the fact that once the relevant peptides are generated by proteasomal cleavage, other cleavage sites within the epitope may be missed¹⁴.

In order to predict which HA-1 peptides may actually be presented by MHC class I molecules, several helpful MHC-peptide binding prediction algorithms¹⁵⁻¹⁷ (<http://bimas.dcrt.nih.gov/>; <http://syfpeithi.bmi-heidelberg.com/>) are available. However, these prediction programs still have significant shortcomings. The contributions to binding of each possible amino acid in every position of the peptide are still only partly defined. Therefore, cognate T cell mH epitopes do not always confirm the predicted MHC class I binding motifs^{18,19}. Molecular modeling systems may partly solve these shortcomings^{20,21}, but actual MHC-peptide binding experiments remain indispensable to confirm the predictions (chapter 3)²². In order to be able to experimentally test the binding capacities of various MHC class I-peptide complexes, we developed easy to perform, reliable competition-based cellular peptide binding assays for 13 prevalent MHC class I alleles, as described in chapter 3. Other peptide binding assays employing either cell-bound MHC class I molecules²⁵⁻²⁸ or solubilized MHC class I molecules²⁹⁻³² are available. Nevertheless, the competition-based cellular peptide binding assay described in chapter 3 was preferred since there is no need to purify HLA class I molecules, or to transfect cells with HLA class I molecules, and no radioactive label is used. Moreover, large panels of HLA-typed human B-cell lines are available as tools for peptide binding to a vast array of HLA molecules.

In addition to proper peptide cleavage and MHC-peptide binding, the stability of the MHC-peptide complex is an important factor in natural antigen presentation. As revealed in chapter 2, rapid MHC-peptide dissociation may result in the absence of antigen cell surface expression. The peptide configuration in context with the MHC class I molecule appears to play an important role in MHC-peptide complex-stability³⁵. Therefore, molecular modeling systems may be of predictive value^{20,21}. However, dissociation experiments will have to confirm the predictions. Finally, before suggesting a newly revealed naturally expressed HA-1 antigen as an epitope for use of immunotherapy, its immunogenic potential needs to be defined.

In chapter 4 we used competition-based cellular peptide HLA binding assays to screen all possible HA-1^H and HA-1^R polymorphic peptides for HLA binding. We describe HLA-A3 binding HA-1 peptides and HLA-B60 binding HA-1 peptides. Since HA-1 peptide binding to HLA-A3 was weak and instable these peptides were unlikely to form HLA-A3 restricted HA-1 epitopes. We discovered, however, the HLA-B60 restricted HA-1^H peptide KECVLHDDL as a novel potential HA-1 T cell epitope.

Immunogenic potential of the HA-1^R counterpart in HLA-B60 was not confirmed although the HA-1^R peptides bound equally well and equally stable to the HLA-B60 molecule as the HA-1^H peptides. T cell lines induced with HLA-B60 binding HA-1^R peptide recognized HA-1^R peptide pulsed HLA-B60 positive target cells but not the non-pulsed target cells positive for HLA-B60/HA-1^R. It is possible that these CTLs are of low affinity. Using antigen presenting cells (APCs) that are transduced with HA-1^R may increase the success-rate of high affinity CTL induction compared with using APCs that are pulsed with HA-1^R peptide⁵⁴. It is also possible that the naturally expressed HLA-B60/HA-1^H complex acts as an altered peptide ligand to antagonize the high avidity HLA-B60/HA-1^R-specific T cells⁵⁵. The antagonistic complex may also cause negative selection of the high avidity HA-1^R specific T cells in the thymus of the HLA-B60/HA-1^{HH} donor^{56,57}. Alternatively, similar to the HLA-A2/HA-1^R ligand, the HLA-B60/HA-1^R ligand may not be expressed on the cell surface. Proteasome cleavage results in combination with the stable binding of the KECVLRDDL peptide to HLA-B60 suggest that the HLA-B60/HA-1^R complex fulfils several criteria to be expressed on the cell surface. However, we have not studied other intracellular processing mechanisms, such as TAP-mediated translocation into the ER³⁸ and TAP-associated binding³⁹. Unfortunately, as yet HLA-B60 HA-1 peptide elution experiments failed to show the presence of the HLA-B60/HA-1^H or HA-1^R peptide on the cell surface. Nevertheless, T cells specific for naturally expressed HLA-B60/HA-1^H were induced. Thus only the actual induction of T cells specific for naturally expressed HLA-B60/HA-1^R would indeed prove the natural immunogenicity of the KECVLRDDL peptide. To this end, as previously mentioned, using HA-1-transduced DCs instead of peptide-pulsed DCs as APC may provide a more efficient CTL induction protocol. cDNA-transduced DCs result in natural processing and constitutive expression of mH antigen HA-1, which is more effective than peptide-pulsed DCs in the generation of HA-1-specific CTLs⁵⁴.

Perhaps other not yet identified MHC class I/HA-1^R complexes are naturally expressed on the cell-surface in a stable fashion which may be capable of triggering an immune response from HA-1^R specific CTLs. However, since we tested the binding of all overlapping nonameric HA-1 peptides containing the H to R polymorphism to 11 prevalent MHC class I molecules, it is unlikely that additional MHC class I restricted HA-1^H or HA-1^R epitopes will be revealed. Moreover, possibly not the H to R polymorphism itself but the resulting ab-

sence of the HA-1^R counterpart on the cell surface is what causes the immunogenicity of the HA-1^H epitope. In that case the HA-1^H and HA-1^R antigens will not act as different epitopes. Therefore, the recently reported additional polymorphisms found within the HA-1 locus (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=23526) form an interesting new field of investigation. Perhaps these additional D to E or G to S polymorphisms result in additional allelic counterparts of HA-1. MHC class I restricted HA-1 peptides containing these polymorphisms may act as additional HA-1 epitopes, which may provide the option of HA-1 directed immunotherapy for patients negative for HA-1^H and/or expressing other MHC class I phenotypes.

7.2 HA-1 directed immunotherapy following UCB SCT

Umbilical cord blood (UCB) SCT is becoming a popular alternative treatment in case of hematological malignancies when no HLA identical SCT donor is available⁴⁰⁻⁴⁵ since reduced severity and reduced incidence of GvHD are observed^{40,41,44-47}. Furthermore, relapse rates following UCB SCT do not seem to be higher than relapse rates following adult SCT⁴¹. However, if leukemia patients relapse after UCB transplantation, donor cells are not available since the anonymous UCB donors cannot be traced. As a consequence these patients cannot be treated with donor lymphocyte infusion (DLI) or adoptive immunotherapy. Thus HA-1^H specific HLA-A2 restricted adoptive immunotherapy is not yet a feasible option for patients who received an UCB SCT. Even if UCB donor cells would be available at time of relapse, the feasibility of this HA-1 specific immunotherapy is questionable since UCB T cells are generally thought to be in a relatively immature state, which may reduce the possibilities for CTL induction⁴⁸⁻⁵¹. However, the generation of both tumour-specific and alloreactive CTLs from UCB has been reported previously⁵²⁻⁵⁴. Furthermore, adult-like fetal CD8 T cell responses against maternal infections have been recently described^{55,56}. Our results further question the naïve phenotype of UCB T lymphocytes.

In chapter 5 we report the generation and functional characterization of hematopoietic cell-specific CTLs from neonate cells directed against HA-1^H in the context of HLA-A2. These UCB CTLs were generated by mimicking the adult CTL induction protocol⁵⁴. Furthermore, these HLA-A2/HA-1^H specific UCB CTLs could also be generated from HLA-A2 HA-1^{RR} typed cord blood directly by FACS sorting. The lytic profile of the UCB derived HA-1 specific CTLs fully resembled the phenotype described for HLA-A2/HA-1^H specific adult CTLs. These results demonstrate that UCB has the capacity to generate a GvL effect following UCB SCT. Thus, including mH antigen typing for the selection of suitable UCB donors for transplantation of HLA-A2/HA-1^H positive leukemic patients may increase the GvL response. Clinical studies should be performed to determine whether HA-1 mismatching has a beneficial effect in UCB trans-

plantation. Nonetheless, the generation of HA-1 specific CTLs from UCB for adoptive immunotherapy is only feasible if sufficient numbers of UCB cells are available in addition to the number of cells necessary for successful transplantation. The cell dose obtained from one UCB donor is low. Furthermore, it is currently not possible to obtain donor cells again for use of DLI at time of leukemia relapse, since UCB is banked anonymously. Studies are ongoing to find ways to expand UCB cells prior to transplantation⁵⁷⁻⁶⁰. This may increase the success-rate of UCB SCT. Furthermore, a portion of UCB cells cryopreserved prior to transplantation may be used for HLA-A2/HA-1^H specific CTL generation. Thus in addition to patients who received an adult SCT, the option of HA-1 specific adoptive immunotherapy may become available to HLA-A2 positive relapsed leukemia patients who received an HLA-A2 matched, HA-1^H mismatched UCB SCT. Since UCB SCT is becoming a popular alternative treatment to adult SCT, this would considerably extend the patient population that can possibly benefit from HA-1 directed immunotherapy.

Importantly, our FACS sorting results suggest that the UCB CTLs are probably expanded from memory precursor cells already present *in vivo*. We suggest that UCB derived HA-1^H specific CTLs could be primed *in utero* against HLA-A2/HA-1^H expressing APCs of the mH antigen mismatched, but HLA-A2 matched mother. CTLs from the mother primed against paternal mH antigens expressed by the child were already found in various multiparous females⁶¹. This mH antigen specific CD8 memory is probably maintained by the long term microchimerism of cells from child origin found in the circulation of the mother^{61,62}. Likewise, priming of mH antigen specific UCB CTLs possibly depends on microchimerism of cells from the mother in the circulation of the child.^{63,64} Cells isolated from four HA-1^{RR} typed UCB samples derived from HA-1^{RR} typed mothers failed to grow *in vitro*, despite a few detectable tetramer-staining CD8⁺ cells. Expanding unprimed CTL precursors thus appeared impossible. Yet, a substantial number of cells isolated from three out of seven HA-1^{RR} typed UCB samples derived from HA-1^H typed mothers stained with the HA-1^{A2} tetrameric complexes. These directly obtained HLA-A2/HA-1^H specific UCB CTLs could be expanded in the absence of HA-1^H antigen. Possibly, once primed by microchimerism of maternal cells, the generated central memory UCB CTLs maintain their HA-1^H specificity by self-renewal⁶⁵. The non-specific expansion resulted in a further enrichment of tetramer staining CD8⁺ cells. Like the *in vitro* generated UCB CTLs, the lytic profile of these HA-1^H specific CTLs derived from UCB directly, resembled the phenotype described for HLA-A2/HA-1^H specific adult CTLs. The majority of tetramer negative CD8⁺ cells expressed CD45RA. In contrast, tetramer staining CD8⁺ cells from two of the three UCB samples clearly expressed lower levels of CD45RA. Low CD45RA expression is indicative of recent antigen exposure, suggesting that HA-1 specific T cell priming has occurred *in utero*. Thus, antigen-experienced circulating T cells specific for maternal mH antigen HA-1 can be detected in cord blood. Therefore, the selection of

HA-1^{RR} typed UCB derived from an HA-1^H typed mother may increase the GvL response. This option will only be feasible if, next to tissue typing of the UCB donors, also tissue typing of their mothers becomes a standard procedure.

It has been shown that HA-1 specific CTLs can also be generated from HLA-A2 negative individuals^{66,67}. Therefore, not only HLA-A2 positive patients who received an HLA-A2 matched HA-1^H mismatched UCB SCT but also who received an HLA-A2 mismatched UCB transplantation may benefit from HA-1 specific immunotherapy. Studies are ongoing to generate CTLs that exclusively recognize MHC class I/HA-1 complexes in an allogeneous setting.

7.3 HA-1 specific TCR transfer

Since *in vitro* generation of HA-1^H specific CTLs is time-consuming and not successful in all donors using the currently available protocols, HA-1 directed immunotherapy is not guaranteed for all HLA-A2 HA-1^H typed patients transplanted with HLA-A2 HA-1^{RR} typed donor stem cells. Therefore, transfer of HA-1 directed anti-leukemic reactivity to CD8⁺ peripheral T lymphocytes by T cell receptor (TCR) transfer might be an alternative strategy to generate HA-1 specific CTLs. For starting generation of HA-1 specific CTLs by TCR transfer much lower cell numbers are acquired than for starting CTL induction. Therefore, particularly in the UCB SCT setting TCR transfer may be a useful option for generating HA-1 specific CTLs, since only a limited number of UCB donor cells may be available for use of adoptive immunotherapy.

In chapter 6 we show that HA-1 specific TCR transfer can redirect both adult and UCB CD8⁺ T cells to antileukemic cytolytic activity. Thus, HA-1 specific TCR transfer may be a useful alternative strategy to generate leukemia-specific adult- or cord blood T cells. However, our results indicate a number of problems of the TCR transfer strategy as well. First, only a limited number of our HA-1 TCR-modified T cells stained HA-1^{A2} tetramer despite good cytolytic activity, an issue that needs to be further clarified. Furthermore, TCR modified T cells generally required more time than the original CTL clones to lyse their target cells. Similar problems were described for HA-2 TCR modified T cells, however optimally functioning HA-2 TCR modified T cells were also found⁶⁸. Possibly, cell surface expression of stable HA-1 specific TCRs following transfer may not correlate with double reporter gene expression due to a number of reasons. For instance, endogenous- and transferred TCRs may compete for cell surface expression. It is conceivable that the separate transferred TCR α and β chains pair with autologous TCR chains of the modified cells⁶⁹. T lymphocytes may contain two autologous TCR α chains⁷⁰ which even enlarges the number of possible TCR α and β combinations following TCR transfer. TCR competition and hybrid pairing of the different TCR α and β chains present in the cell following TCR transfer may thus explain our results. HA-1 TCR clustering could

be hampered by the other TCRs expressed on the cell surface or may be impaired by the low numbers of HA-1 TCRs on the cell surface. As a consequence tetramer staining may be decreased and the appropriate activation and lytic function of the T cells may require more time.

The overall drawback of the currently available TCR transfer technology is TCR hybrid pairing that may result in the formation of TCRs of unknown specificities⁷¹. These newly formed TCRs are a possible hazard when using the TCR modification strategy for treatment of hematological malignancies, since severe autoimmune reactions or graft versus host responses may occur. Another potential mechanism by which TCR gene transfer might result in autoimmunity is the genetic modification of ignorant self-specific T cells, as these T cells may become auto-reactive after triggering of the transferred TCR⁶⁹. Likewise, TCR transduced naive alloantigen-specific cord blood T cells from an HLA mismatched donor, may induce a severe graft versus host response in the patient upon transplantation. However, a first survey of autoimmunity in a mouse model of TCR gene transfer, in which the donor and recipient were fully MHC matched, did not give any clear indication of autoimmune pathology. This indicates that these two mechanisms are not likely to result in large-scale graft versus host effects after TCR transfer⁷². The development of strategies that can prevent the formation of hybrids or suppress endogenous TCR expression remains nevertheless desirable. Thus, prior to using this strategy for treatment of hematological malignancies, mechanisms of TCR formation and -triggering following TCR transfer into random peripheral T cells should be studied more extensively.

Transducing $\gamma\delta$ T cells with TCR α and $-\beta$ chains may prevent the problem of hybrid pairing since hybrid pairing of TCR γ or $-\delta$ chains with TCR α or $-\beta$ chains is not possible. The numbers of $\gamma\delta$ T cells in the circulation are very low⁷³, however only low numbers of T cells are necessary to perform TCR transfer. Alternatively, to prevent hybrid pairing the TCR chains may be modified *in vitro* in order to restrict their pairing capacity to each other. Nevertheless, the function of T cells modified with such redirected TCR chains remains unclear and needs to be studied extensively⁶⁹.

The problem of triggering unknown ignorant or naïve TCRs can be circumvented by TCR transfer into established memory T cells containing endogenous CMV specific TCRs⁷⁴. The resulting T cells display dual specificity against both hematopoiesis restricted mH antigen and CMV. Stimulating these dual specific T cells with CMV antigen results in higher CMV specificity, while stimulating these cells with mH antigen results in higher mH specificity. However, since CMV specific memory T cells are required for this strategy, this is not an option when using cord blood cells. Furthermore, although the number of possible hybrid formations is restricted using this strategy, hybrid formation of TCRs of unknown specificity may still remain a problem. Perhaps, co-transduction of a suicide gene together with the TCR α and $-\beta$ chains may provide a tool to eradicate the modified T cells if adverse effects occur⁷⁵.

Another use of mH antigen HA-1 specific TCR transfer may be the modification of CD4⁺ T helper (Th) cells of a known specificity, in order to increase the priming efficiency of HA-1 specific CTLs. In our protocols, priming for HA-1 CTLs can be improved by adding Th cells to the CTL cultures (chapter 5). Currently the Th cells are produced using recall antigens (DKTP vaccine) (unpublished) or by non-specific stimulation of CD4⁺ T cells with CD3/CD28 coated beads (chapter 5). Gene transfer of HLA-A2 restricted HA-1^H specific TCRs into Th cells with a known specificity may result in the generation of Th cells that can specifically produce helper cytokines in response to the HA-1^H expressing HLA-A2 positive APCs. These helper cytokines specifically produced in response to the HLA-A2/HA-1^H complex may improve HA-1^H specific CTL priming. Since the Th1 type cytokines IFN- γ and IL-2 are key players in CTL induction, this TCR transfer may be most useful when using Th1 type CD4⁺ T cells. Additional transduction of these CD4⁺ Th cells with the CD8 α and - β chains may, in addition, lead to cytolytic activity of these cells in response to the HLA-A2/HA-1^H complex. Thus transduction of for instance tetanus toxoid (TT)-specific CD4⁺ Th cells with both the HLA-A2 restricted HA-1^H specific TCR and the CD8 α and - β chains, may result in TT-specific T cells capable of producing helper cytokines as well as lysing target cells in response to the HLA-A2/HA-1^H complex.

In conclusion, in order to increase the HA-1 specific adoptive immunotherapy success-rate, HA-1 specific TCR transfer may be a useful alternative strategy. Not only to rapidly generate HA-1^H specific HLA-A2 restricted adult- or UCB CTLs, but perhaps also to generate CD4⁺ Th cells which can increase the priming efficiency of HA-1^H specific CTLs. Like induction of HA-1^H specific CTLs, the generation of these CTLs by TCR transfer is only useful in HA-1^{RR} typed donor T cells since HA-1^H positive donor T cells may be destroyed by transduced HA-1^H specific TCRs.

Concluding remarks

All possible options for HA-1 specific immunotherapy presented in this thesis require an HA-1^H mismatch between patient and donor. Providing this HA-1^H mismatch, some leading points are now available to extend the patient population that can benefit from HA-1 specific adoptive immunotherapy in the future.

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Abbreviations



aa	amino acid
ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
APC	antigen presenting cell
β_2 M	β_2 -microglobulin
B-LCL	B-lymphoblastoid cell line
BMT	bone marrow transplantation
CB	cord blood
CB-MNC	cord blood mononuclear cells
CBT	cord blood transplantation
CLL	chronic lymphoid leukemia
CML	chronic myeloid leukemia
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DLI	donor lymphocyte infusion
dNGF-R	truncated form of nerve growth factor receptor
EBV-LCL	Epstein-Barr Virus transformed B lymphoblastoid cell line
eGFP	enhanced green fluorescent protein
ER	endoplasmatic reticulum
E:T	effector:target
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Fl	fluorescein
GAP	GTPase-activating protein
GvHD	graft versus host disease
GvL	graft versus leukemia
HLA	human leukocyte antigen
HS	human serum
IFN-	interferon-
IL-	interleukin-
IMDM	Iscove's modified Dulbecco's medium
MACS	magnetic activated cell sorting
MF	mean fluorescence
mH	minor histocompatibility
mHag	minor histocompatibility antigen
MHC	major histocompatibility complex
moab	monoclonal antibody
MoDC	monocyte derived dendritic cell
MTA	maternally transmitted antigen
NIMA	non-inherited maternal antigen
NK	natural killer
ORF	open reading frame
PBMC	peripheral blood mononuclear cells

PBS	phosphate buffered saline
PCR	polimerase chain reaction
PHA	phytohemagglutinin
SC	stem cell
SCT	stem cell transplantation
TAP	transporter associated with antigen processing
TCL	T (lymphocyte) cell line
TCR	T cell receptor
Th	helper T lymphocyte
TNF	tumour necrosis factor
TT	tetanus toxoid
UCB	umbilical cord blood

Summary



Allogeneic stem cell transplantation (SCT) from a related or unrelated donor is a well-established and effective therapy for advanced hematological malignancies. This therapy may –besides the required graft versus leukemia (GvL) response– initiate graft versus host disease (GvHD). In order to prevent GvHD, T cell depletion of the stem cell graft was introduced. Unfortunately, this not only resulted in a dramatic reduction of GvHD, but also in an increase of graft rejection and leukemia relapse rates. Relapsed leukemia patients can be successfully treated with donor lymphocyte infusion (DLI). The beneficial GvL response after DLI is however associated with GvHD. Differences in the major histocompatibility complex (MHC) molecules between donor and recipient cause this graft versus host reaction in case donor and recipient are MHC mismatched, however, in MHC identical settings, GvHD is still frequently initiated. In these MHC identical settings incompatibility between the so-called minor histocompatibility (mH) antigens is associated with GvHD induction. Human mH antigens consist of peptides derived from naturally processed intracellular proteins and are presented by MHC molecules. Incompatibilities between mH antigens are generally caused by amino acid polymorphisms in these self-proteins but can also be caused by gene deletion.

Several mH antigens were found to be expressed only by certain cell types, while others are expressed ubiquitously. This differential tissue distribution of mH antigens may be an important feature in targeting specific cell types using mH specific donor cytotoxic T lymphocytes (CTLs). For example, the mH antigen HA-1 was found to be expressed by cells of hematopoietic origin only. Therefore, HA-1 may be targeted specifically to induce a GvL response. The immunogenic mH antigen HA-1 was identified as the HLA-A2 restricted non-amer peptide VLHDDLLEA derived from the di-allelic KIAA0223 gene. Two nucleotide differences in the KIAA0223 sequence resulted in a single amino acid sequence difference between the immunogenic CTL epitope VLHDDLLEA (HA-1^H) and the VLRDDLLEA (HA-1^R) counterpart. HA-1^H is the most ideal candidate for immunotherapy of hematological malignancies since this mH antigen is highly immunogenic, hematopoietic restricted, expressed in high levels on leukemic cells and its frequency is 69% among the HLA-A2 positive population. Large numbers of HA-1^H specific HLA-A2 restricted donor CTLs can potentially be generated *in vitro* for the use of adoptive cellular immunotherapy.

Selectively infusing these HA-1^H specific donor CTLs may mediate a strong GvL effect with a low risk for GvHD. However, The HA-1^H specific immunotherapy is currently feasible only for HLA-A2 HA-1^{HH} or HA-1^{HR} patients who relapsed following an SCT from an HLA-A2 HA-1^{RR} donor. Thus, the feasibility of this therapy depends both on the presence of recipient-donor disparity for the mH antigen HA-1 and on HLA-A2 expression. In addition, the therapy is not guaranteed for all of these patients. For instance, the anonymous umbilical cord blood (UCB) donors cannot be traced again for use of DLI or adoptive immunotherapy following transplantation. Moreover, the success rate of HA-1^H

specific HLA-A2 restricted CTL induction is donor dependent. This thesis describes several attempts and possibilities to extend the patient population that can benefit from HA-1 specific immunotherapy in the future.

Previously described peptide elution studies could not detect HA-1^R expression in the context of HLA-A2; i.e. beyond 5 copies per cell. In chapter 2, we examined several possible causes for the absence of cell surface HLA-A2/HA-1^R expression in order to evaluate the option of HA-1^R directed immunotherapy. Immunotherapy directed towards the HA-1^R counterpart would make the HA-1 specific therapy available for HA-1^R typed patients who relapsed following an SCT from a HA-1^H typed donor. However, chapter 2 reveals that HLA-A2/HA-1^R specific CTLs are not useful for immunotherapy since the natural presentation of HA-1^R in HLA-A2 is unstable. Other not yet identified MHC class I/HA-1^R complexes that are naturally expressed on the cell-surface in a stable fashion, might be capable of triggering an immune response from HA-1^R specific CTLs. Nevertheless, possibly HA-1^R peptides are not capable of serving as natural T cell epitopes in any setting. Therefore, the recently reported additional polymorphisms found within the HA-1 locus form an interesting new field of investigation. Additional allelic counterparts in the HA-1 gene may result in additional MHC class I restricted HA-1 epitopes which may provide the option of HA-1 directed immunotherapy for patients negative for HA-1^H.

Furthermore, we investigated whether the HA-1^{H/R} polymorphic region contains peptides that can be presented by MHC class I molecules other than HLA-A2. To this end, competition-based peptide binding assays for 13 prevalent MHC class I alleles were developed, as described in chapter 3. In chapter 4, using this competition-based peptide-binding assay, we discovered the HLA-B60 restricted HA-1^H peptide KECVLHDDL as a novel additional HA-1 epitope. Immunogenic potential of the HA-1^R counterpart in HLA-B60 was not confirmed. However, proteasome cleavage results and the stable binding of both the KECVLHDDL and the KECVLRDDL peptide to HLA-B60 suggested that the HLA-B60/HA-1^R complex is expressed on the cell surface. Using HLA-B60 APCs that are transduced with HA-1^R may increase the success-rate of high affinity HLA-B60 restricted HA-1^R specific CTL induction compared with using APCs that are pulsed with HA-1^R peptide.

In chapter 5 we report the generation and functional characterization of hematopoietic-specific CTLs from UCB directed against HA-1^H in the context of HLA-A2. We suggest that including mH antigen typing for the selection of suitable UCB donors for transplantation of HLA-A2/HA-1^H positive leukemic patients may increase the GvL response following UCB transplantation. Furthermore, like for patients treated with adult SCT, the option of HA-1 specific immunotherapy may become available to HLA-A2 positive relapsed leukemia patients who received an HLA-A2 matched, HA-1^H mismatched UCB SCT. Yet, sufficient numbers of UCB donor cells need to be available in order to be able to generate HLA-A2/HA-1^H specific UCB CTLs for use of immunotherapy. The

cell dose obtained from one UCB donor, however, is low. Expanding UCB cells prior to transplantation may provide sufficient numbers of cells to increase the success-rate of UCB SCT. In addition, cryopreservation of a portion of these UCB cells prior to transplantation may provide donor cells at time of relapse, which can be used for HLA-A2/HA-1^H specific UCB CTL generation.

In chapter 6 we show that HA-1 specific TCR transfer can direct non-HA-1 TCR expressing adult and UCB CD8⁺ T cells to hematopoietic-specific cytolytic activity. Thus, HA-1 specific TCR transfer may be a useful alternative strategy to generate HA-1^H specific HLA-A2 restricted adult- or UCB CTLs. However, only a limited number of TCR-modified cells stained HA-1^{A2} tetramer. Also TCR-modified cells generally required more time than the original CTL clones to lyse their target cells. This may be caused by low cell surface expression of stable HA-1 specific TCRs. Endogenous- and transferred TCRs possibly compete with each other for cell surface expression. Furthermore, the separate transferred TCR chains may pair with the autologous TCR chains of the modified cells. This hybrid pairing may not only result in a low concentration of HA-1 specific TCRs but also in the formation of hybrid TCRs of unknown specificities. In addition, ignorant self-specific T cells may become autoreactive after triggering of the transferred TCR. Thus, several intracellular mechanisms should be studied extensively before the option of TCR transfer becomes clinically available.

In conclusion, some important features of the polymorphic mH antigen HA-1^{H/R} locus are now available, which may lead to extension of the patient population that may benefit from HA-1 specific immunotherapy.

Samenvatting



Een effectieve therapie waarmee leukemiepatiënten doorgaans worden behandeld is allogene stamceltransplantatie. Voordat de stamcellen uit het beenmerg van de donor worden getransplanteerd, worden de leukemie cellen in de patiënt gedood door middel van bestraling en/of chemotherapie. Ook de gezonde bloedcellen van de patiënt worden bij deze behandeling gedood. Na transplantatie zullen de van de donor afkomstige stamcellen groeien en differentiëren tot rijpe bloedcellen, zodat het hematopoietische (= bloedvormende) systeem in de patiënt herstelt. Vervolgens kunnen bepaalde witte bloedcellen, de T lymfocyten oftewel T cellen, van de donor de nog levende leukemie cellen van de patiënt herkennen en doden: het zogenaamde graft versus leukemia (GvL) effect. De donor T cellen aanwezig in het stamcel transplantaat kunnen echter ook gezonde cellen en weefsels van de patiënt als vreemd herkennen, wat kan leiden tot graft versus host disease (GvHD). Om het ontstaan van GvHD te voorkomen kan het stamcel transplantaat vóór de transplantatie worden ontdaan van de T cellen. Helaas resulteert dit niet alleen in afname van GvHD maar ook in toename van afstotingsverschijnselen en terugkeer van de leukemie. Bij terugkeer van de leukemie kunnen patiënten worden behandeld met het toedienen van donor afweercellen, oftewel donor lymphocyte infusion (DLI). De beoogde respons tegen leukemie na DLI is echter geassocieerd met het ontstaan van GvHD.

T cel receptoren (TCRs) op T cellen herkennen eiwitfragmenten (peptiden) van lichaamsvreemde eiwitten (proteïnen). Deze zijn voorbehandeld door een cel en worden op het celoppervlak gepresenteerd door een ander molecuul. Dit molecuul wordt in de mens human leucocyte antigen (HLA) genoemd en is gecodeerd in het major histocompatibility complex (MHC). Met name verschillen in MHC/HLA moleculen tussen transplantaat en ontvanger zijn de oorzaak van het ontstaan van GvHD. Daarom wordt er zoveel mogelijk getransplanteerd met stamcellen van een donor waarvan de MHC moleculen nagenoeg identiek zijn aan die van de ontvanger. Ook in een dergelijke MHC identieke situatie kan desondanks GvHD ontstaan. In deze situatie is de afweerreactie gericht tegen verschillen in andere moleculen dan MHC moleculen. Deze moleculen worden de minor histocompatibility (mH) antigenen genoemd. mH antigenen zijn eiwitfragmenten afkomstig van lichaamseigen eiwitten in de cel, die door MHC moleculen worden gepresenteerd op het celoppervlak. Verschillen tussen deze mH antigenen worden voornamelijk veroorzaakt door kleine verschillen in de aminozuren waaruit de antigenen zijn opgebouwd, polymorfismen genaamd. In sommige gevallen ontstaat het verschil in mH antigenen presentatie tussen donor en ontvanger echter doordat het gen dat het mH antigenen codeert niet tot expressie wordt gebracht door ofwel de donor ofwel de ontvanger.

Sommige mH antigenen worden alleen tot expressie gebracht door bepaalde specifieke celtypes terwijl andere mH antigenen door ieder celtype tot expressie worden gebracht. Deze verschillen in expressie kunnen van belang zijn om de

afweerreactie door donor T cellen specifiek te kunnen richten tegen bepaalde celtypes, zonder andere celtypes aan te tasten. Het mH antigeen HA-1 wordt bijvoorbeeld alleen tot expressie gebracht door hematopoïetische cellen, dus ook door leukemie cellen. Een specifieke afweerreactie tegen HA-1 zou dan ook kunnen leiden tot een GvL effect. Van het HA-1 eiwit zijn twee varianten geïdentificeerd die in één aminozuur verschillen, een H of een R (dit wordt geschreven als HA-1^H en HA-1^R). Ieder mens bezit één van de twee varianten of beide. Na transplantatie wordt de H variant als vreemd herkend door T cellen afkomstig van een donor die alleen de R variant heeft. Deze H variant is als mH antigeen geïdentificeerd wanneer het als eiwitfragment gepresenteerd wordt door het MHC molecuul HLA-A2. HLA-A2 komt in 49% van de Caucasische populatie voor. 69% van de HLA-A2 positieve populatie brengt ook HA-1^H tot expressie. Leukemie patiënten met deze weefsel typering kunnen wellicht geholpen worden door immunotherapie met donor T cellen die specifiek gericht zijn tegen HA-1^H in HLA-A2. Aanzienlijke aantallen cytotoxische (= celdodende) T cellen met deze specificiteit kunnen worden gekweekt voor deze zogenaamde adoptieve cellulaire immunotherapie.

Het selectief toedienen van HA-1^H specifieke T cellen zou dus een sterk GvL effect kunnen genereren met slechts een laag risico op GvHD. Deze HA-1 specifieke adoptieve immunotherapie is echter alleen beschikbaar voor HLA-A2 positieve patiënten die HA-1^H tot expressie brengen en die getransplanteerd zijn met cellen van een donor die ook HLA-A2 positief is maar geen HA-1^H tot expressie brengt. De haalbaarheid van deze therapie is dus zowel afhankelijk van dit HA-1 verschil tussen patiënt en donor als van HLA-A2 expressie. Tijdens dit promotie onderzoek is gezocht naar mogelijkheden om de patiënten populatie die van HA-1 specifieke immunotherapie zou kunnen profiteren te vergroten.

Tijdens eerder onderzoek is expressie van de HA-1^R variant in combinatie met HLA-A2 niet gevonden. In hoofdstuk 2 wordt een aantal mogelijke oorzaken voor de afwezigheid van HLA-A2/HA-1^R op het celoppervlak onderzocht. De mogelijkheid van immunotherapie gericht tegen de HA-1^R variant zou immers de HA-1 specifieke therapie beschikbaar maken voor HA-1^R positieve patiënten die getransplanteerd zijn met cellen afkomstig van een HA-1^R negatieve donor. De natuurlijke presentatie van HA-1^R door HLA-A2 bleek echter extreem instabiel. Daardoor vervalt de mogelijkheid van immunotherapie gericht tegen HA-1^R in combinatie met HLA-A2. Desondanks worden andere nog niet geïdentificeerde HA-1^R eiwitfragmenten wellicht stabiel gepresenteerd door andere MHC moleculen. Een dergelijke combinatie zou een afweerreactie van HA-1^R specifieke donor T cellen kunnen uitlokken. Het is echter ook mogelijk dat geen enkel HA-1^R fragment kan dienen als antigeen. In dit geval kunnen de recent gevonden andere aminozuur polymorfismen op verschillende posities in het HA-1 gen van belang zijn. Deze 'nieuwe' polymorfismen resulteren wellicht in een grotere variatie van HA-1 eiwitfragmenten die worden gepresenteerd

door MHC moleculen en die kunnen worden herkend door donor T cellen. Deze grotere variatie zou immunotherapie mogelijk kunnen maken voor HA-1^H negatieve patiënten.

De toepassing van HA-1 specifieke immunotherapie zou in de toekomst kunnen worden uitgebreid door de therapie ook mogelijk te maken voor patiënten die geen HLA-A2 tot expressie brengen. Hiertoe is voor verschillende HA-1^H- en HA-1^R eiwitfragmenten onderzocht of deze kunnen worden gepresenteerd door andere MHC moleculen dan HLA-A2. In hoofdstuk 3 wordt de ontwikkeling van het peptide-MHC-bindings experiment beschreven dat hiervoor werd toegepast. Dit experiment is ontwikkeld voor 13 verschillende MHC moleculen en is gebaseerd op competitie voor MHC-binding tussen een HA-1 fragment en een eiwitfragment waarvan al bekend is dat het bindt aan het MHC molecuul van interesse. Hoofdstuk 4 beschrijft vervolgens de ontdekking van HA-1 eiwitfragmenten waarvan zowel de H als de R variant binden aan HLA-B60. Bovendien bleek het mogelijk om een afweerreactie van donor T cellen specifiek te richten tegen een natuurlijk HA-1^H fragment gepresenteerd door HLA-B60. Een dergelijke afweerreactie werd niet gevonden tegen de natuurlijke HA-1^R variant in HLA-B60. Desondanks wijzen bepaalde bevindingen erop dat ook de HA-1^R variant door HLA-B60 op het celoppervlak kan worden gepresenteerd. Daarom zou het optimaliseren van de kweektechnieken wellicht kunnen leiden tot een afweerreactie specifiek gericht tegen natuurlijk HA-1^R in HLA-B60.

In hoofdstuk 5 konden cytotoxische T cellen specifiek gericht tegen HA-1^H in HLA-A2 worden verkregen uit HLA-A2 positief HA-1^H negatief navelstrengbloed. Het typeren voor zowel HLA- als mH antigeen expressie lijkt dus niet alleen van belang voor het selecteren van een geschikte volwassen donor, maar ook voor het selecteren van een passende navelstreng donor. Transplantatie van HLA-A2/HA-1^H positieve leukemie patiënten met HLA-A2 positief HA-1^H negatief navelstrengbloed zou kunnen leiden tot een toename van het GvL effect. Bovendien zou HA-1 specifieke immunotherapie in de toekomst mogelijk kunnen worden voor HLA-A2/HA-1^H positieve leukemie patiënten die getransplanteerd zijn met HLA-A2 positief HA-1^H negatief navelstrengbloed. Het kweken van HLA-A2/HA-1^H specifieke cytotoxische T cellen uit navelstrengbloed voor gebruik bij immunotherapie vergt echter een groot aantal cellen, boven op het aantal dat nodig is voor succesvolle transplantatie. Het celaantal dat wordt verkregen uit één navelstreng is over het algemeen laag. Wellicht zal het expanderen van navelstrengbloed vóór de transplantatie leiden tot voldoende aantallen cellen om het transplantatiesucces te laten toenemen. Verder zou het invriezen van een gedeelte van deze navelstrengbloedcellen vóór de transplantatie kunnen voorzien in donor cellen wanneer de leukemie terugkeert. Hieruit kunnen dan HLA-A2/HA-1^H specifieke cytotoxische T cellen worden gekweekt.

Hoofdstuk 6 laat zien dat het mogelijk is om HA-1 specifieke T cel receptoren over te brengen naar zowel volwassen als navelstrengbloed T cellen. Deze T cellen vertonen vervolgens cytotoxische respons tegen leukemie cellen. In tegenstelling tot het kweken van HLA-A2/HA-1^H specifieke T cellen is voor het verkrijgen van HLA-A2/HA-1^H specifieke T cellen door TCR overdracht slechts een klein aantal cellen nodig. TCR overdracht zou dus zowel in geval van transplantatie met volwassen stamcellen als met navelstrengbloed een bruikbare alternatieve strategie kunnen vormen om het welslagen van HA-1 specifieke immunotherapie te bevorderen. Vooralsnog moeten er nog diverse hordes genomen worden. Verschillende aanwijzingen werden namelijk gevonden voor de suggestie dat de TCR-gemodificeerde T cellen slechts een klein aantal HA-1 specifieke T cel receptoren op hun oppervlak tot expressie brengen. Het is mogelijk dat de TCRs die al aanwezig waren in de T cellen competitie aangaan met de HA-1 specifieke TCRs die zijn ingebracht, voor expressie op het celoppervlak. Verder is het mogelijk dat de beide ketens waaruit de HA-1 specifieke TCR bestaat, apart paren met de TCR ketens al aanwezig in de cel. Door deze zogenaamde hybride paringen komt slechts een laag aantal complete HA-1 specifieke TCRs op het celoppervlak tot expressie. Bovendien worden hierdoor hybride TCRs gevormd waarvan de specificiteit onbekend is. Daarnaast kan het voorkomen dat niet-geactiveerde TCRs in de cel die specifiek zijn voor eigen gezonde cellen of weefsels, toch actief worden na activatie van de ingebrachte TCR. Om destructie van gezonde cellen tegen te gaan zullen verschillende mechanismen in de cel dus uitgebreid bestudeerd moeten worden. Zowel de formatie van hybride TCRs met onbekende specificiteit als de activatie van zelfspecifieke TCRs moeten worden uitgesloten voordat de mogelijkheid van TCR overdracht beschikbaar wordt voor gebruik in de praktijk.

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Curriculum Vitae



De schrijfster van dit proefschrift is geboren op 6 september 1974 in Leiderdorp. Zij behaalde haar HAVO diploma in 1991 en haar VWO diploma in 1993, beide aan de Louise de Coligny Scholen Gemeenschap in Leiden. Vervolgens heeft zij een jaar doorgebracht in Driebergen alwaar zij het Propedeutisch Jaar volgde van de Vrije Hogeschool. In 1994 ging zij Biomedische Wetenschappen studeren aan de Universiteit Leiden. Tijdens haar studie doorliep zij verschillende stages: aan de afdeling Pathologie/Heelkunde van het Leids Universitair Medisch Centrum in de groep van Dr. P. Kuppen, aan de afdeling Medical Microbiology van de Edinburgh University in Schotland in de groep van Prof. Dr. M. Norval, aan de afdeling Parasitologie van het Leids Universitair Medisch Centrum in de groep van Dr. A. Waters en aan de afdeling Immunohematologie en Bloedbank van het Leids Universitair Medisch Centrum in de groep van Prof. Dr. F. Claas. Het doctoraal diploma werd in augustus 1999 behaald. Hierna volgde het promotieonderzoek dat is beschreven in dit proefschrift. Zij was hiervoor van september 1999 tot maart 2004 aangesteld als Assistent In Opleiding (AIO); aan de afdeling die inmiddels was omgedoopt tot Immunohematologie en Bloedtransfusie (hoofd Prof. Dr. C. Melief) van het Leids Universitair Medisch Centrum. Het promotieonderzoek werd verricht in de groep van Prof. Dr. E. Goulmy, onder haar begeleiding en die van Dr. T. Mutis en Dr. A. van Halteren. Sinds maart 2005 is zij werkzaam als Scientist Immunology bij Crucell Holland B.V.

