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A Single Polymorphic Residue Within the Peptide-Binding Cleft of MHC Class I Molecules Determines Spectrum of Tapasin Dependence

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Different HLA class I alleles display a distinctive dependence on tapasin for surface expression and Ag presentation. In this study, we show that the tapasin dependence of HLA class I alleles correlates to the nature of the amino acid residues present at the naturally polymorphic position 114. The tapasin dependence of HLA class I alleles bearing different residues at position 114 decreases in the order of acidity, with high tapasin dependence for acidic amino acids (aspartic acid and glutamic acid), moderate dependence for neutral amino acids (asparagine and glutamine), and low dependence for basic amino acids (histidine and arginine). A glutamic acid to histidine substitution at position 114 allows the otherwise tapasin-dependent HLA-B4402 alleles to load high-affinity peptides independently of tapasin and to have surface expression levels comparable to the levels seen in the presence of tapasin. The opposite substitution, histidine to glutamic acid at position 114, is sufficient to change the HLA-B2705 allele from the tapasin-independent to the tapasin-dependent phenotype. Furthermore, analysis of point mutants at position 114 reveals that tapasin plays a principal role in transforming the peptide-binding groove into a high-affinity, peptide-receptive conformation. The natural polymorphisms in HLA class I H chains that selectively affect tapasin-dependent peptide loading provide insights into the functional interaction of tapasin with MHC class I molecules.

Materials and Methods
DNA constructs

The cDNA encoding human tapasin was subcloned into the pcDNA3.1/(Hyg) vector (Invitrogen, Carlsbad, CA). Site-directed mutants of B2705 with H to D at position 114 (B27H114D), H to N at position 114 (B27H114N), H to R at position 114 (B27H114R), D to Y at position 116 (B27D116Y), and the other substitution mutants of B4402 were made by changing the codons by PCR with Pfu DNA polymerase (Stratagene, La Jolla, CA). All HLA cDNAs and their mutagenized derivatives were inserted into the mammalian expression vector pcDNA3.1 (Invitrogen).
Stable cell lines and Abs

Tapasin expression was restored in 721.220 cells by transfection with the cDNA encoding tapasin, and transfectants were selected with 0.2 μg/ml hygromycin, giving rise to the 721.220.Tpn cell line. The HLA-G-specific mAb G233 was a gift from Dr. Y. Loke (University of Cambridge, Cambridge, U.K.). Polyclonal rabbit K455 Ab reacts with MHC class I H chains and β2m in both assembled and nonassembled forms (15). The mouse mAb HLA-B-Ab-1 (NeoMarkers, Fremont, CA) recognizes only HLA-B alleles and mAb W6/32 recognizes only the complex of H chain and β2m. The anti-tapasin Ab (Rgpa6B11) was a gift from Dr. P. Cresswell (Yale University, New Haven, CT). FITC-conjugated goat anti-mouse IgG Abs and HRP-conjugated streptavidin were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and Pierce (Rockford, IL), respectively.

Pulse-chase and immunoprecipitation

Cells (5 × 10⁶) were transfected by electroporation (Gene Therapy Systems, San Diego, CA), starved for 40 min in medium lacking methionine, labeled with 0.1 mCi/ml [35 S]methionine (TranS-label; NEN, Boston, MA) and incubated for 30 min at 26 °C. Cells were washed three times with 0.1% Nonidet P-40 in PBS. Proteins were eluted from the beads by boiling in SDS sample buffer and separated by 12% SDS-PAGE. The gels were stained, exposed to BAS film for 1 h, and then analyzed with Phosphor Imaging System BAS-2500 (Fuji Film, Tokyo, Japan). For endoglycosidase H (endo-H) treatment, immunoprecipitates were digested with 5 μg/ml endo H (Roche, Indianapolis, IN) at 37°C overnight in 50 mM sodium acetate (pH 5.6), 0.3% SDS, and 150 mM 2-ME (Sigma-Aldrich).

Flow cytometry

The surface expression of MHC class I molecules was determined by flow cytometry (FACSCalibur; BD Biosciences, Mountain View, CA). Cells (1 × 10⁶) were washed twice with cold PBS containing 1% BSA and incubated for 1 h at 4°C with a saturating concentration of primary Ab. Normal mouse IgG Ab was used as a negative control for each test. Cells were washed twice with cold PBS containing 1% BSA and then stained with FITC-conjugated goat anti-mouse IgG Abs for 30 min. A total of 10,000 gated events were collected by the FACSCalibur cytometer and analyzed with CellQuest software (BD Biosciences).

Coimmunoprecipitation and Western blot analysis

Cells were lysed in 1% digitonin in buffer containing 25 mM HEPES, 100 mM NaCl, 10 mM CaCl₂, and 5 mM MgCl₂ (pH 7.6) supplemented with protease inhibitors. Lysates were precleared with protein G-Sepharose (Amersham Pharmacia Biotech) at 1 h at 4°C. For immunoprecipitation, samples were incubated with the appropriate Abs for 2 h at 4°C before protein G-Sepharose beads were added. Beads were washed four times with 0.1% digitonin, and bound proteins were eluted by boiling in SDS sample buffer. Proteins were separated by 12% SDS-PAGE, transferred onto a nitrocellulose membrane, blocked with 5% skim milk in PBS with 0.1% Tween 20 for 2 h, and probed with the appropriate Abs for 4 h. Membranes were washed three times with PBS with 0.1% Tween 20 and incubated with HRP-conjugated streptavidin (Pierce) for 1 h at 4°C. Immunoblots were visualized with ECL detection reagent (Pierce).

Microsomes and peptide-loading assay

Microsomes from 721.220 and 721.220.Tpn cells expressing B2705, B4402, or their mutant HLA class I H chains, with or without tapasin, were prepared and purified as previously described (16). Biotinylated peptides GRIDKPILK (ligand for B2705) and AEIDKTVGY (ligand for B4402) were conjugated to the photoreactive cross-linker N-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS; Pierce) as described (17). For the peptide-loading assay, reporter peptides, with or without various concentrations of unlabeled peptides, were mixed with 15 μl of microsomes (concentration of 60 A₂₈₀/ml) in a total volume of 50 μl of RM buffer (250 mM sucrose, 50 mM triethanolamine-HCl, 50 mM potassium acetate, 2 mM magnesium acetate, 1 mM DTT, and 10 mM ATP). The mixture was incubated for 30 min at 26°C in a flat-bottom 96-well tissue culture plate. Samples were maintained on ice during a 3-min exposure to shortwave (365 nm) ultraviolet irradiation. After centrifugation, membranes were washed once with cold RM buffer and lysed with 1% digitonin, and the cross-linked proteins were immunoprecipitated with mAb HLA-B Ab-1. Precipitates were separated by 12% SDS-PAGE and transferred to an immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated with HRP-conjugated streptavidin for 1 h at 4°C, and biotinylated proteins were visualized by using ECL Western blotting reagent (Pierce). Peptide translocation was determined after incubating microsomes with biotin-conjugated reporter peptides in the absence of competitor for 30 min at 26°C with or without 1 mM ATP. Microsomal membranes were recovered by centrifugation at 75,000 × g for 10 min through a 0.5 M sucrose cushion in cold RM buffer. After washing with cold RM buffer twice, the membrane pellet was directly dissolved in sample buffer. The samples were analyzed on tricine/SDS-PAGE, appropriate for resolution of low mass polypeptides as described (18), and probed with HRP-conjugated streptavidin. The relative densities of the peptide bands were determined by use of an imaging densitometer (GS-700; Bio-Rad, Richmond, CA) and Multi-Analyst densitometer software (Bio-Rad).

Results

Variable dependence of HLA class I molecules on tapasin for their cell surface expression

Among different HLA class I alleles, dependence on tapasin for class I expression is variable. B4402, and to a lesser extent B0801, are tapasin-dependent for cell surface expression, whereas B2705 is tapasin-independent (3). To evaluate whether these allele-specific differences in tapasin dependence could be further extended to other MHC class I molecules, we examined the cell surface expression of 10 HLA class I alleles in 721.220 and 721.220.Tpn cells. In the absence of tapasin, a spectrum of surface expression of MHC class I alleles was consistently evident in pools of transfected cells (Fig. 1a). High levels of surface expression were observed for B2705, B2702, A0210, A2401, B0801, and B5401 alleles. In contrast to these alleles, >5-fold lower surface expression was observed for B4402, B3501, A3001, and, interestingly, HLA-G Ag, a nonclassical MHC class I molecule (Fig. 1a, middle column). Significantly, expression of tapasin fully restored the surface expression of B4402, B3501, A3001, and HLA-G Ag (Fig. 1a, middle column). Surface levels of B0801 and B5401 were also increased, albeit to a lesser extent, upon expression of tapasin (Fig. 1a, right column). However, expression of tapasin did not affect the surface levels of B2705, B2702, A0210, and A2401 (Fig. 1a, left column). These results indicate that the degree to which class I surface expression is affected by tapasin is indeed allele-dependent.

Residue 114 determines spectrum of tapasin dependence of HLA class I molecules

As tapasin shows little polymorphism (10), variable dependence on tapasin should be explained by amino acid differences among the respective class I alleles. These differences are mainly located in the α1α2 domain of MHC class I molecules. To define the amino acids potentially involved in determining tapasin dependence, we, therefore, aligned the sequences of the α1α2 domain of HLA class I molecules (Fig. 1b). Interestingly, tapasin-independent HLA class I alleles contain histidine or arginine at position 114, whereas tapasin-dependent alleles contain acidic amino acid residues, such as aspartic acid or glutamic acid. Alleles that are weakly dependent on tapasin contain neutrally charged asparagine or glutamine at this position. Database analysis reveals that position 114 of all HLA class I molecules is represented by one of the following six amino acids: histidine, arginine (basic), aspartic acid, glutamic acid (acidic), and asparagine, glutamine (neutral).

To test whether the side chain of the amino acid at position 114 governs the extent of tapasin dependence of HLA class I alleles, we constructed substitution mutants and examined their dependence on tapasin for surface expression. Surprisingly, B44D114H was no longer tapasin-dependent and instead displayed a tapasin-independent phenotype for their surface expression (Fig. 2a).
B44D114N and B27H114N, in which residue 114 was replaced by the neutral-charged asparagine, fell between the B4402 and B2705 in the spectrum of tapasin dependence. The reciprocal mutation, B27H114D, rendered the otherwise tapasin-independent B2705 allele dependent on tapasin for surface expression. As arginine, a basic amino acid, is also found in some MHC class I alleles, such as A0101, A0301, A1101, or A2902, we substituted arginine for histidine in B2705 (B27H114R) and examined its dependence on tapasin for surface expression. In the absence of tapasin, the surface expression of B27H114R was comparable to the level observed for wild-type B2705 and was not affected by expression of tapasin (Fig. 2b), indicating that arginine can functionally replace histidine at position 114 in respect to conferring tapasin independence on MHC class I alleles. These results indicate that the tapasin dependency of HLA alleles is determined by the nature of the amino acid at position 114.

**Differential effect of tapasin on maturation of HLA class I alleles is exerted by residue 114**

To investigate the mechanism by which the amino acid at position 114 influences dependence of MHC class I molecules on tapasin for surface expression, we compared the intracellular maturation and transport of wild-type HLA class I alleles with their respective point mutants in the absence or presence of tapasin. We used pulse-chase analysis to estimate the extent of intracellular transport of MHC class I molecules by examining their acquisition of endo-H resistance. In the absence of tapasin, the majority of B4402 H chains remained sensitive to endo-H digestion, even after 90 min (Fig. 3a, upper panel), reflecting complete retention of these molecules in the ER over this period. This finding suggests that the impaired intracellular transport of B4402 molecules in the absence of tapasin accounts for their lower levels of surface expression. Conversely, in the presence of tapasin, B4402 molecules had become endo-H-resistant by this time, consistent with their efficient expression at the cell surface (Fig. 3a, lower panel). Pulse-chase experiments from tapasin-negative and -positive cells revealed comparable acquisition of endo-H resistance by B4402 and B44D114H (Fig. 3b), indicating its tapasin independence for normal intracellular transport. Without tapasin, the maturation kinetics of B2705 and B27H114D were essentially the opposite of the maturation kinetics of B4402 and B44D114H, respectively. After a 90-min chase, B2705 had become resistant to endo H irrespective of tapasin, indicating its transport out of the ER (Fig. 3c). At the 90-min chase, B27H114D remained sensitive to endo H in the absence of human tapasin but had become resistant to endo H in its presence (Fig. 3d). In the absence of tapasin, B44D114N, in which aspartic acid is replaced with the neutrally charged asparagine at position 114 of B4402, was efficiently transported out of the ER (Fig. 3e, upper panel) at a rate that was similar, albeit lower, to the rate observed for B44D114H. Tapasin slightly promoted the transport...
of B44D114N (Fig. 3e, lower panel). The intracellular maturation and transport results correlate with the cell surface expression of MHC class I molecules. From these experiments, we have established that the amino acid residue at position 114 governs a spectrum of dependence on tapasin for biochemical maturation and surface expression.

Residue 114 influences the association of HLA class I alleles with tapasin

To further understand the molecular basis for the critical role of residue 114 in defining the tapasin dependence of HLA class I molecules, we examined by communoprecipitation the interaction of wild-type HLA and its mutant derivatives with tapasin in the loading complex. Remarkably, B44D114H, in comparison with wild-type B4402, showed an 8-fold lower affinity for tapasin (Fig. 4a, lanes 1 and 2), suggesting that introduction of a basic amino acid residue at position 114 disrupts its interaction with tapasin. B44D114N exhibited weaker association with tapasin than did B4402 but exhibited stronger association with tapasin than did B44D114H (Fig. 4a, lane 3). In contrast, B27H114D had an affinity for tapasin that was even higher than the tapasin affinity of the wild-type B2705 in this and repeating experiments (Fig. 4b, lanes 1 and 2); B27H114N fell between B27H114D and B2705 in tapasin affinity. To confirm that the observed differential binding of HLA class I molecules to tapasin was not due to differences in sample loading, we reacted the same blots with the K455 Ab and verified that each lane contained a comparable amount of MHC class I molecules (Fig. 4, a and b, lower panels). Several studies have shown that positions 115 and 116 affect association of HLA alleles with chaperones and that the interaction of chaperones with HLA class I H chains is cooperative (11–14). We wanted to confirm the effect of residue 116 on the association of HLA class I H chains with chaperones and thereby validate our coprecipitation system. Substitution of aspartic acid to tyrosine at position 116 caused disparate binding of tapasin by B2705 and B4402. In the case of 2705, the substitution (B27D116Y) significantly decreased tapasin (Fig. 4b, lane 4), whereas the B44D116Y mutant had a stronger association with tapasin (Fig. 4a, lane 4). In general agreement with the previous reports (12, 14), these data confirm the importance of position 116 for chaperone association and indicate that position 116 indeed affects the association with tapasin. Taken together, our findings indicate that position 114 influences tapasin association and that the residue 114-mediated tapasin dependence of HLA class I alleles correlates with the strength of tapasin interaction.

Position 114 determines the peptide-loading characteristics of HLA class I molecules

In the absence of tapasin, the impaired intracellular transport and, consequently, the low surface expression of B4402 and B27H114D might be due to their inability to load peptides. To determine the influence of residue 114 on peptide loading as a function of tapasin, we examined peptide loading into MHC class I molecules by using the reporter peptide. We incubated intact microsomes derived from B4402 and B44D114H transfectants with or without tapasin and AEIDKVTGY peptide, a high-affinity ligand for B4402 (19), in the presence of the competitors at different concentrations. The amount of tapasin-dependent peptide loading was determined by measuring the level of incorporation of labeled peptide into W6/32-reactive class I complexes. We were surprised at the marked differences in the ability of B4402 and B44D114H to load peptide in the absence of tapasin. When no competitor was added, the level of binding of the reporter peptide by B4402 was only 25% of the binding observed in B44D114H (Fig. 5a, lane 0). Furthermore, the reporter peptides loaded into B4402 were completely out-competed by a lower concentration (1.6 μM) of unlabeled peptide, whereas as much as 12.8 μM of unlabeled peptide was not even sufficient for completely out-competing reporter peptide binding to B44D114H. However, in the presence of tapasin, no discernible difference in the peptide-binding ability of B4402 and B44D114H was seen (Fig. 5b). Analysis of B2705 and B27H114D as to tapasin dependence for peptide loading revealed that wild-type B2705 resembles B44D114H, whereas B27H114D behaves like wild-type B4402. In the absence of tapasin, B2705 efficiently binds the reporter peptide, GRIDK PILK, a high-affinity ligand for B2705 (20). A high concentration (12.8 μM) of unlabeled peptide was not sufficient to out-compete
the reporter peptide binding (Fig. 5c, upper panel). In contrast, substitution of aspartic acid for histidine at position 114 of B2705 dramatically abolished its ability to load peptides (Fig. 5c, lower panel). However, expression of tapasin greatly enhanced the ability of B27H114D to load peptides to the same level as observed for wild-type B2705 (Fig. 5d, lower and upper panels, respectively). These results indicate that B4402 molecules are highly dependent on tapasin for efficient peptide loading, but the aspartic acid-to-histidine substitution at position 114 renders the molecules independent of tapasin for peptide loading. Accordingly, the impaired intracellular transport and reduced surface expression of B4402 and B27H114D in the absence of tapasin are likely the result of their inability for peptide loading.

To further investigate whether the differential peptide loading observed in the absence of tapasin might be due to differences in the luminal availability of the peptide, we quantitated the amount of reporter peptides that were translocated into the ER lumen by UV irradiation time point. Comparable amounts of peptides were recovered between B2705 and B27H114D (Fig. 5e, lanes 1 and 2) and between B4402 and B44D114H (Fig. 5e, lanes 3 and 4). Therefore, we exclude the notion that luminal availability of the peptide plays an important role in dictating class I loading in

**FIGURE 3.** Tapasin-dependent effect of residue 114 on intracellular transport of MHC class I molecules. 721.220 (tapasin-negative) and 721.220.Tpn (tapasin-positive) cells were transfected with B4402 (a), B44D114H (b), B2705 (c), B27H114D (d), or B44D114N cDNAs (e). Cells were labeled and chased for the indicated times. Cell lysates were immunoprecipitated with mAb HLA-B Ab-1, and then digested with endo H (+) or mock digested (−) for 16 h. Endo-H-resistant (r) and endo-H-sensitive (s) proteins bands are indicated.
tapasin-deficient cells. In control experiments in which ATP was omitted, little peptide was recovered (Fig. 5e, lanes 5 and 6). Because peptide translocation via TAP requires ATP (21), we conclude that the modified reporter peptides entered the ER lumen in a TAP-dependent manner. Overall, our data clearly show that functional polymorphism in tapasin dependence for efficient peptide loading of MHC class I molecules is defined by the presence of different amino acids at position 114.

Discussion
Based on the available class I crystal structures, some considerations on the peculiar behavior of position 114, an integral part of the D pocket of the peptide-binding groove, may be discussed. The general structure of different MHC class I molecules is similar, diverging only at the polymorphic residues, which are relatively few and located mostly in the peptide-binding groove (22). A slight structural variation affecting this region can dramatically change the requirements for binding peptides and might result in a different set of bound peptides. Our findings reveal that tapasin dependence and the nature of the side chain of amino acid residue 114 are clearly correlated (the “tapasin-rule”). HLA class I alleles with acidic amino acids at position 114 associate strongly with tapasin and are dependent on tapasin for high-affinity peptide loading and surface expression. The negatively charged side chain of aspartic acid or glutamic acid might pose steric hindrances in the peptide-binding groove that would interfere with the strong binding of peptides. Upon interaction with tapasin, the peptide-binding cleft transits to the open, peptide-receptive form. In contrast to tapasin-dependent HLA class I alleles, the peptide-binding groove of MHC class I molecules containing basic residues at position 114 seemingly forms the open state to fit the appropriate peptides, regardless of tapasin. Accordingly, these HLA class I alleles are capable of binding a broad spectrum of the peptide repertoire without the assistance of tapasin and are subsequently transported to the cell surface. In corroboration with this concept, HLA class I alleles with a neutral-charged amino acid display an intermediate level of tapasin dependence for surface expression (3). These findings intimate that the dependence upon tapasin for loading a repertoire of peptides of sufficient stability to allow egress from the ER might reside in the ability of the D pocket to facilitate the loading of peptides or perhaps to initiate a peptide-induced conformational change in the class I molecule that signals release from the loading complex. We predict that the tapasin rule might also be imposed upon other nonclassical MHC class I molecules, such as HLA-E, HLA-F, as well as the unexamined other classical MHC class I molecules.

Recently, Williams et al. (23) observed that unlike tapasin-dependent B4402, B4405 (tyrosine at position 116), which was assumed to be identical to B4402 (aspartic acid at position 116) apart from the polymorphism at position 116, is tapasin-independent for the cell surface expression. This observation led them to conclude that amino acid residue 116 determines the tapasin dependence of cell surface expression. This result contrasts with our observations with B44D116Y. We found that the single amino acid change of tyrosine for aspartic acid at position 116 does not change the phenotype of B4402 for tapasin dependence (data not shown) and that B4402 and B2705, which share the same amino acid residue at position 116, exhibit different phenotypes for tapasin dependence in their surface expression (Fig. 1). Likewise, HLA-G and A2401
It is intriguing how Williams et al. (23) could detect the absence of the N-terminal 48 amino acids and the entire type for tapasin dependence (Fig. 1). In fact, the structures of B4405 and B4402 have tyrosine at position 116 but they display the opposite phenotype for tapasin dependence (Fig. 1). In fact, the structures of B4402 and B4405 are quite different. In comparison with B4402, B4405 is devoid of the N-terminal 48 amino acids and the entire α3 region, including the transmembrane and cytoplasmic domains (24). It is intriguing how Williams et al. (23) could detect the putative “soluble” B4405 proteins at the cell surface by FACS analysis. On the basis of our findings and the findings of others, we propose that residue 114 acts as a major determinant of tapasin dependence for efficient peptide loading, maturation, and surface expression of HLA class I alleles while residue 116 can also affect association of certain MHC class I molecules with the components of the loading complex, albeit, in unpredictable manner.

Several studies have shown that TAP association of MHC class I molecules requires tapasin (2, 25–27), as expected if tapasin bridges MHC class I molecules to TAP. Therefore, a spectrum of residue 114-dependent dependence of HLA class I alleles on tapasin might be an indirect effect of the residue on TAP-related function. However, our data argue against this possibility. First, residue 114 appears to have no effect on the function of TAP in translocating peptides into the ER lumen (Fig. 5e). Second, as loading of peptides onto B44D114H and B2705 proceeds normally in the absence of tapasin (Fig. 5, a and c), bridging of MHC class I molecules to TAP, which is one of the proposed functions of tapasin, appears to be unnecessary for enhanced peptide loading, which is consistent with a previous report (3).

What is the clinical relevance of our findings on tapasin dependence of MHC class I alleles? HLA alleles that encode tapasin-independent molecules might have evolved in response to evolutionary pressures exerted by microbial pathogens. Adenovirus protein E19 targets tapasin to prevent Ag presentation and in turn to evade CTL recognition (28). The flexibility offered by tapasin independence might permit certain MHC class I molecules to continue presenting viral Ags even when tapasin function is disrupted. Peptide loading that is independent of tapasin, although beneficial during infection with such microorganisms, might also result in the up-regulated presentation of poorly tolerized self-peptides in an inflammatory context. Such up-regulation might be a novel mechanism for triggering autoimmunity responses. This hypothesis might be particularly relevant to the development of inflammatory spondyloarthritides, known to be strongly associated with the “tapasin-independent” HLA-B27 alleles (29). Differences in the residues at positions 114 and 116 are likely to contribute to allele-specific recognition by different CTL clonotypes, changing the requirements for binding peptides and modifying the set of bound peptides. This might provide a possible molecular mechanism by which a molecular mismatch involving positions 114 and 116 of MHC class I H chain influences the outcome of unrelated bone marrow transplantation in donor-recipient pairs, otherwise matched for HLA-DRB and -DQ loci; patients with substitution at positions 114 and 116 are at increased risk for transplant-related mortality (30). The description of the natural polymorphisms in HLA class I H chains that selectively affect tapasin-dependent peptide loading provides insights into the functional interaction of tapasin with MHC class I molecules and indicates that manipulation of class I sequences can affect MHC class I maturation and cell surface expression.

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References


968 DETERMINANT FOR TAPASIN DEPENDENCE OF MHC CLASS I MOLECULES
CORRECTIONS


In the original article, both the concepts and language in the last paragraph of the Discussion were taken without attribution from the previous publications (References 3 and 9). We deeply regret and apologize for this lack of appropriate attribution.


In Figure 2B, the mismatch repair enzyme expression for osteoarthritis (OA) and rheumatoid arthritis (RA) was compared in a bar graph. The designation for RA and OA were reversed in the figure. The corrected figure is shown below.