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Relevance of HLA Expression Variants in Stem Cell Transplantation

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1. Introduction

Matching the donor and recipient for class I and II human leukocyte antigens (HLA) is pivotal to the success of allogeneic hematopoietic stem cell transplantation (HSCT). Transplantation across HLA barriers will lead to the development of T-cell responses to the mismatched HLA molecules, resulting in T-cell–mediated graft-versus-host disease (GvHD) or graft rejection in patients with insufficient immune suppression. The accuracy of testing and matching criteria has an important impact on the transplant outcome, but exact matching across multiple HLA loci (e.g., HLA-A, HLA-B, HLA-C, and HLA-DRB1) is a challenging task. Today, serological HLA diagnostic tests are being replaced by DNA-based typing methods considering only selected regions of the genes. Therefore, HLA null alleles or expression variants bearing their variation outside of these regions may be misdiagnosed as normally expressed variants, resulting in HLA mismatches that are highly likely to stimulate allogeneic T cells and trigger GvHD. This chapter will address the relevance, genetics, prevalence and diagnosis of HLA expression, variants of HLA class I loci and will discuss their clinical implications for transplantation.

2. The human major histocompatibility complex

The human major histocompatibility complex (MHC), also referred to as the human leukocyte antigen (HLA) complex, is encoded on the short arm of chromosome 6 (6p21) and is extremely polymorphic (Parham et al. 1988). HLA class I molecules are expressed on most nucleated cells. The HLA class I region comprises the gene loci for the heavy chains of the three classical human leukocyte antigens, HLA-A, -B, and -C. They consist of a heavy chain (44 kDa) and a non-covalently bound β2 microglobulin (β2m) light chain (12 kDa) encoded by chromosome 15. The heavy chain is made up of three extracellular domains: α1, α2, and α3. The highly polymorphic region of HLA class I molecules is located in the DNA and amino acid sequences of the α1 and α 2 domains, which form the peptide-binding groove. Endogenous 8 to 12 amino acid peptides are presented to CD8+ cytotoxic T lymphocytes (CTLs) (Natarajan et al. 1999). The α3 domain is mainly invariant and contains the binding site for the co-receptor CD8. Because of the MHC's role in recognizing pathogenic and cancerous peptides, these genes are under high environmental pressure to be very polymorphic. A total of 4,946 HLA class I alleles have been identified to date (http://www.ebi.ac uk/imgt/hla; released April 2011).

HLA class I molecules are stabilized by disulfide bonds located in the α2 and α3 domains between cysteine (C) residues at amino acid positions 101/164 and 203/259. These bonds are essential for the correct processing and function of the molecules (Solheim 1999). Amino acid substitutions in these crucial C residues are likely to cause aberrant expression of the respective HLA class I molecules and may also change the affinity of the peptide-binding groove towards endogenous peptides (Warburton et al. 1994; Hirv et al. 2006; Hinrichs et al. 2009; Hinrichs et al. 2010).

HLA class II molecules (DR, DQ, DP) are mainly expressed on hematopoietic cells (macrophages, dendritic cells, T cells and B cells). The heterodimers are formed by two membrane-bound chains (α and β), each consisting of two domains (α1/α2 or β1/β2, respectively) encoded by two genes co-located in the centromeric part of the MHC. The antigenic peptide (up to 30 amino acids) is presented to CD4+ T helper cells (T_h cells) in a cleft formed by the outermost α1 and β1 domains. Nearly all of the polymorphisms occur at exon 2 of the respective A or B genes. Peptides presented by HLA class II molecules are derived from exogenous proteins as well as from epitopes of plasma membranes or endosomes (Rudensky et al. 1991; Chicz et al. 1993; Sant 1994). The nonpolymorphic β2 domain contains the binding site for the T cell co-receptor CD4. More than 1,457 HLA class II alleles have been identified to date (http://www.ebi.ac uk/imgt/hla; released April 2011).

2.1 Peptide presentation by HLA

The ability to recognize and distinguish between self and non-self is primarily mediated by T lymphocytes, which survey the protein environment of cell surfaces for binding partners, i.e. for signs of foreign invasion. T cells do not recognize proteins directly; instead, they recognize imprints of ongoing protein metabolism in the form of peptides presented by HLA molecules. This phenomenon is called MHC restriction. The biological function of HLA molecules is to present antigenic peptides to T cells. Therefore, HLA molecules play a central role in T cell-mediated adoptive immunity. MHC class I molecules present peptides from endogenously synthezised proteins, whereas MHC class II molecules present peptides from incorporated exogenous proteins. All of these peptides originate from foreign or host cell proteins and are generated by proteasomal cleavage (class I pathway) or lysosomal processing (class II pathway). It has been estimated that about 0.5% of presented peptides are bound to MHC molecules, whereas more than 99% are ignored. Consequently, peptide binding to HLA is the single most selective event involved in antigen processing and presentation (Yewdell, Norbury, and Bennink 1999; Yewdell and Bennink 2001). A T cell-mediated immune response occurs when the T-cell receptor recognizes a specific peptide-MHC complex and thus identifies cells that have been infected by intracellular parasites or viruses or cells containing abnormal proteins (e.g., tumor cells). The peptides beeing part of a certain peptide-MHC complex triggering T-cell recognition are important tools for diagnosis and treatment of infectious, autoimmune, allergic and neoplastic diseases (Ferrari et al. 2000; Haselden, Kay, and Larche 2000; Singh 2000; Wang, Phan, and Marincola 2001).

Different polymorphic HLA molecules have different peptide binding specificities (Falk et al. 1991; Sette et al. 1994; Bade-Doeding et al. 2007; Bade-Doeding et al. 2011). Peptides presented by MHC class I molecules are derived from cytoplasmic proteins by proteolytic degradation in the proteasome. Therefore, the MHC class I presentation pathway is often called the cytosolic or endogenous pathway. The MHC class I crystal structure features a

unique peptide-binding groove at the outer polymorphic α2 and α3 domains (Bjorkman et al. 1987, Madden et al. 1991). This groove can be subdivided into six pockets (A-F) of different size, shape, and function (Garrett et al. 1989; Matsumura et al. 1992). A pocket is defined as a unit having an affinity for a certain peptide side chain (e.g., affinity of pocket A for peptide position P1 and pocket B for P2). Some pockets have a well-shaped structure with an affinity for only one side chain, whereas others have an affinity for a group of side chains. In some cases, the boundaries between pockets are unclear. The most important residues and positions of a peptide are known as anchor residues and anchor positions. The identity and spacing of these primary anchors constitutes the peptide motif of an HLA specificity (Sette et al. 1987; Sette et al. 1989; Jardetzky et al. 1991; Ruppert et al. 1994; Rammensee, Friede, and Stevanoviic 1995). A typical peptide is 8 to 12 amino acids in length and binds in the peptide-binding groove, exhibiting an extended conformation with its terminal amino group bound to a pocket at one end of the groove and its terminal carboxyl group bound to a pocket at the other end of the groove.

Peptide binding motifs generally contain two to three anchor positions (Rammensee, Friede, and Stevanoviic 1995). Other features such as secondary anchors and disfavored residues have also been described as playing an important role in defining the peptide-MHC interaction (Ruppert et al. 1993). The peptide-binding cleft of HLA class II molecules is formed by the outer α1 and β1 domains. Since it does not narrow at the ends, it can accommodate longer peptides containing up to 30 but usually 13 to 17 amino acids. The peptides presented by class II molecules are derived from extracellular proteins internalized by endophagocytosis and degraded in an endocytic compartment. Hence, the MHC class II-dependent pathway of antigen presentation is called the endocytic or exogenous pathway.

2.2 HLA nomenclature and typing methods

According to the World Health Organization (WHO) Committee on Nomenclature for Factors of the HLA System (Holdsworth et al. 2009), each HLA allele name has a unique number corresponding to up to four sets of digits separated by colons. The 2-digits before the first colon describe the type, which often corresponds to the serological antigen carried by an allotype. The next set of digits are used to list the subtypes, numbers being assigned in the order in which DNA sequences have been determined. Broad families of alleles are clustered into serotypes (e.g., HLA-A1).

There are two levels of typing: low-resolution (2-digits) and high-resolution (at least 4-digits). Low-resolution typing delivers results equivalent to serological typing and can be achieved by serological (microlymphocytotoxicity test) or molecular techniques. Due to its simplicity and low cost, serologic typing is still used in some laboratories. High-resolution typing can only be achieved by DNA-based techniques allowing classification of the individual alleles within each serotype (e.g., HLA-A*01:01). A number of HLA typing methods based on PCR technology have been developed. PCR with sequence-specific primers (PCR-SSP), PCR followed by sequence-specific oligonucleotide probing (PCR-SSO) and PCR followed by sequencing-based typing (PCR-SBT) are currently the most commonly used molecular methods for low- and high-resolution HLA typing. These methods have displaced serology in most laboratories because of a much greater accuracy.

2.2.1 Types and nomenclature of HLA expression variants

To label HLA alleles with an alternative expression pattern the WHO Nomenclature Committee for Factors of the HLA System defined suffixes ('N', 'L', 'S', 'C', 'A', 'Q'), that are

added to an allele name to indicate its expression status (Holdsworth et al. 2009). Alleles shown to be not expressed ('Null' alleles) are given the suffix 'N'. The alteration does not necessarily imply the lack of production of an internal partial product which might be a T-cell target (Elsner and Blasczyk 2004). HLA alleles with 'Low' cell surface expression of an intact antigen compared to normal levels are indicated using the suffix 'L'. The suffix 'S' is used to denote an allele specifying a protein which is exclusively expressed as a 'Secreted' molecule but not as a cell surface protein. A 'Q' suffix is used when the expression of an allele is 'Questionable' given that the mutation seen in the allele has previously been shown to affect normal expression levels. The suffix 'C' is used to denote an allele product found in the 'Cytoplasm' but not on the cell surface, and the suffix 'A' indicates 'Aberrant' expression.

Currently, 197 HLA class I alleles (168 N, 5 L, 24 Q and 1 S allele) and 21 HLA class II alleles (all null alleles) with variant expression are listed in the IMGT/HLA database on the HLA nomenclature website (www.ebi.ac.uk/imgt/hla; released April 2011). As of April 2011, no alleles have been named with a 'C' or 'A' suffix. Most of these alleles carry mutations causing stop codons, leaving no doubt about their non-expression. Examples include HLA-A*02:82N, HLA-A*23:08N, HLA-A*24:132N, HLA-B*14:07N, HLA-B*39:40N, HLA-B*46:07N, HLA-B*56:190N, or HLA-C*06:49N. In the case of HLA-A*03:03N, a frame deletion is responsible for non-expression (Lienert et al. 1996).

Only four HLA-A alleles (HLA-A*01:01:38L, HLA-A*02:01:01:02L, HLA-A*24:02:01:02L, HLA-A*30:14L) and one HLA-B allele (HLA-B*39:01:01:02L) with low-expression patterns have been identified up to now (Balas et al. 1994; Magor et al. 1997; Laforet et al. 1997; Dunn et al. 2004; Hirv et al. 2006; Perrier et al. 2006). Low expression of these alleles is usually associated with a low expression of the corresponding mRNA. However, the alteration causing the low expression of HLA*A-30:14L is not associated with a reduced mRNA level, but rather seems to result from the loss of the disulfide bond between the cysteine residues at positions 101 and 164 in the α2 domain (Hirv et al. 2006; Hinrichs et al. 2009).

The only soluble secreted allele (S) known so far is HLA-B*44:02:01:02S (Dubois et al. 2004). This HLA-B44 variant was typed as a null allele by microlymphocytotoxicity, whereas the B*44:02:01:01 allele was identified by PCR-SSP. DNA sequencing revealed a single nucleotide difference at the end of intron 4 in the acceptor splicing site, leading to a splicing error characterized by the deletion of exon 5 (transmembrane domain of the HLA antigen).

All known HLA class I Q alleles (7 HLA-A, 9 HLA-B and 8 HLA-C) and the HLA-A*30:14L allele have cysteine residue mutations at amino acid position 101 or 164 affecting the 101/164 disulfide bridge in the α2 domain. Point mutations altering codon 101 have been described for HLA-C*02:25Q and HLA-C*03:22Q (Middleton et al. 2006). In the case of HLA-A*02:293Q, HLA-A*11:50Q, HLA-A*30:14L (Hirv et al. 2006), HLA-A*32:11Q (Tang et al. 2006), HLA-B*15:218Q, HLA-B*35:65Q (Elsner et al. 2006), HLA-B*37:16Q, HLA-B*39:38Q (Tang et al. 2006), HLA-B*40:133Q, HLA-C*04:59Q, HLA-C*07:121Q, HLA-C*12:42Q, HLA-C*15:32Q and HLA-C*16:16Q, point mutations in codon 164 result in a replacement of the Cys residue, causing disruption of the disulfide bond in the α2 domain. HLA-A*30:14L is the only one of these alleles described as having a low expression pattern not affecting the corresponding mRNA levels (Hirv et al. 2006; Hinrichs et al. 2009). There are no known alleles with an amino acid mutation at positions 203 or 259 affecting the bridge in the α3 domain.

2.3 HLA in transplantation

The best donor is an HLA genotypically matched sibling identified by family typing. When no identical sibling donor is available, transplantation of stem cells from an HLA-matched unrelated donor can result in comparable disease–free survival, particularly for good-risk patients (Petersdorf et al. 2004; Petersdorf 2007; 2008). Nevertheless, unrelated transplantation is associated with a higher frequency of post-transplant complications than in genotypically matched sibling HSCT, mainly because of undefined HLA incompatibilities. The negative impact of HLA mismatches on the outcome of hematopoietic stem cell transplantation has been demonstrated in a variety of studies (Mickelson et al. 2000; Ottinger et al. 2003; Schaffer et al. 2003). Most allele mismatches affect differences in the T-cell receptor contact area of the heavy chain or the peptide-binding site causing a change in the peptide binding repertoire both leading to a T cell-mediated allorecognition.

HistoCheck (www.histocheck.org) is an online tool which helps clinicians and researchers visualize the amino acid substitutions of HLA alleles so that they can make informed judgments about their functional similarity (Elsner et al. 2004). Because exact HLA matching is often not possible, it is important to understand which alleles are the most similar. *HistoCheck* provides crystallography-based 3-dimensional (3D) visualizations of the allelic mismatches by highlighting amino acid mismatches, positions, and functions. The user is provided with dissimilarity scores (DSSs) for the amino acids involved as well as an over-all DSS for the two alleles. However, scoring HLA mismatches by HistoCheck has not been shown to predict clinical outcome in unrelated hematopoietic stem cell transplantation.

Several large-scale studies have shown that high-resolution matching of patients and unrelated donors significantly improves post-transplant survival (Bray et al. 2008), the incidence and severity of acute and chronic GVHD (Morishima et al. 2002; Morishima et al. 2007), and engraftment (Petersdorf et al. 2001; Flomenberg et al. 2004; Lee et al. 2007; Petersdorf 2008). Regarding cord blood transplantation, several studies have shown that the degree of HLA match is important as well, but a large cell dose may be at least equally important (Laughlin et al. 2004; Rocha, Sanz, and Gluckman 2004; Arcese et al. 2006; Eapen et al. 2007).

The National Marrow Donor Program (NMDP¸ www.marrow.org) proposed minimum HLA matching requirements for adult donors for HLA-A, -B, -C and -DRB1 (8/8) typed, at high resolution by DNA-based methods and cord blood units (CBU) for HLA-A, -B, (low resolution) and -DRB1 (high resolution) (Table 1) (Bray et al. 2008; Kamani et al. 2008).

Considering HLA allele and haplotype frequencies can be very useful when interpreting typing results and finding appropriate donors. Simply knowing that a patient's haplotype is extremely rare can prevent futile registry searches. Considering allele frequency alone is insufficient, because a rare allele can be acceptable when it is found in its most common haplotype. Being aware of rare alleles and haplotypes is also an important factor in quality control. Furthermore, typing results in registries are often incomplete. In the case where there are two matching donors, but each donor typing is incomplete with respect to different alleles, then haplotype frequencies can help choose the donor who is most likely to be an exact match. To overcome these limitations the new matching algorithm *HapLogic* (www.marrow.org) and *Haplocheck* (www.haplocheck.org) were developed. *HapLogic* a new enhanced matching algorithm that automatically identifies the donors or CBUs with the highest potential to match the patient, was established by the NMDP to accelerate and improve the efficiency of searches. The new matching algorithm analyzes the haplotypes of

millions of donors on NMDP's *Be The Match Registry*. HapLogic uses advanced logic to predict a donor's or CBU's high-resolution match and builds upon mathematical formulas that predict DR match in AB donors (Hurley et al. 2006).

HLA locus	**Tissue type patient?**	**Match donor and patient?**
A	Yes, allele level	Yes
B	Yes, allele level	Yes
C	Yes, allele level	Yes
DRA	No	No
DRB1	Yes, allele level	Yes
DRB3, 4, and 5	Yes (DRB1 association)	Unknown
DQA1	No	No
DQB1	Yes (DRB1 association)	Uncertain
DPA1	No	No
DPB1	No	Uncertain

Table 1. HLA tissue typing recommended by the NMDP (from www.marrow.org) (Bray et al. 2008; Kamani et al. 2008)

The web tool *HaploCheck* is addressing this chance by ranking typing results based upon haplotype frequencies. The user enters the typing results for a patient, for which the cis/trans phase is unknown. The result is a list of separated haplotypes, ordered by frequency. Very rare alleles and associations are highlighted to inform the user of potential problems when searching registries, or to identify potential typing errors. For the case that a single mismatch is unavoidable, the user is presented with a list of mismatch-containing haplotypes and their frequencies. This can not only prevent futile registry searches, but also enable the clinician to make decisions about accepted mismatches before initiating a registry search.

3. Prevalence and allogenicity of HLA class I expression variants

Few investigators have systematically addressed the prevalence of HLA null and alternatively expressed alleles, which has been shown to be about 0.003% and 0.3%, respectively (Noreen et al. 2001; Elsner and Blasczyk 2004; Smith et al. 2005). Considering that most studies indicate that the prevalence of these alleles is around 1 per 1000 individuals, these alleles are not particularly rare. Consequently, it was recommended that laboratories typing unrelated bone marrow patients and donors should have a strategy to identify these expression variants (Elsner and Blasczyk 2004).

HLA null and expression variants are typically identified by the discrepancy between serological and molecular typing results. As molecular typing techniques have nearly displaced serological methods and are focusing on selected regions of the HLA genes many expression variants are likely to be overlooked. In solid organ transplantation, HLA expression variants are not considered in the matching procedure. In allogeneic HSCT, expression variants make an essential difference and can strongly affect transplant-related mortality since HLA mismatches are the major cause of severe GvHD or graft rejection. Thus, in contrast to solid organ transplantation, excluding HLA expression variants is required in the matching process for HSCT (Elsner and Blasczyk 2004; Hirv et al. 2006; Hinrichs et al. 2009).

Overlooking an HLA null allele in the donor would result in a T cell-mediated allorecognition of the recipient's HLA and may lead to the development of acute severe GvHD (Elsner and Blasczyk 2004). In the reverse setting (recipient null allele, donor expressed allele), allogeneic recognition of the recipient's stem cells may lead to their destruction and subsequent graft failure. Accordingly, mismatches between expressed and non-expressed HLA variants should be avoided in HSCT. In case of a recipient with an HLA null allele having no HLA-identical donor with the same null variant, matching must be performed as if the patient would be homozygous for the expressed allele of the respective HLA locus (Figure 1).

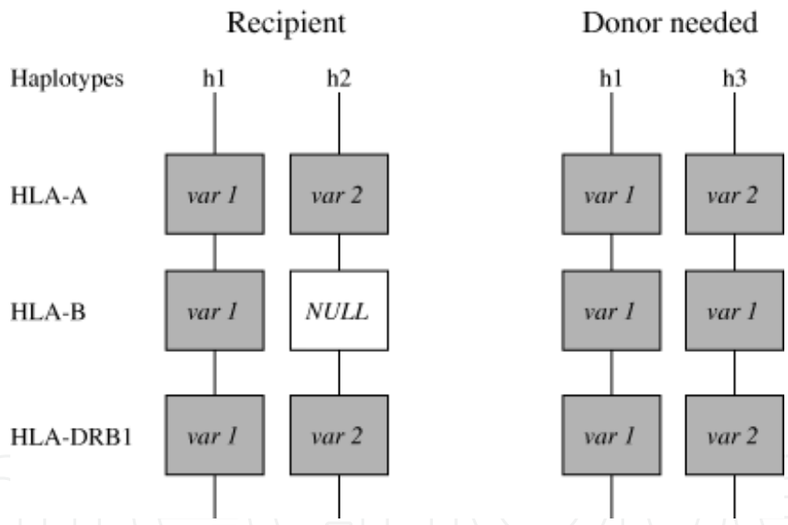

Fig. 1. Haplotypes in a recipient-donor combination with a null allele in one of the recipient's haplotypes. Shadowed boxes indicate normally expressed variants. The recipient carries an HLA-B null allele (white box). In the donor search the recipient's haplotype h2 has to be 'replaced' by haplotype (h3) containing the expressed HLA-B allele (var1). However, such a haplotype may be rare and a matching donor hard to find (Elsner and Blasczyk 2004).

On the other hand, an incomplete HLA molecule may be generated, as has been shown for HLA-B*44:02:01:02S, which might be presented via the indirect allogeneic recognition pathway (Magor et al. 1997; Dubois et al. 2004). Provided that the HLA-derived peptides fit into the peptide-binding groove and are capable of triggering a strong T-cell response, they may act as minor histocompatibility antigens (mHags). This could also apply to those HLA

expression variants where the transcription of a truncated mRNA is known and/or translation is probable. It also shows that premature stop codons do not automatically lead to the interruption of transcription (Balas et al. 1994; Laforet et al. 1997; Magor et al. 1997; Dunn et al. 2004; Hirv et al. 2006; Perrier et al. 2006; Eiz-Vesper, Blasczyk, and Horn 2007). In the light of countless non-HLA mHags this is probably of inferior importance.

4. Characterization of HLA expression variants by cytokine-induced HLA secretion

Because of the clinical importance of expresion variants an HLA secretion assay was designed capable of discriminating between low-expression (L) and non-expressed (N) HLA variant alleles and assigning questionably expressed (Q) alleles to either group (Hinrichs et al. 2009).

All of the aforementioned HLA class I alleles with an unknown expression profile (Q alleles; 7 HLA-A, 9 HLA-B and 8 HLA-C) and HLA-A*30:14L, have a mutation of cysteine residue 101 or 164 affecting disulfide bridge 101/164 in the α2 domain. Because HLA-A*30:14L is the only one of these alleles described to have a low expression pattern with no effect on mRNA levels (Hirv et al. 2006; Hinrichs et al. 2009), A*30:14L was used as an expression model. HLA-A*30:14L was reported to be non-expressed under normal conditions and to show weak aberrant expression after cultivation of the corresponding B-lymphoblastoid cell line at 30°C (Hirv et al. 2006).

HLA-A*30:14L was originally identified in a patient suffering from chronic myeloid leukemia (Hirv et al. 2006). The sequence of this allele is identical to that of HLA-A*30:01 except for a transversion at nucleotide position 563 in exon 3 (guanine to cytosine substitution), resulting in a replacement of cysteine by serine at position 164, impairing disulfide bridge formation in the α2 domain of the mature polypeptide. This alteration of the secondary structure presumably decreases expression, rendering HLA-A*30:14L basically undetectable by serology.

Human cell lines (HEK293, C1R and K562) expressing recombinant soluble HLA (sHLA) molecules (Table 2) were incubated with interferon (IFN)-γ and/or tumor necrosis factor (TNF)-α (Hinrichs et al. 2009). These pro-inflammatory cytokines are known to enhance the expression of HLA molecules by affecting the interaction of DNA-binding proteins with the HLA-A promoter regions, resulting in the increased transcription of heavy and light chain genes (Girdlestone 1996; Gobin et al. 1997; Gobin et al. 1998; Gobin et al. 1999; Johnson 2003). In addition, these cytokines induce the transcription of proteasome subunits, peptide transporters and chaperones that promote the expresson of HLA class I molecules by providing peptides for presentation (Ma et al. 1997; Lankat-Buttgereit and Tampe 2002).

Expression of soluble HLA-A*30:14L and HLA-A*30:01 was measured in the supernatants of transfected and untransfected cells incubated with or without IFN-γ and/or TNF-α using a W6/32 and anti-β2-microglobulin-based sandwich ELISA (Figure 1) (Bade-Doeding et al. 2007). HLA-A*30:14L was not detected in the supernatant of unstimulated transfectants. Stimulation with IFN-γ and/or TNF-α increased HLA-A*30:14L secretion to detectable levels and increased HLA-A*30:01 expression up to 8-fold, but did not result in any difference between mRNA levels of HLA-A*30:14L and A*30:01 (Figure 2).

Day		Expression level (ng/ml)		
		HEK293	C1R	K562
1	HLA-A*30:01	31.3 ± 10.3	98.0 ± 14.7	155.8 ± 73.9
	HLA-A*30:14L	0	3.2 ± 1.7	0
3	HLA-A*30:01	383.2 ± 56.5	225.8 ± 177.5	143.5 ± 40.8
	HLA-A*30:14L	1.8 ± 1.4	0	0
7	HLA-A*30:01	160.4 ± 3.2	253.6 ± 16.1	175.9 ± 74.7
	HLA-A*30:14L	0	9.7 ± 4.9	0

Table 2. Soluble HLA-A*30:01 and HLA-A*30:14L expression levels (ng/ml) in the supernatant of three transfected cell lines (HEK293, C1R and K562) after 1, 3 and 7 days of incubation.

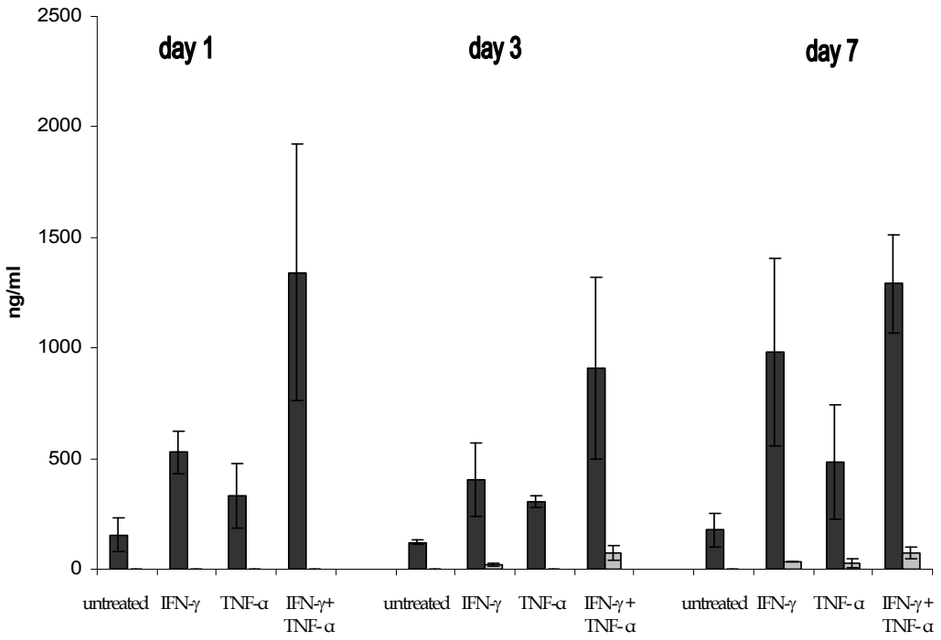

Fig. 2. Secretion of soluble HLA-A*30:01 and HLA-A*30:14L by transfected K562 cells

Expression of mRNA transcripts of both alleles was determined by real-time PCR. For control, Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell line (B-LCL) expressing HLA-A*30:14L was established from cells of the patient's mother (genotype HLA-A*30:14L,*02:01) (Hirv et al. 2006). The positive control was a B-LCL expressing HLA-A*30:01 (genotype HLA-A*30:01,*02:01). In both B-LCLs and HEK293 cells, the mRNA level of HLA-A*30:14L was nearly identical to that of HLA-A*30:01 (Figure 3). This finding suggests that the mRNA transcription rate of sHLA-A*30:14L is not affected by the mutation at nucleotide position 563 (G->C). The mRNA levels of both alleles clearly increased in response to combined stimulation with IFN-γ and TNF-α. In view of this lack of any difference in mRNA transcription, the protein expression defect is most likely caused by the missing disulfide bond in the α2 domain.

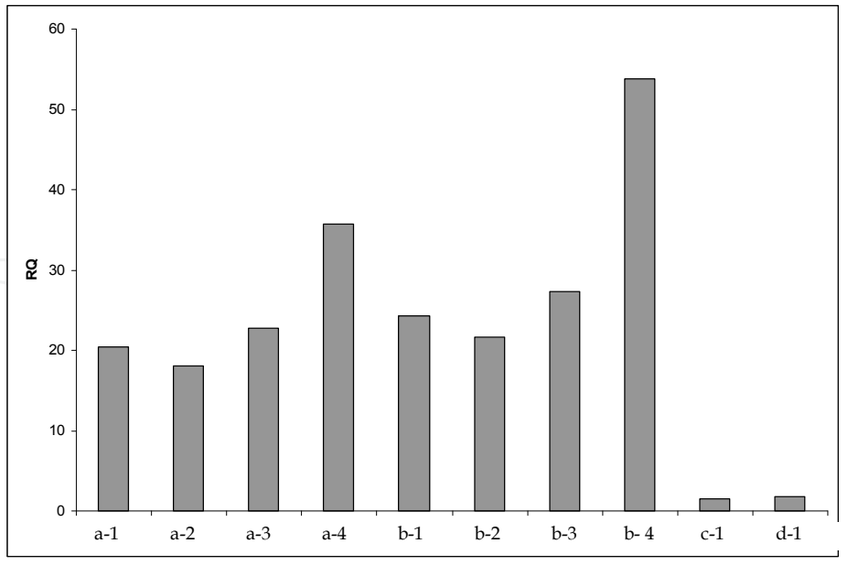

Fig. 3. Detection of mRNA levels of HLA-A*30 alleles in B-LCLs and HEK293 cells

mRNA expression of the HLA-A*30 alleles in transfected cell lines and B-LCLs was determined by real-time PCR. Shown are representative results for HEK293 cells measured after 3 days of culture in the presence ("treated") or absence ("untreated") of the cytokines IFN-γ and/or TNF-α. Data were acquired using a probe specific for the HLA-A30 sequence. Similar results were achieved for all transfected cell lines. Lanes: a) sHLA-A*30:01-transfected HEK293, b) sHLA-A*30:14L-transfected HEK293, c) EBRCC-256 (HLA-A*30:01), d) EBRCC-1818 (HLA-A*30:14L), 1 untreated, 2 IFN-γ-treated, 3 TNF-α-treated, 4 IFN-γ plus TNF-α-treated

The observation that HLA-A*30:14L protein accumulates inside the cells indicates that HLA-A*30:14L translation is not affected. Consequently, the lack of protein secretion in the supernatant is best explained by post-translational instability of the HLA-A*30:14L molecules because of the missing disulfide bridge (Hinrichs et al. 2009). Based on these findings, it is likely that the intracellular enriched HLA-A*30:14L protein is a major substrate for proteasomal cleavage and that it provides a flood of peptide fragments presented to cytotoxic T lymphocytes. As a result of this indirect surface expression by the presentation of peptide fragments, it is possible that GvHD or graft rejection might be promoted in the event of mismatching (Benichou 1999). Consequently, considering HLA-A*30:14L as null allele is, in case of a mismatch with any other HLA-A allele, potentially more dangerous in terms of GvHD and graft rejection than a mismatch with its most related allele HLA-A*30:01. Indeed, mistyping HLA-A*30:14L as an N allele has led to a severe GvHD in a patient transplanted with hematopoietic stem cells from an HLA-A*02:01 homozygous donor (Hirv et al. 2006).

In recent studies, the cytokine-based HLA secretion assay was used to classify the expression patterns of HLA-A*32:11Q (Tang et al. 2006) and HLA-B*35:65Q (Elsner et al. 2006). Both alleles undergo cysteine substitution at amino acid position 164 and thus lack the disulfide bond between the cysteine residues at amino acid positions 101 and 164 in the α2 domain of the mature protein. This interferes with HLA maturation inside the ER and therefore impairs cell surface expression. In concordance with the results of Hinrichs

et al. (Hinrichs et al. 2009), IFN-γ and TNF-α increased the expression of the HLA expression variants, making HLA-A*32:11Q and HLA-B*35:65Q distinctly detectable. Compared to HLA-A*32:01 and HLA-B*35:01, the variants have very weak protein levels, indicating a low expression status. Consequently, they should be handled as low expression variants (L alleles).

5. The nature of peptides presented by HLA class I expression variants

The functional integrity of HLA low-expression variants is a prerequisite for considering them as essential in hematopoietic stem cell donor and recipient matching to diminish the risk of serious complications such as GvHD or graft rejection. HLA class I molecules present endogenous peptides 8-12 amino acids in length to CD8+ cytotoxic T lymphocytes (Natarajan et al. 1999). Most amino acid polymorphisms of different HLA class I molecules are located in the peptide-binding region shaped by parts of the α1 and α2 domains; these polymorphisms determine the characteristics of presented peptides. Peptide motifs have been reported for the most common HLA-A and B alleles and for some rare variants. Importantly, differences in peptide binding among the alleles of a serological group have also been described (Prilliman et al. 1999; Bade-Doeding et al. 2011,). Identification and comparison of allele-specific peptide-binding motifs provide important information for donor-recipient matching and prediction of HLA subtype allogenicity in allogeneic HSCT.

In order to determine the functionality of HLA low-expression alleles, peptides from recombinant truncated HLA-A*30:14L molecules secreted in the supernatant of a human cell line were eluted and sequenced (Hinrichs et al 2010). The suitability of the monoclonal anti-HLA class I antibody W6/32 for purifying recombinant HLA-A*30:14L molecules suggested its proper folding and assembly. Presumably, more soluble HLA-A*30:14L is produced and secreted into the supernatant that might not be correctly folded because of the lack of a disulfide bridge in the α2 domain.

Edman pool sequencing of eluted peptides corroborated the hypothesis that peptides are presented by HLA low expression variants and showed idential peptide motifs in HLA-A*30:01 and HLA-A*30:14L confirming the previously described peptide motif of A*30:01 (Lamberth et al. 2008; Sidney et al. 2008). The C-terminal position (PΩ) was identified as a primary anchor position. The preferred residues of the HLA-A*30 peptide epitopes at this position are lysine (K), valine (V) or arginine (R). The preference for lysine as the top amino acid at the PΩ position of the bound peptides, like described by positional scanning combinatorial peptide libraries (PSCPL) analysis, could be consolidated by the obtained peptide sequence data (Lamberth et al. 2008; Sidney et al. 2008). Position P3 of the peptides was identified as a primary-secondary anchor showing a high preference for the basic amino acids K and R. Six amino acids are reportedly favored at position P2: phenylalanine (F), serine (S), threonine (T), valine (V), isoleucine (I) or leucine (L).

The size of the obtained peptides ranged from 8 to 14 amino acids, but most had a length of 9 to 10 aa. The sequences of 200 HLA-A*30:01 ligands and of 100 HLA-A*30:14L ligands were identified. The following three peptide epitopes (3%) were presented by both HLA-A*30:01 and HLA-A*30:14L: 1) VLDTPGPPV, a nonameric peptide derived from titin (isoform N2-A, aa position 19783-19791), a protein of human muscle ultrastructure and

elasticity; 2) EITALAPSTMK, an 11-mer peptide derived from human muscle protein ACTA1 (actin, alpha 1, skeletal muscle; aa position 301-311); and 3) DNIQGITKPAIR, a 12-mer peptide derived from a histone protein (HIST2H4A; aa position 25-36) (Table 3).

Peptide position	1	2	**3**	4	5	6	7	8	**9**	**10**	**11**	**12**	Source
Ligand	V	L	D	T	P	G	P	P	**V**				Titin (TTN titin isoform N2-A)
	E	I	T	A	L	A	P	S	T	M	**K**		Actin (ACTA1)
	D	N	I	Q	G	I	T	K	P	A	I	**R**	Histone (HIST2H4A)

Table 3. Shared peptide epitopes of HLA-A*30:14L and HLA-A*30:01

To verify the presentation of naturally presented peptides from recombinant HLA-A*30:01/30:14L molecules, peptide binding was analyzed by flow cytometry (Storkus et al. 1993; Zeh et al. 1994; Maeurer et al. 1996) in three EBV-transformed B-LCLs expressing either HLA-A*30:14L,*02:01 (Ulm-241539), HLA-A*30:01,*02:01 (EBRCC-256) or HLA-A*02:01 (EBRCC-2296) (Warburton et al. 1994; Hirv et al. 2006; Hinrichs et al. 2009; 2010). Acid treatment of the cell lines resulted in the dissociation of the naturally bound peptides and the release of β2 microglobulin from the HLA class I heavy chain. The HLA class I molecules were then reconstituted by adding fluorescein isothiocyanate (FITC)-labeled HLA peptide ligands and recombinant β2 microglobulin. The synthetic FITC-labeled peptide EITALAK(FITC)PSTMK (HLA-A*30:01/30:14L) and the immunodominant HLA-A*02:01-restricted CMVpp65$_{495-503}$ peptide (NLVPMK(FITC)VATV) were used. Reconstitution of HLA with the HLA-A*30 ligand mounted up to 51% (Ulm-241539) and 74% (EBRCC-256), respectively, compared to 25% for the HLA-A*02:01 homozygous cell line (EBRCC-2296). Binding on cells expressing the normal HLA-A*30:01 allele was higher than on those expressing HLA-A*30:14L, the low expression variant (Figure 4). The results confirm that the A*30 peptide previously isolated binds to HLA-A*30 on the cell surface. Peptide binding was found for the A*30:01 specific peptide on the HLA-A*30:14L-expressing cell line (Ulm-241539), indicating the stability of HLA-A*30:14L cell surface expression.

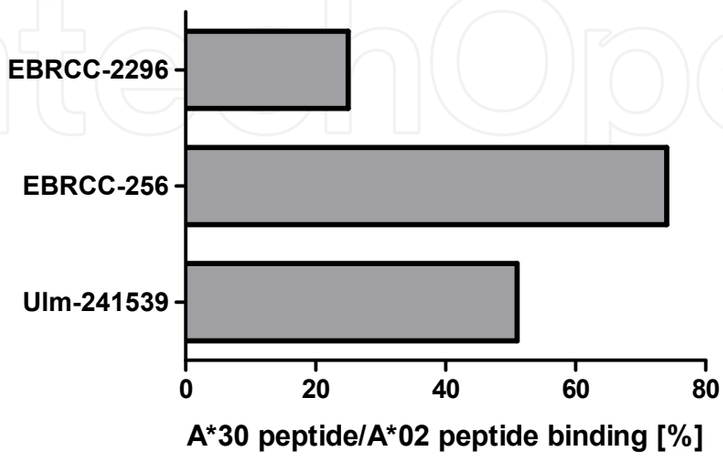

Fig. 4. Relative A*30/A*02:01 peptide-binding intensities for different HLA-expressing B-LCLs

Homology-based modeling for each HLA-A*30 alleles with the shared 9, 11 and 12-mer peptide epitopes revealed only marginal differences between the two HLA-A*30 alleles. The HLA-A*30:01 and HLA-A*30:14L models were essentially identical with the Cys164 Ser substitution, but simply adopted an alternate rotamer conformation upon breakage of the disulfide bond. Therefore, only the HLA-A*30:14L model is illustrated (Figure 5).
Although the models look identical and the alleles appear to bind identical peptides, the Cys164Ser variation could potentially generate additional flexibility within the peptide-binding groove, thereby influencing binding kinetics, particularly in peptides of lower affinity. Such an effect could stimulate a T-cell immune response and have serious implications in allogeneic HSCT.

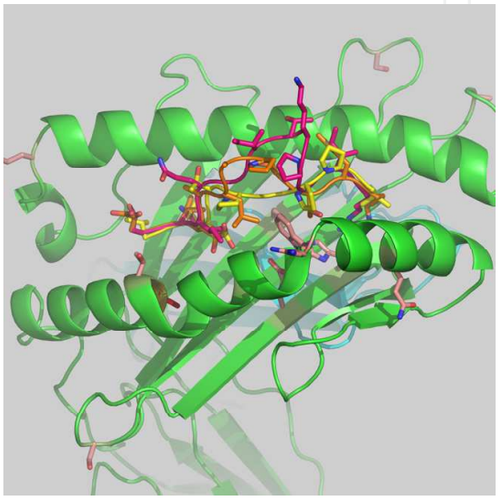

Fig. 5. Homology-based model of HLA-A*30:14L with the three shared peptide ligands

Modeling of the HLA-A*30:01 and HLA-A*30:14L structures was carried out using the SCWRL homology-based modeling server (Wang, Canutescu, and Dunbrack 2008) while employing the crystal structure of the closely related HLA-A*11:01 (1Q94) as a template. Peptide templates for 9-mer (1Q94), 11-mer (2BVO) and 12-mer (3BW9) were superimposed and merged with the HLA-A*30:14L model. Peptide mutagenesis was then performed using DeepView (Guex and Peitsch 1997) and the rotamer library to find the best side chain orientations with minimum steric clashes. Each model was then subjected to energy minimization using DeepView software. The graphics program PyMOL (http://www.pymol.org) was used to generate the structural models.

6. Conclusions

Since HLA mismatches are the main cause of severe GvHD and graft rejection, misinterpretation of HLA null alleles and expression variants as irrelevant could strongly affect transplant-related mortality.
The cytokine-based HLA secretion assay can be used to distinguish between low-expressed and non-expressed HLA alleles in order to classify alleles with a currently undefined expression status (questionable alleles, Q) as well as to re-classify certain alleles which have been assigned as null variants (N). Additionally, discrimination between cytokine inducible and non-inducible defect alleles may be important in allotransplant settings in which a

cytokine storm usually occurs following pre-transplant myeloablative conditioning or post-transplant immunosuppressive therapy.

The fact that the monoclonal anti-HLA class I antibody W6/32 is a conformational antibody implies that only correctly folded, β2 microglobulin-assembled and peptide-loaded MHC complexes can be detected. This suggests that parts of soluble HLA-A*30:14L molecules are assembled correctly and secreted by transfectants. Presumably, more soluble HLA-A*30:14L is produced and secreted into the supernatant, but it might not be correctly folded because of the lack of the disulfide bridge in the α2 domain. Therefore, these molecules are not detected by the conformational anti-HLA-ABC mAb. This assumption arose after comparing mRNA and associated protein levels of HLA-A*30:14L and HLA-A*30:01 alleles (Hinrichs et al. 2009). Additionally, it was found that HLA-A*30:14L accumulates inside the cells; therefore, it might be a major substrate for proteasomal cleavage and could provide a flood of peptide fragments presented to cytotoxic T lymphocytes. As a result of this indirect allorecognition pathway, GvHD or graft rejection might be promoted in the event of a severe mismatch.

It was shown for the first time that an HLA low expression allele (HLA-A*30:14L) presents peptides with identical features to those of its most closely related relative, HLA-A*30:01 (Hinrichs et al. 2010). The results indicate that a mismatch at amino acid position 164 might be permissive. Therefore, mismatching of these alleles will presumably be of low allogenicity in allogeneic HSCT. The fact that a low expression variant is not only functional and able to present peptides, but also shares epitopes with its related variant leads to the conclusion that low expression variants need to be considered in donor selection as permissive or non-permissive mismatches, respectively. Increasing knowledge of the expression behavior of HLA expression variants, such as L and Q alleles, will help to improve HLA allogenicity prediction algorithms by delivering proof that these variants are fully functional. Taking all relevant factors into account, the results shown allow to predict the immunogenicity of aberrantly expressed alleles in a transplant setting.

In the case of HLA-A*30:14L misinterpreting it as a null allele is, in case of a mismatch with any other HLA-A allele, potentially more dangerous in terms of GvHD and graft rejection according to the direct and indirect allo-recognition pathway than a mismatch with its most related allele HLA-A*30:01. Indeed, mistyping HLA-A*30:14L as an N allele has led to a severe GvHD in a patient transplanted with hematopoietic stem cells from an HLA-A*02:01 homozygous donor (Hirv et al. 2006).

In order to predict the relevance of similar alleles with disulfide bridge rearrangements (e.g., HLA-A*32:11Q and B*35:65Q) in allogeneic HSCT, it is important to know their surface expression as well as their peptide binding of HLA variants. From a clinical perspective, HLA variants with similar disulfide bridge variations need to be considered as functionally active in an allogeneic HSCT setting as long as the opposite has not been shown.

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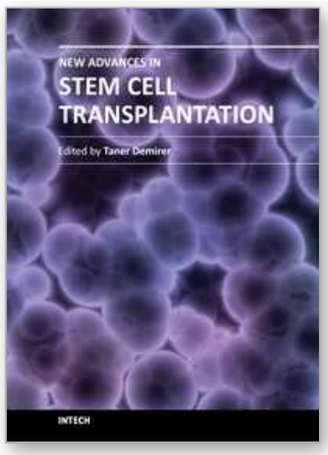

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This book documents the increased number of stem cell-related research, clinical applications, and views for the future. The book covers a wide range of issues in cell-based therapy and regenerative medicine, and includes clinical and preclinical chapters from the respected authors involved with stem cell studies and research from around the world. It complements and extends the basics of stem cell physiology, hematopoietic stem cells, issues related to clinical problems, tissue typing, cryopreservation, dendritic cells, mesenchymal cells, neuroscience, endovascular cells and other tissues. In addition, tissue engineering that employs novel methods with stem cells is explored. Clearly, the continued use of biomedical engineering will depend heavily on stem cells, and this book is well positioned to provide comprehensive coverage of these developments.

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