Position 156 influences the peptide repertoire and tapasin dependency of human leukocyte antigen B*44 allotypes

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ABSTRACT

Background
Polymorphic differences between donor and recipient human leukocyte antigen class I molecules can result in graft-*versus*-host disease due to distinct peptide presentation. As part of the peptide-loading complex, tapasin plays an important role in selecting peptides from the pool of potential ligands. Class I polymorphisms can significantly alter the tapasin-mediated interaction with the peptide-loading complex and although most class I allotypes are highly dependent upon tapasin, some are able to load peptides independently of tapasin. Several human leukocyte antigen B*44 allotypes differ exclusively at position 156 (B*44:02^{156Asp}, 44:03^{156Leu}, 44:28^{156Arg}, 44:35^{156Glu}). From these alleles, only the high tapasin-dependency of human leukocyte antigen B*44:02 has been reported.

Design and Methods
We investigated the influence of position 156 polymorphisms on both the requirement of tapasin for efficient surface expression of each allotype and their peptide features. Genes encoding human leukocyte antigen B*44 variants bearing all possible substitutions at position 156 were lentivirally transduced into human leukocyte antigen class I-negative LCL 721.221 cells and the tapasin-deficient cell line LCL 721.220.

Results
Exclusively human leukocyte antigen B*44:28^{156Arg} was expressed on the surface of tapasin-deficient cells, suggesting that the remaining B*44/156 variants are highly tapasin-dependent.
Our computational analysis suggests that the tapasin-independence of human leukocyte antigen B*44:28^{156Arg} is a result of stabilization of the peptide binding region and generation of a more peptide receptive state. Sequencing of peptides eluted from human leukocyte antigen B*44 molecules by liquid chromatography-electrospray ionization-mass spectrometry (LTQ-Orbitrap) demonstrated that both B*44:02 and B*44:28 share the same overall peptide motif and a certain percentage of their individual peptide repertoires in the presence and/or absence of tapasin.

Conclusions
Here we report for the first time the influence of position 156 on the human leukocyte antigen/tapasin association. Additionally, the results of peptide sequencing suggest that tapasin chaperoning is needed to acquire peptides of unusual length.

Key words: HLA polymorphism, peptide-loading complex, tapasin, peptide-binding motif.

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The online version of this article has a Supplementary Appendix.

Introduction

Following hematopoietic stem cell transplantation or solid organ transplantation, polymorphic differences between donor and recipient human leukocyte antigen (HLA) class I molecules can lead to transplant rejection via graft-*versus*-host disease.[1] Extensive clinical data have demonstrated that the risk of graft-*versus*-host disease strongly correlates with the number of HLA mismatches and that both the type of amino acid (AA) substitution and location within the HLA molecule can directly influence transplantation outcome.[2] Certain polymorphisms within the peptide-binding region (PBR) of HLA class I molecules can, therefore, influence which allogenic peptides are selectively bound and subsequently recognized as self or non-self by the effector T-lymphocytes that survey HLA-peptide complexes on antigen-presenting cells.

The loading of optimal peptides into empty HLA class I molecules is a complicated process and is reviewed in more detail by Wearsch *et al.*[3] Initially, proteasomally digested peptides are transported into the endoplasmic reticulum via the transporter associated with antigen processing (TAP) and are then loaded onto HLA class I molecules with the assistance of the peptide-loading complex (PLC). The transmembrane glycoprotein tapasin functions within this multimeric PLC as a disulfide-linked heterodimer with the thiol oxidoreductase ERp57,[4] to stabilize the empty class I molecule and promote the selection of high affinity peptides.[5] Certain HLA class I polymorphisms within the PBR appear not only to influence the peptide repertoire, but also to determine the requirement for the PLC-mediated acquisition and optimal loading of peptides for the given HLA class I molecule.[6] Whereas most class I allotypes associate strongly with the PLC and are highly dependent upon tapasin for the effective presentation of high affinity peptides and cell surface expression, others can acquire peptides without assistance from the PLC but are then suboptimally loaded.[7] The role of the PLC and tapasin in optimizing and loading potential ligands does, therefore, appear to be critical for the immunorecognition of pathogens. Many viruses have developed strategies to perturb the association of HLA class I molecules with the PLC or PLC function itself to avoid immune recognition. For example, the US3 protein from human cytomegalovirus retains tapasin-dependent molecules within the endoplasmic reticulum thus preventing the presentation of allogeneic/immunogenic epitopes to the immune system, while tapasin-independent molecules remain unaffected.[8]

Approximately 25% of the Caucasian population possess alleles of the HLA-B*44 type. This allelic group can be further distinguished into subtypes based on the AA at position 156 (B*44:02^{156Asp}, 44:03^{156Leu}, 44:28^{156Arg}, 44:35^{156Glu}), located in the center of the α2 domain of the PBR. AA 156 is part of pockets D and E within the PBR and binds the peptides at position 3 and 7.[9] AA 156 also has a structural role by influencing the conformation of the PBR. This was demonstrated in the crystal structures of HLA-B*44:02 and B*44:03 with the same epitope.[10,11] The peptide-binding motifs have also been determined for each of these allotype.[12] Clinically, HLA-B*44:02 and B*44:03 alleles are known to represent a non-permissive transplantation scenario[13] and are associated with strong alloreactive T-cell responses and acute graft-*versus*-host disease. Furthermore, substitutions at position 156 were demonstrated to modify T-cell alloreactivity *in vitro* for HLA-A2 subtypes[14-16] and HLA-B35[17,18] while polymorphism at this position also appears to be responsible for acute graft-*versus*-host disease in HLA-C mismatched donor-recipient pairs.[19]

Position 156 is known to be one of the most non-permissive transplantation mismatches. Despite this, certain alleles that differ at position 156 also appear to share the same peptide-binding motif (e.g. B*44:02^{156Asp} and B*44:03^{156Leu}). It has also been demonstrated that the resulting mismatch leads to disparity in the derived peptide repertoire and subsequently in the cytotoxic T-lymphocyte recognition of the different peptide-HLA landscapes.[10] The obvious question is: if position 156 is involved in the PLC/HLA association, does polymorphism at position 156 affect tapasin dependency by influencing the structure and property of the PBR and subsequently the peptide repertoire? We, therefore, sought to investigate the mode of peptide loading for the B*44/156 mismatched variants.

Design and Methods

Design of lentiviral vectors

For surface expression of full length HLA-B44, cDNA from an HLA-B*44:02 positive donor (exon 1 through exon 7) was amplified by polymerase chain reaction (PCR) using the primers HLA-B1-TAS (5` GA-GATGCGGGTCACGGCG 3`) and HLA-B-TAAS-E7 (5` TCAAGCTGTGAGAGACACATCAG 3`). The PCR product was ligated into the eukaryotic expression vector pcDNA3.1V5/His using the pcDNA3.1V5/His TA-cloning Kit (Invitrogen, Karlsruhe, Germany). The recombinant pcDNA3.1V5/His/B*44:02 vector was then used as a template for the lentiviral pRRL.PPT.SFFV.mcs.pre/B*44:02 vector. The HLA-B*44:02 insert was ligated into the pRRL.PPT.SFFV.mcs.pre vector and the ligation was verified by sequencing using an ABI PRISM 377 sequencer (Applied Biosystems, Darmstadt, Germany). All HLAB*44/156 variants used in this study were produced by site directed mutagenesis using the Quik-Change Multi Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) with the pRRL.PPT.SFFV.mcs.pre/B*44:02 vector as the template.

For expression of truncated HLA-B44 variants, cDNA (exon 1 through exon 4) from an HLA-B*44:02 positive donor was amplified by PCR using the primers HLA-B3-TAS (5' gag atg cgg gtc acg gca c 3') and HLA-E4-WAS (5' cca tct cag ggt gag ggg ct 3'). Lentiviral pRRL.PPT.SFFV.mcs.pre vector was generated by following the cloning procedure described above.

Tapasin silencing was carried out by cloning stable short-hairpin RNA (shRNA) expression cassettes into the lentiviral expression vector pLVTHm/si containing enhanced green fluorescent protein (GFP) as the reporter gene.

Production of lentiviral particles in HEK293T cells

HEK293T cells were transfected by adding 1 mg/mL polyethyleneimine (Sigma Aldrich Chemie Gmbh, Munich, Germany) in DMEM medium (Invitrogen) followed by incubation for 20 min before adding the plasmids to 6×10^6 HEK293T cells (10 μg of transfer vector, packaging plasmid: 5 μg psPAX2; envelope plasmid: 5 μg pDM2G). The medium was changed 24 h and 48 h post-transfection, virus-containing supernatant was removed, passed through a 0.45 μm filter (Millipore GmbH, Schwalbach, Germany) and concentrated by centrifuging overnight at 16°C at 10,000 rpm. The lentiviral pellet obtained was dissolved in RPMI 1640 (Invitrogen).

Lentiviral particles for tapasin silencing were produced by transfecting 5×10^6 HEK293T cells with 10 μg of plasmid encoding for tapasin-specific shRNA, 9 μg of plasmid psPAX2 and 3 μg of

pMD2G. pLVTHm/si, psPAX2, and pMD2G were provided by D. Trono (Lausanne, Switzerland). Following exchange of the medium, supernatant containing lentiviral vector was collected, filtered and concentrated by centrifuging overnight at 16°C at 10,000 rpm. The lentiviral pellet was resuspended in RPMI 1640 medium (Invitrogen).

Transduction of cells from B-lymphoblastoid cell lines

Cells from the lymphoblastoid cell lines (LCL) 721.220 (HLA^-, $tapasin^-$) and LCL 721.221 (HLA^-, TPN^+) were lentivirally transduced by adding the dissolved lentiviral-pellet to the cells in the presence of 8 μg/mL protamine sulfate (Sigma-Aldrich) followed by incubation for 8 h. Transduced B cells were cultured in complete RPMI 1640 media.

Analysis of HLA-B44 surface expression on cells from B-lymphoblastoid cell lines

Cell surface expression of HLA-B*44 was assessed by flow cytometry using an anti-Bw4-FITC labeled antibody (OneLamda, BmT GmbH, Meerbusch-Osterath, Germany). The cells were washed twice with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and then incubated with 10 μL of the antibody stock for 20 min at 4°C. The cells were washed twice with PBS/0.5% BSA and then analyzed using FacsCanto A (BD Biosciences, Heidelberg, Germany).

Real time polymerase chain reaction for analysis of mRNA levels

The expression levels of B*44-specific mRNA or tapasin-specific mRNA after silencing were confirmed by real time PCR. Following transduction, total RNA was isolated from the recombinant B-LCL cells (RNeasy mini kit, Qiagen, Hilden, Germany). RNA was reverse transcribed to cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR for HLA-B44 or tapasin was performed using the ONEStep Plus real-time PCR system (Applied Biosystems). The relative C_T method (described in User Bulletin N. 2, ABI PRISM 7700 Sequence Detection System, pp. 11–15) as implemented in the Steponeplus software was used to calculate the relative mRNA level of the target gene normalized to β-actin in each sample. All values are expressed as fold-changes relative to the appropriate untreated cell controls (set to 1.0) and are representative of more than one experiment.

Verification of soluble HLA expression

Expression of V5-tagged, soluble HLA-B44 (sHLA) molecules was quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) in which anti-HLA-A-B-C W6/32 (Serotec, Düsseldorf, Germany)[20,21] and anti-V5 (Invitrogen) monoclonal antibodies were employed as capture antibodies. Horseradish peroxidase (HRP)-conjugated anti-β2 microglobulin monoclonal antibody (DAKO, Hamburg, Germany) served as the detection antibody. B-LCL clones with the highest sHLA expression were used for large-scale production.

Large-scale production of soluble HLA molecules and affinity purification

sHLA-producing clones were cultured and expanded in bioreactors *CELLine* (Integra, Fernwald, Germany). The conditioned media were first centrifuged for 20 min at 1,200 rpm to remove cell debris and the supernatant was then filtered through a 0.45 μm filter (Sartorius, Göttingen, Germany) and stored at –20°C. The supernatants were thawed, adjusted to pH 8.0 and sHLA-B44 purified using N-hydroxysuccimide-activated *HiTrap* columns coupled with monoclonal antibody W6/32, using a BioLogic DuoFlow system (Bio-Rad, Hercules, USA). Trimeric complexes (class I heavy chain, β2 microglobuin and peptide) were eluted using 0.1 M glycine/HCl buffer (pH 2.7).

Characterization of HLA-B*44-derived peptides

To distinguish between peptides of low and high affinities, purified trimeric elution fractions were filtered (10 kD MWCO; Millipore, Schwalbach, Germany) and the peptides detected in the flow-through were considered to be of low affinity. The retentate containing trimeric complexes was then treated with 0.1% trifluoroacetic acid (TFA) to elute the high affinity peptides from sHLA-B44 complexes. The peptides were then separated by filtration through a 10 kD MWCO YM membrane (Millipore).

Flow-through fractions containing the low or high affinity peptides were subjected to mass spectrometry using an Eksigent nano-LC Ultra 2D HPLC coupled to an Orbitrap ion trap (Thermo Fischer, Waltham, Massachusetts, USA) providing a very high mass accuracy (< 5 ppm). Database queries were carried out using Mascot software[22] incorporating the IPI human and the respective decoy databases.

Computation analysis

The HLA-B*44 mutants was modeled using the 1M6O structure[10] as a template and mutating 156Asp to the other 19 amino acids. Modeling was performed using DeepView[23] and the internal rotamer library to find the best side chain orientations with minimum steric clashes. Each model was then subjected to energy minimization as implemented in DeepView. The graphics program PyMOL (*http://www.pymol.org*) was used to generate all structure figures.

Antibodies used for immunprecipitation and western blotting

Rabbit polyclonal antibodies against TAP1 (#ADI-CSA-620), ERp57 (#ADI-SPA-585) and tapasin (#ADI-CSA-625J) were purchased from Enzo Life Sciences GmbH (Lörrach, Germany). Rabbit polyclonal calreticulin antibody (#ABR-01176) was purchased from Dianova GmbH (Hamburg, Germany) and rabbit polyclonal ERAP1 antibody (#PA5-15021) from Thermo Scientific (Bonn, Germany). Mouse anti-V5 HRP-conjugated antibody (#MCA1360P) recognizing the C terminal V5 epitope of the soluble HLA-B*44 molecules was purchased from ABD Serotec (Düsseldorf, Germany). Unconjugated primary antibodies were detected using goat-anti rabbit HRP-conjugated secondary antibody (#166-2408EDU) (Bio-RAD, Munich, Germany).

Western blotting

To detect components of the PLC, LCL 721.221 cells were washed twice with PBS, centrifuged at 1,200 rpm for 5 min and the cell pellets were resuspended in PBS. Sodium dodecylsulfate (SDS) sample loading buffer (Invitrogen) and reducing agent (Invitrogen) were added and the samples were boiled at 95°C for 10 min. The proteins were separated by 4-12% SDS-polyacrylamide gel electrophoresis (PAGE) (Invitrogen) and electrophoretically transferred to PVDF membrane (0.45 μm pore size, Invitrogen). Non-specific binding sites were blocked by incubating the membranes for 1 h in 3% skimmed milk powder in PBS. Following this, the blots were incubated for 1 h at room temperature with rabbit polyclonal primary antibodies against TAP1, tapasin, ERp57, CRT and ERAP1 proteins. The primary antibodies were diluted in PBS containing 3% skimmed milk powder. The blots were washed twice with PBS-T (0.05% Tween). Bound antibodies were detected by incubating the blots with HRP-conjugated secondary antibody diluted in blocking solution, followed by a washing step with PBS-T (0.05% Tween). Protein bands were

visualized using TMB blotting substrate (KEM-EN-TEC Diagnostics, Köln, Germany).

Immunoprecipitation

To verify that sHLA molecules are associated with the PLC, immunoprecipitation was performed. In brief, 5×10^6 cells were washed twice with PBS and centrifuged at 5,500 rpm for 5 min at 4°C. The cell pellet was resuspended in 200 μL lysis buffer containing 1% (w/v) digitonin in PBS and Complete EDTA-free Protease Inhibitor (Merck, Darmstadt, Germany). The cells were lysed for 30 min on ice and then centrifuged at 13,000 rpm for 15 min at 4°C. Following centrifugation, the supernantant was precleared by incubating for 1 h with protein-A sepharose beads (GE Healthcare Europe GmbH, Munich, Germany) and then immunoprecipitated for 1 h with protein-A sepharose beads covalently coupled to TAP1 rabbit polyclonal antibody at 4°C. The beads were washed twice with 1 mL of ice-cold 0.1% digitonin in PBS, once each with 1 mL of 0.1% digitonin/450 mM NaCl, 10 mM Tris (pH 7.4) and 10 mM Tris (pH 7.4). Bound proteins were eluted by boiling the beads with SDS sample loading buffer (without reducing agent) at 100°C for 10 min. Proteins were separated by 4-12% Bis-Tris SDS-PAGE and electrophoretically transferred to PVDF membrane (0.45 μm pore size, Invitrogen). Membranes were incubated for 1 h in blocking solution [3% (w/v) skimmed milk powder in PBS] at room temperature. The blots were incubated for 1 h with peroxidase-conjugated (Thermo Scientific) primary antibodies to tapasin, CRT, ERp57 and soluble MHC class I heavy chain diluted in blocking solution. Following this, the membranes were washed three times with PBS/0.05% Tween for 10 min each. Bands were visualized using Roti-Lumin substrate (Roth, Karlsruhe, Germany).

Results

Effect of tapasin on the surface expression of B*44/156 variants

In LCL 721.221 cells, B*44-specific mRNA transcripts for all of the transduced B*44/156 variants could be detected by real-time PCR. Flow cytometric analysis showed that all the transduced B*44/156 variants, with the exception of B^*44^{156Gly}, were expressed on the cell surface (Figure 1A,C). It is unclear why the 156Gly variant could not be expressed on the surface of LCL 721.221 cells. However, it seems likely that this substitution might impair the correct folding of the molecule and hence its surface expression.

In LCL 721.220 cells lacking tapasin, B*44-specific mRNA transcripts could be detected in all recombinant B*44/156 variants. Flow cytometric analysis showed the absence of B*44 surface expression on all of the transduced B*44/156/LCL 721.220 variants with the exception of B^*4428^{156Arg} (Figure 1), illustrating that only B^*4428^{156Arg} can be considered as a tapasin-independent allele. The absence of the B*44 molecules on the surface of LCL 721.220 cells is attributed to a disrupted assembly of the HLA molecules with peptides.

Silencing of tapasin expression in recombinant B*44:02- or B*44:28-expressing LCL721.221 cells

To validate that the surface expression of HLA-B*44 variants in LCL 721.221 cells is due to the presence of tapasin, we performed a reciprocal experiment by silencing tapasin in recombinant LCL 721.221 cells expressing the respective HLA-B*44 variants. We constructed a VSV-G pseudotype lentiviral vector for the stable delivery of a tapasin-specific shRNA or a non-specific shRNA as a control.

These vectors also encoded enhanced GFP as a reporter gene. A mean of more than 75% GFP-expressing cells was obtained after transduction of the LCL 721.221 cells expressing HLAB*44:02 or B*44:28 on their surface. Two weeks after transduction, real-time PCR demonstrated a reduction of as much as 95% of tapasin mRNA levels in LCL721.221/B*44:02 or LCL721.221/B*44:28 cells treated with shRNA specific for tapasin as compared to the tapasin mRNA levels in non-transduced cells. Accordingly, a reduction in HLA class I surface expression of as much as 90% was observed in the LCL721.221/B*44:02 cells expressing tapasin-specific shRNA, as demonstrated by flow cytometry (Figure 2). No reduction of HLA class I surface expression was observed for the LCL721.221/B*44:28 cells, suggesting that the B*44:28 variant could be expressed on the cell surface without the assistance of tapasin.

Peptide features and peptide repertoire

The mass-spectrometer used for this study (LTQ Orbitrap) has a very high mass accuracy in the range of 10 ppm. To maximize the probability of peptide identification, we selected only those peptides that had a delta value of 0.0 Da and excluded all other peptides. sHLA-B*44:02 molecules were expressed in LCL 721.221 cells (HLA^-/$tapasin^+$) and their derived peptides were distinguished as low or high affinity peptides (see *Design and Methods*). A total of 17 peptide sequences of low binding affinity are given in *Online Supplementary Table S1A* while 194 peptide sequences of high affinity are given in *Online Supplementary Table S1B*. Interestingly, some peptides of a non-canonical length (13-19 AA) were recovered from B*44:02 after TFA treatment and these were subsequently sequenced.

Another interesting finding is that no low binding peptides were recovered from sB*44:28/LCL 721.221 cells (HLA^-/$tapasin^+$). However, after acidification (TFA treatment), 79 peptides could be identified (*Online Supplementary Table S1C*). The profile of tapasin-independently loaded peptides derived from sHLA-B* 44:28 molecules expressed in LCL 721.220 cells (HLA^-/$tapasin^-$) shows 24 peptides of low affinity and 44 peptides of high affinity (*Online Supplementary Table S1D, E*).

Peptides shared between the sHLA alleles that selected peptides from different sources (LCL 721.221 or LCL 721.220 cells) and loaded the peptides through different pathways (with or without the assistance of tapasin) are presented in Table 1. Sixteen such shared peptides were defined.

Additionally, six proteins showed differential processing, such as N- or C-terminal alteration of their processed peptides and subsequent differential selection by the investigated B*44 variants (Table 2).

Distribution and frequency of amino acids in the HLA-B*44 derived peptides

Comparison of the frequency of given amino acids present at P1-7 and at the C-terminus of the eluted peptides demonstrated that the glutamate anchor residue at P2 was conserved between the two allotypes (Figure 3). Although sHLA-B*44:28 favored bulky hydrophobic residues at the C-terminal, there was a complete absence of phenylalanine residues and tryptophan and tyrosine residues were preferred in the absence of tapasin. In contrast, sHLAB*44:02 molecules in the presence of tapasin showed a preference

for phenylalanine at the C-terminal. Although the 156Arg was able to contact the peptide at position 5, there were no distinct differences in the proportion of amino acids selected at this position, consistent with an interaction with the main chain.

Computational analysis

To investigate the structural basis of tapasin-dependency on AA position 156 within the HLA heavy chain, HLA-B*44 mutants were generated using the 1M6O structure[10] as a template in which residue 156Asp was mutated to the other 19 amino acids (Figure 4). From these models, it is clear that only 156Lys and 156Arg substitutions appear long enough to be able to contact the main-chain carbonyl group at P5 of the peptide. The modeling illustrates that the lysine NZ is 3.2Å from the P5 carbonyl oxygen. In comparison the substitution of arginine at this position shows distances of 2.9 Å and 3.3 Å for the NH1 and NH2 amino groups to the P5 carbonyl oxygen. Furthermore, the NE of the arginine is only 3 Å away from the OD2 carboxyl side

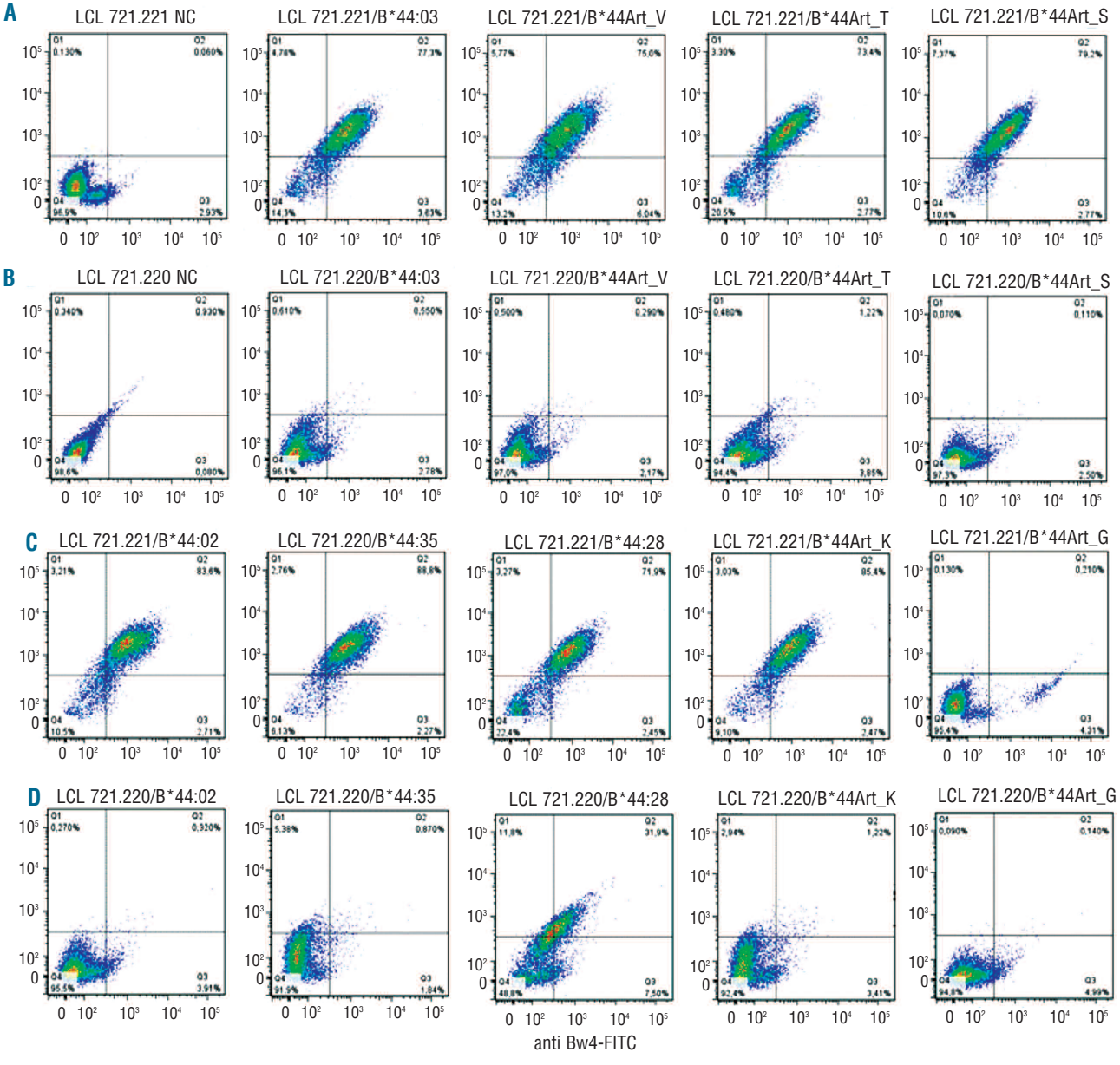

Figure 1. HLA-B*44 expression on the surface of LCL 721.221 and LCL 721.220 cells. Expression of B*44/156 variants in 721.221 (Tapasin+) and 721.220 (Tapasin-) cells. Flow cytometric analysis for cells stained with anti Bw4-FITC and w6/32-PE labeled monoclonal antibodies. An example for all 20 AA that were exchanged at position 156, here we show the FACS plots for two AA of each group (representative of three separate experiments). (A) and (B) show the FACS plots for the non-polar hydrophobic AA - 156Leu (represented by B*44:03), 156Val (artificial B*44/156 molecule) and polar neutral AA - 156Thr, 156Ser (artificial B*44/156 molecules) in 721.221 and 721.220 cells, respectively. (C) and (D) show the FACS analysis for the acidic AA - 156Asp (represented by B*44:02), 156Glu (represented by B*44:35) and for basic AA 156Arg (represented by B*44:28), 156Lys in 721.221 and 721.220 cells, respectively. All the natural and artificial B*44/156 molecules with the exception of 156Gly were expressed on the surface of LCL 721.221 cells. Only B*44:28^{156Arg} was exclusively expressed on the surface of LCL 721.220 cells lacking tapasin.

chain of 114Asp forming an additional and perhaps critical contact that maintains stability, forming a network of hydrogen-bonded residues (156Arg, 114Asp and 97Arg) which tethers the α-2 helix to the floor of the PBR. The modeled AA at position 156 clearly support the *in vitro* results by showing that 156Arg for B*44:28 would increase the stability of the binding cleft and enable non-optimized peptides to form peptide-HLA complexes that are stable and able to reach the cell surface.

Lymphoblastoid cell line 721.221 cells contain all of the minimum essential components of the peptide-loading complex

We performed western blots to confirm that LCL 721.221 cells express all of the minimum components required for peptide loading, using lymphoblastoid B cells from a healthy blood donor as a positive control. We demonstrated that LCL 721.221 cells express ERAP1, TAP, tapasin, ERp57 and CRT (Figure 5A).

*Soluble HLA-B*44 molecules are associated with the peptide-loading complex*

We performed immunoprecipitation and western blots to demonstrate the association of sHLA-B*44 molecules with the PLC in our transduced cells. Lymphoblastoid B cells from a healthy blood donor were used as a positive control for the components of the PLC, while LCL 721.221 cells were used as a negative control. LCL 721.221 cells expressing sHLA-B*44 molecules with a C-terminal V5-tag were then compared. Cell lysates were first immunoprecipitated with an anti-TAP1 antibody covalently conjugated to protein-A-sepharose beads and bound proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Subsequent immunoblotting of the membranes with HRP-conjugated antibodies specific to individual proteins of the PLC confirmed that V5 tagged recombinant sHLA*B44 molecules are associated with the PLC components (tapasin, ERp57 and CRT) (Figure 5B).

Discussion

The function of tapasin is to stabilize the PBR of the HLA molecule against irreversible denaturation and to maintain it in a peptide-receptive state before peptides are selected and loaded.[3] Our data presented here demonstrate that only the HLA-B*44:28^{156Arg} variant can acquire peptides independently of tapasin and that AA position 156 is unambiguously responsible for the HLA/tapasin interaction within B*44 subtypes. Research to date has shown

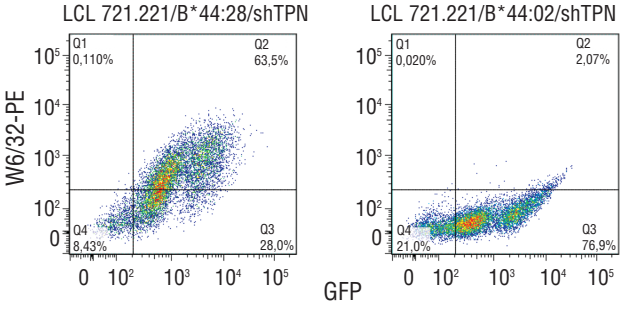

Figure 2. Tapasin (TPN) silencing results in the lack of surface expression of B*44:02 but not B*44:28 on LCL 221.721 cells. Surface expression of HLA-B*44:02 and B*44:28 on LCL 221.721 cells transduced with shRNA targeting tapasin. Flow cytometric analysis for GFP (reporter gene from the pLVTHm/si plasmid) and w6/32-PE staining shows both B*44:02^{156Asp} and B*44:28^{156Arg} to be GFP-positive, but only B*44:28^{156Arg} to be positive for w6/32-PE staining, thereby indicating that B*44:28^{156Arg} can be expressed on the cell surface even in the absence of tapasin.

Table 1. Shared epitopes.

Peptide position												Protein source	Presenting allele				
													B*44:02 (+TPN)		B*44:28 (+TPN)	B*44:28 (-TPN)	
1	2	3	4	5	6	7	8	9	10	11	12		low	high	high	low	high
A	**E**	E	L	E	R	Q	G	**Y**				199 kDa prot.	+	+	-	-	-
A	**E**	I	R	S	L	V	T	**W**				Interferon-induced prot.	+	-	-	+	+
S	**E**	E	D	L	K	V	L	**F**				Isof. 1 of Polypyrimidine	-	+	+	-	-
A	**E**	D	E	L	F	N	R	**Y**				Nuclear pore complex prot.Nup107	-	+	-	-	+
S	**E**	D	E	I	K	K	A	**Y**				DnaJ homolog subfamily C member 7	+	+	-	-	-
S	**E**	L	E	K	T	F	G	**W**				Isof. LYSp100-A of Nucl. body prot.	+	+	-	-	-
A	**E**	K	A	V	T	K	E	E	**F**			similar to 40S ribosomal prot. SA	+	+	-	-	-
A	**E**	D	S	V	M	D	H	H	**F**			Isof. 1 Plasminogen activ. inhibitor	+	+	-	-	-
E	**E**	D	A	A	L	F	K	A	**W**			Isof. 2 of Interferon reg. factor 4	+	+	-	+	+
E	**E**	A	D	G	G	L	K	S	**W**			F-actin capping prot. subunit alpha-1	+	+	-	-	-
N	**E**	D	N	G	I	I	K	A	**F**			60S ribosomal prot. L4	+	+	-	-	-
G	**E**	D	V	E	T	S	K	K	**W**			Isof. 2 of Endothelial factor 1	+	+	+	+	+
V	**E**	D	P	T	N	D	H	I	**Y**			Isof. 1 of Activ. signal subunit 3	+	+	-	-	-
E	**E**	V	H	D	L	E	R	K	**Y**			20 kDa prot.	+	+	-	-	-
Q	**E**	L	Q	E	I	N	R	V	**Y**			Similar to annexin A2 isof.1	+	+	+	-	-
E	**E**	V	D	L	S	K	D	I	Q	H	**W**	Ribonucleoside-diphosphate reductase	+	-	-	+	+

*Anchors for the corresponding B*44 subtypes are printed in bold. The columns on the right indicate the HLA-B*44 variants from which the respective peptides were recovered. Information about the cell type from which the respective peptides were selected (+TPN = LCL 721.221 cells; -TPN = LCL 721.220 cells) is given in brackets. TPN: tapasin.*

that tapasin interacts with the strand/loop (AA residues 128-136) directly below the first segment of the α2-helix (AA residues 138-149) of HLA class I molecules.[3] Based on its position and orientation, residue 156 is unlikely to contact tapasin directly. Similarly, tapasin-dependent B*44:02 and tapasin-independent B*44:05 alleles with a micropolymorphic difference at residue 116 also appear unlikely to contact tapasin directly. Molecular dynamics study of these two alleles has indicated that in the absence of peptide, this micropolymorphism influences the stability of the first segment of the α2-helix (which contacts the C-terminus of the peptide).[24] Although AA residue 156 is not a part of the first segment of α2-helix, it probably influences the strand/loop region that tapasin interacts with and, in a similar manner

Table 2. Differentially selected peptides by B*44 subtypes.

Peptide	Protein source	Length	Presenting allele				
			B*44:02 (+TPN)		B*44:28 (+TPN)	B*44:28 (-TPN)	
			low	high	high	low	high
R **E** E D A A L F K A **W** I	Isof. 2 of Interferon reg. factor 4	11-mer	-	+	-	-	-
E **E** D A A L F K A **W**	Isof. 2 of Interferon reg. factor 4	10-mer	-	+	-	-	-
S **E** E A E I I R K **Y**	Poly [ADP-ribose] polymerase 1	10-mer	-	+	-	-	-
E **E** A E I I R K **Y**	Poly [ADP-ribose] polymerase 1	9-mer	-	+	-	-	-
Q **E** E I N E V K T **W**	16 kDa prot.	10-mer	-	+	+	-	-
E **E** I N E V K T **W**	16 kDa prot.	9-mer	-	+	-	-	-
L **E** E L Y T K K L **W**	HSPC027	10-mer	-	+	-	-	-
E **E** L Y T K K L **W**	HSPC027	9-mer	-	+	-	-	-
A **E** F K E A F Q L **F**	Isof. Non-muscle of myosin light polypeptide	10-mer	-	+	-	+	-
A **E** F K E A F Q **L**	Isof. Non-muscle of myosin light polypeptide	9-mer	-	+	-	-	-
E **E** G P D V L R **W**	Sec23 homolog B	9-mer	-	+	-	+	+
S **E** E G P D V L R **W**	Sec23 homolog B	10-mer	-	-	-	+	+
A **E** S E E G P D V L R **W**	Sec23 homolog B	12-mer	-	+	-	-	-

*Anchors for the corresponding B*44 subtypes are printed in bold.The columns on the right indicate the HLA-B*44 variants from which the respective peptides were recovered.The information about the cell type from which the respective peptides were selected (+TPN = LCL 721.221 cells; -TPN = LCL 721.220 cells) is given in brackets.TPN: tapasin.*

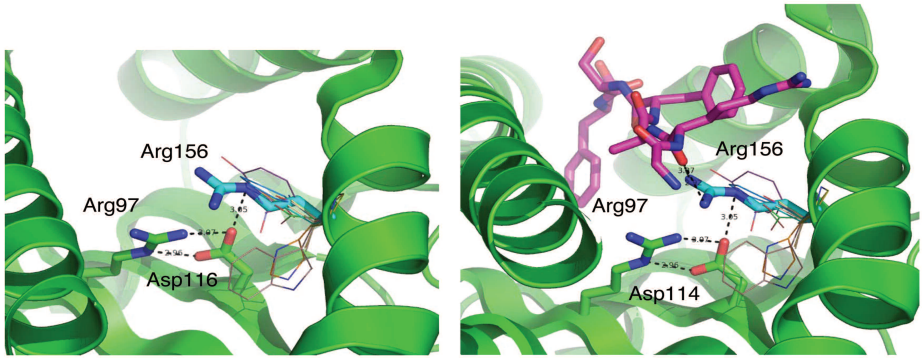

Figure 3. B*44/156 substitution models. We modeled all amino acids at position 156 with their best rotamer; the results indicate that only arginine and possibly lysine could contact the peptide backbone influencing the stability of the complex. We took the B*44:02 structure (1M6O)[10] and modeled all 20 amino acids at position 156 fitting the best side chain rotamer. Arg156 shows increased hydrogen bonding both to residue Asp114 and to the peptide backbone. This is likely to increase stability of the HLA-peptide complex.

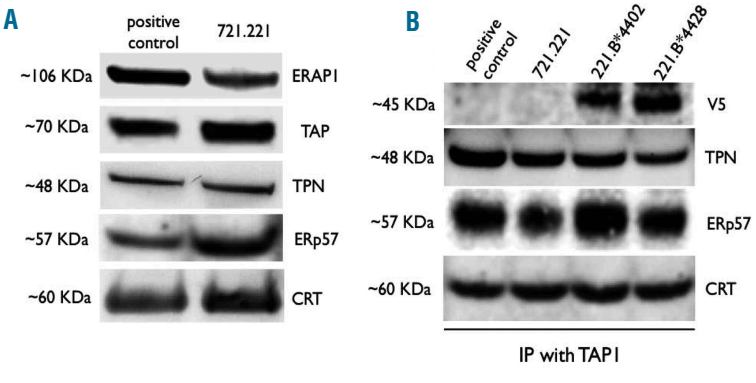

Figure 4. Western blot analysis of LCL 721.221 cells and sHLA-B*44 expressing cells. (A) LCL 721.221 cells contain all of the minimum essential components of the PLC. The western blot analysis of lymphoblastoid B-cells and 721.221 cells using antibodies against the components of the PLC – ERAP1, TAP, tapasin (TPN), ERp57 and CRT confirmed that 721.221 cells possess all of the minimum components of the PLC. (B) sB*44 molecules are associated with the PLC. Lymphoblastoid B-cells (positive control), LCL 721.221 cells, LCL 721.221/sHLA-B*44:02 and LCL 721.221/sB*44:28 cells were immunoprecipitated with anti-TAP1 antibody covalently conjugated to protein-A-sepharose beads. Eluted proteins were resolved by SDS gel, transferred to a PVDF membrane and immunoblotted with HRP-conjugated antibodies against V5 tagged recombinant sHLA*B44 protein and the components of the PLC – TPN, ERp57 and CRT.

to residue 116, affects the stability/dynamics of the unloaded MHC molecule.

After demonstrating the link between tapasin-independency and AA position 156 in B*44 variants, we sought to answer the following questions: (i) How do B*44:02^{156Asp} and B*44:28^{156Arg} differ with respect to their bound peptide features? (ii) Are different subsets of peptides acquired in the absence of tapasin chaperoning? We, therefore, sequenced the peptides derived from B*44:02 (from LCL 721.221 cells) that were acquired with the assistance of tapasin and hence through the optimization machinery of the PLC. These peptides were further subdefined as either low or high affinity bound peptides. The anchor motifs identified were similar to those previously described[12,25,26] illustrating exclusively E for the P2 position and Y, F, W for the PΩ position of the bound peptides (Figure 3) although the individual peptides reflect an alternative source. Overall, more than 200 peptides were recovered from the B*44:02 subtype, which is highly dependent on the presence of tapasin. From these recovered peptides, most of the high affinity peptides were found to be longer than the canonical length (≥12) of amino acids, with the longest recovered peptide being a 19-mer (Table 3). However, one limitation of this approach that should be taken into account is that LCL 721.221 and LCL 721.220 cells have a slightly different genetic background and thus a slightly different proteomic content.

HLA-B*44:28 was shown to acquire the peptides in a tapasin-independent manner to optimize the selection and binding of the peptide cargo. Based on this observation, we expected not to see any significant differences among the B*44:28 derived peptides acquired in the presence and absence of tapasin (LCL 721.221 and LCL 721.220 cells, respectively). However, we found significant differences between these sets of peptides, both in their attributed binding affinity and in the length of the derived peptides.

While the peptide repertoires of sHLA-B*44:02 and sHLAB*44:28 display subtle differences, suggesting an alternate antigen presentation pathway, the core binding motifs are strongly retained. The presence of low affinity peptides isolated from HLA-B*44:28 expressed in tapasin-deficient LCL 721.220 cells *versus* their absence when derived from tapasin-sufficient LCL 721.221 cells, suggests that tapasin still plays a role in optimizing the HLA-B*44:28 cargo.

Peptides of low affinity could not be recovered from B*44:28 molecules that were matured in the presence of tapasin, supporting our computational analysis that clearly indicates a role for 156Arg in increasing the stability of the peptide-HLA complex through contacts with both Asp114 and the peptide backbone at P5. Although the binding affinity of the peptide is not indicative of the relative immunogenicity of the peptide, higher affinity peptides generally extend the half life of the peptide-HLA complex at the cell surface and thus the time available for T-cell receptor recognition. Additional studies into the affinities of the eluted peptides from HLA-B*44:02 and HLA-B*44:28 will help to further elucidate the effect of tapasin.

We observed that peptides could only be eluted from sB*44:28 molecules after TFA treatment, suggesting that they represent high affinity ligands with a long half life. The majority of these peptides are 9-11 AA in length and no peptides greater than 13 residues in length could be recovered. In contrast, those peptides derived from B*44:28 molecules that were matured in the absence of tapasin show the features of both low and high affinity peptides, with the majority being 9-11 residues in length for both sets (*Online Supplementary Table S1D,E*). Irrespective of the source, the anchor motifs are the same for all B*44:28 peptides and reflect a similar binding motif as seen with the B*44:02 allele. This is expected since the position of the mismatch (AA 156) is described to be part of the specificity pockets C, D, E9 and thus not in contact with either the P2 or PΩ position of a given peptide.

From our molecular models, it appears that both arginine and lysine substitutions at position 156 would contact the peptide main-chain at the P5 position of the peptide, however lysine-substituted molecules were not detected on the surface of the recombinant LCL 721.220 (HLA^{+}/tapasin^{-}) cells (Figure 1). Considering that the enthalpy value of peptides binding to HLA molecules is relatively small and that it is likely to be very sensitive to entropy changes involving both the solvent and the protein, certain high entropy AA such as lysine may not, therefore, be favored at the binding interface. Indeed, lysine residues although predominantly surface exposed are statistically less favored than arginine to be in protein:protein interactions.[27]

The modeling illustrated in Figure 3 is based on a peptide-bound crystal structure (1M6O). Our results indicate that the HLA-B*44:28^{156Arg} variant stabilizes the binding groove in its empty state, thus negating the contribution of the PLC and allowing independent loading of high affinity peptides. How this stability is achieved are difficult to predict even with detailed molecular dynamics simulations. The structures of B*44:02 (1M6O) and B*44:05 (1SYV) have been studied by such simulations to illustrate the differences between peptide-bound and peptide-free conformations in order to understand the tapasin-independence of the HLAB*44:05^{116Tyr} micropolymorphism.[24] In this study the floor of the peptide-binding groove showed only small conformational fluctuations both in the absence and presence of peptides but this remained sufficient to influence the peptide C-terminal binding region (F pocket) and generate a more stable peptide receptive state. For the HLAB*44:28^{156Arg} variant, the interaction between Arg156 and Asp114 on the floor of the peptide-binding groove is

Table 3. Analysis of the length of HLA-B*44-derived ligands.

Allele/ derivation/ affinity		B*44:02 (+TPN) low	B*44:02 (+TPN) high	B*44:28 (+TPN) high	B*44:28 (-TPN) low	B*44:28 (-TPN) high
(N) total number of peptides		N= 17	N= 194	N= 79	N= 24	N= 44
length of ligands (AA)	19		1			
	16		2			
	15		1			
	14		1			
	13		4	3		
	12		21	11	2	2
	11	1	47	23	5	9
	10	11	54	24	11	17
	9	4	63	18	6	16
	8	1				

*This table represents the length (8 to 19 residues) and number of eluted peptides specific for each of the individual B*44 alleles.*

also able to generate the same stable peptide receptive state. It would, therefore, be interesting in the future to compare the simulated molecular dynamics of both sets of allelic variants.

The results of the peptide analysis suggest that tapasin chaperoning is needed to acquire peptides longer than the canonical length (>12 AA). We also describe for the first time the evidence that AA position 156 can influence both the conformation of a mismatched molecule (tapasin-dependency) and the peptide repertoire that will be displayed on the cell surface to the immune system (peptide affinity and length). Clinically, our data provide a molecular under standing of potential immunological episodes and will help us to further define permissive and non-permissive mismatches within the B*44 alleleic group.

Whether tapasin-dependency or tapasin-independency is advantageous or not is likely to depend on the combination of HLA-A, -B, and C- alleles of an individual. An appreciation of the interaction between tapasin, HLA class I molecules and peptide loading may, therefore, be important not only during viral infections, but also while considering transplantation scenarios. While it has been suggested that tapasin-independency might permit certain MHC class I molecules to present viral antigens upon disruption of the PLC, how this would translate to a transplantation scenario and whether an allelic mismatch could be beneficial to overcome an infection remain to be determined. On the other hand, tapasin-independent peptide loading might result in the presentation of poorly tolerated self-peptides that could trigger auto-immune responses. Clinically, our data provide a molecular understanding of potential immunological episodes and will help to further define permissive and non-permissive mismatches within the B*44 alleleic group.

Authorship and Disclosures

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