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Anti-HLA-E mAb 3D12 mimics MEM-E/02 in binding to HLA-B and HLA-C alleles: Web-tools validate the immunogenic epitopes of HLA-E recognized by the antibodies

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ABSTRACT

HLA-E shares several peptide sequences with HLA-class Ia molecules. Therefore, anti-HLA-E antibodies that recognize the shared sequences may bind to HLA-class Ia alleles. This hypothesis was validated with a murine anti-HLA-E monoclonal antibody (mAb) MEM-E/02, which reacted with microbeads coated with several HLA-B and HLA-C antigens. In this report, the hypothesis was reexamined with another mAb 3D12, considered to be specific for HLA-E. The antibody binding is evaluated by measuring mean fluorescence index [MFI] with Luminex Multiplex Flow-Cytometric technology. The peptide-inhibition experiments are carried out with synthetic shared peptides, most prevalent to HLA-E and HLA-Ia alleles. The results showed that mAb 3D12 simulated MEM-E/02 in recognizing several HLA-B and HLA-C antigens. Both 3D12 and MEM-E/02 did not bind to HLA-A, HLA-F and HLA-G molecules. As observed with MEM-E/02, binding of 3D12 to HLA-E is inhibited by the peptides sequences ^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142}. Decrease in binding of mAb 3D12 to HLA class Ia, after heat treatment of antigen coated microbeads, supports the contention that the epitope may be located at the outside of the "thermodynamically stable" α-helix conformations of HLA-E. Several sequence and structure-based web-tools were employed to validate the discontinuous epitopes recognized by the mAbs. The scores obtained by these web-tools distinguished the shared peptide sequences that inhibited the mAb binding to HLA-E. Furthermore, ElliPro web tool points out that both mAbs recognize the conformational discontinuous epitopes (the shared inhibitory peptide sequences) in the secondary structure of the HLA-E molecule. The study favors the contention that the domain of the shared inhibitory peptide sequences may be the most immunogenic site of HLA-E molecule. It also postulates and clarifies that amino acid substitution on or near the binding domains may account for the lack of cross reactivity of 3D12 and MEM-E/02 with HLA-A, HLA-F and HLA-G molecules.

1. Introduction

HLA-E (low polymorphic and highly conserved non-classical HLA class Ib molecules) is ubiquitously transcribed in all human tissues (Wei and Orr, 1990), over expressed and shed into circulation upon inflammation (Coupel et al., 2007; Lin et al., 2009) and neoplastic transformation (Malmberg et al., 2002; Derre et al., 2006; Bianchini et al., 2006; Kren et al., 2010). HLA-E shares several peptide sequences with HLA-Class Ia molecules[1] (Ravindranath et al., 2010a), with exception of two peptide sequences ^{65}RSARDTA^{71} and ^{143}SEQKSNDASE^{152} (Ravindranath et al., 2010b). Since HLA-E shares several peptide sequences with HLA class Ia molecules, it is hypothesized that the monoclonal antibodies raised against HLA-E may cross react with HLA-Ia molecules, provided they recognize the shared peptide sequences. In support of the hypothesis, we have shown that anti-HLA-E mAb, MEM-E/02, considered specific for HLA-E (Menier et al., 2003; Lo Monaco et al., 2008, 2010), bound to several HLA-Ia molecules (HLA-B &-C) coated on to microbeads (Ravindranath et al., 2010a). The binding of MEM-E/02 to β2m-free HLA-E and to HLA-Ia molecules was inhibited dosimetrically by the shared epitope sequences ^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142}, but not by the peptide sequence (^{126}LNEDRSWTA^{135}) in between the two epitopes (see Fig. 4D in Ravindranath et al., 2010a). Instead, the middle sequence enhanced the binding, suggesting that the epitope recognized by MEM-E/02 may be discontinuous. Examination of the 3D structure and location of the peptides revealed

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[1] ^{47}PRAPWMEQE^{55}, ^{59}EYWDRETR^{65}, ^{90}AGSHTLQW^{97}, ^{108}RFLRGYE^{123}, ^{115}QFAYDGKDY^{123}, ^{117}AYDGKDY^{123}, ^{126}LNEDLRSWTA^{135}, ^{137}DTAAQIS^{143}, ^{137}DTAAQI^{142}, ^{157}RAYLED^{162}, ^{163}TCVEWL^{168}, ^{182}EPPKTHVT^{190}.

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that the sequence ^{115}QFAYDGKDY^{123} at the terminal region α2-helix is exposed in β2m-free soluble HLA-E and masked by β2m in intact HLA-E. HLA-E antibodies binding to HLA-E specific sequences could be HLA-E-specific, but those binding to shared sequences are capable of cross-reacting with class Ia molecules. Thus, murine anti-HLA-E mAbs (MEM-E/02, MEM-E/6, MEM-E/07 and MEM-E/08) developed against such soluble recombinant HLA-E heavy chains cross-reacted with several HLA-Ia molecules (Ravindranath et al., 2010a). The HLA-Ia alleles recognized by these mAbs are remarkably identical.

The biological significance of the finding is elucidated when the pattern of HLA-Ia reactivity of the human sera (anti-HLA-E positive) closely paralleled to that of mAb MEM E/02 and when the binding of the human sera to HLA-E and HLA-Ia alleles were inhibited by the shared epitope sequences (^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142}). These observations suggested that these sequences may be the most immunogenic site in HLA-E (Ravindranath et al., 2010c).

Anti-HLA-E mAb (mAb 3D12), generated by immunization of HLA-B27 transgenic mice with recombinant HLA-E purified from AEH cells (*Lymphoblastoid cell line LCL 721.221 cells transfected with HLA-E gene*), is also considered to be specific for HLA-E (Lee et al., 1998). In order to reconfirm the hypothesis. This investigation examines whether mAb 3D12 recognizes shared peptides sequences or HLA-E specific peptide sequences, using shared peptide sequences as inhibitors. The affinities of 3D12 and MEM-E/02 for HLA-Class Ia and Ib alleles were compared.

Several sequence and structure based web-tools (http://tools.immuneeptiope.org/tools) are used to predict that the epitope sequences recognized by the mAbs. These tools also allow visualization of the epitopes in 3D-structure of HLA-E. In the present study, these tools are utilized to validate the experimental findings on the discontinuous epitopes of anti-HLA-E mAbs, derived from the peptide inhibition experiments.

2. Materials and methods

2.1. Monoclonal anti-HLA-E antibodies

This investigation includes four murine mAbs (MEM-E/02, MEM-E/06, MEM-E/07, MEM-E/08; Affinity Bioreagents (ABR, Golden, Colorado) and mAb 3D12 (eBioScience, Cat. No.: 14-9953-82, Lot # E032916, www.ebioscience.com). The specificity of mAb 3D12 was tested for cross-reactivity with other HLA class Ia alleles.

2.2. Immunoassay with single antigen beads

To simultaneously detect the binding of minimal quantities of murine mAbs to HLA-E, HLA-F, HLA-G, HLA-A, HLA-B and HLA-C alleles, multibead multiplex immunoassay is used, as described earlier (Ravindranath et al., 2010a,c). The single antigen beads (HLA class Ia) are obtained from One Lambda Inc. (Canoga Park, CA, USA). The HLA-class Ib molecules are coated on microbeads at One Lambda. Data generated with Luminex Multiplex Flow Cytometry (LABScan 100) are analyzed using computer software, as reported earlier (Ravindranath et al., 2010a). The HLA-Ia microbeads supplied by One Lambda (Canoga Park, CA, USA) have inbuilt negative control beads (Human serum albumin coated) and positive control beads (human IgG-coated). For HLA-E, we have separately included positive and negative control beads. Each experiment is done in duplicate. For each analysis, a minimum of 100 beads are counted. The Trimmed Mean is obtained by trimming a percent off the high and low ends of a distribution of fluorescence intensity and finding the mean of the remaining distribution. Isotype control is run simultaneously. Trimmed mean cut-off MFI is 500. To express the values of anti-HLA antibodies at different dilutions, the sample specific fluorescent value (trimmed MFI) for each bead are normalized and used for analysis.

Four different 'Trimmed means' of MFI are obtained. They are (1) for MFI obtained with mAbs against HLA class I alleles, (2) for MFI of the negative control beads (used for each mAb), (3) for MFI of PE-conjugated 2nd antibody only, tested on HLA-Ia coated beads. (4) For MFI of negative control beads (for PE-conjugated 2nd antibody). From these four different Trimmed Mean MFIs, the normalized trimmed MFI is calculated as follows:

$$[\text{MFI of}(1) - \text{MFI of}(2)] - [\text{MFI of}(3) - \text{MFI of}(4)].$$

For heat treatment, the beads are incubated in the assay buffer at 37 °C for 30 min and exposed to freshly diluted antibodies at room temperature. Origin Graphics Software® is used to plot the data. Basic statistical analyses are carried out with Excel software.

2.3. Immune epitope database (IEDB) analysis resource: antibody epitope prediction (AEP)

Two systems of web-tools are utilized: (1) to determine the frequency of the linear sequence and (2) to predict discontinuous and conformational epitopes, based on 3D protein structure. Recently, a new structure-based tool for the prediction of antibody epitopes is proposed, called ElliPro (Ponomarenko et al., 2008). Essentially, ElliPro is based on the geometrical properties of the protein structure and its usefulness in predicting the protein–antibody interaction. Each method has its own limitations. Parameters such as hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity of polypeptides chains have been correlated with the location of the epitopes. AEP tutorial recommends that when computing the score for a given residue *i*, the amino acids in an interval of the chosen length, centered around residue *i*, are considered. In other words, for a window (sequence) size n, the $i - (n-1)/2$ neighboring residues on each side of residue *i* were used to compute the score for residue *i*. Unless specified, the score for residue *i* is the average of the scale values for these amino acids. In general, a window size of 5–7 is appropriate for finding regions that may potentially be antigenic. We have applied as many methods as possible to ascertain and compare the immunogenicity scores of the peptides inhibited the binding of MEM mAbs between species. The web-tool system # 1 is supported by several methods that include:

(1) Chou and Fasman beta turn prediction which predicts beta turns in protein secondary structures (Chou and Fasman, 1978).
(2) Karplus and Schulz flexibility scale based on a flexibility scale is similar to classical calculation, except that the center is the first amino acid of the six amino acids window length, and there are three scales for describing flexibility instead of a single one (Karplus and Schulz, 1985).
(3) Kolaskar and Tongaonkar antigenicity scale makes use of physicochemical properties of amino acid residues and their frequencies of occurrence in experimentally known segmental epitopes was developed to predict antigenic determinants on proteins. Application of this method to a large number of proteins has shown by the authors that the method can predict antigenic determinants with about 75% accuracy which is better than most of the known methods (Kolaskar and Tongaonkar, 1990).
(4) Parker hydrophilicity prediction (Parker et al., 1986): in this method, hydrophilic scale based on peptide retention times during high-performance liquid chromatography (HPLC) on a reversed-phase column was constructed. A window of seven residues was used for analyzing epitope region. The corresponding value of the scale was introduced for each of the seven

Table 1
Reactivity of different anti-HLA-E monoclonal antibodies with non-classical HLA-Ib molecules (HLA-E, HLA-F & HLA-G). All values presented under HLA columns refer to MFI.

Monoclonal ID		Lot & dilutions	HLA-E	HLA-F	HLA-G
MEM-E/02	Lot # 2010	1/300 (n = 2)	15,833	14	31
	Lot # 2009	1/300 (n = 3)	8179	n/t	177
		1/300 (n = 3)	8585	n/t	0
MEM-E/06	Lot # 2010	1/300 (n = 2)	7658	5123	11
	Lot # 2009	1/300 (n = 3)	7422	n/t	111
MEM-E/07	Lot # 2010	1/300 (n = 2)	19,080	677	3370
	Lot # 2009	1/300 (n = 3)	10,584	n/t	1769
		1/1000 (n = 2)	2814	n/t	143
MEM-E/08	Lot # 2010	1/300 (n = 2)	13,022	2150	358
	Lot # 2009	1/300 (n = 3)	10,725	n/t	3238
		1/1000 (n = 2)	5435	n/t	735
3D12		1/800	6819	0	0
		1/1600	5909	0	0

(n/t: not tested).

residues and the arithmetical mean of the seven residue value was assigned to the fourth (i + 3), residue in the segment. The detailed steps to carry out the assessment of antigenic epitope are described in detail in http://tools.immuneeptiope.org/tools. The scores of each 5-mer or 6-mer residues can be recorded.

We have also utilized ElliPro (a terminology derived from Ellipsoid and Protrusion) web-tool (http://tools.immuneeptiope.org/tools/ElliPro) that implements modified version of Thornton's method (Thornton et al., 1986) which allows the prediction and visualization of antibody epitopes in protein sequences based on 3D-structures (Ponomarenko et al., 2008). In this study, we will be comparing ElliPro (the second system) with the sequences analyses listed above (the first system). The requirements of ElliPro are (1) protein sequence, (2) protein structure and (3) 4 letter code of Protein Data Base ID (PDB ID). We have used "3BZF" as PDB ID of the human non-classical MHC molecule HLA-E. The other PDB IDs tested include "1MHE" and "1KPR", which represent HLA-E molecules with one and two amino acids less, respectively. Based on the total number of amino acids in the sequence, we can select the appropriate PDB ID for the 3D structure.

3. Results

3.1. mAb 3D12 cross reacts with HLA-B and HLA-C but not with HLA-A, HLA-F or HLA-G

Both anti-HLA-Es, MEM-E/02 and 3D12 recognized HLA-E strongly and failed to recognize HLA-F and G (Table 1). On the other hand, MEM-E/06 recognized both HLA-E and HLA-F strongly and MEM-E/07 recognized HLA-E strongly but HLA-G about six fold less (Table 1). The two lots of MEM-E0/8 differed in their affinity for HLA-F and HLA-G, although the antibody reacted well with HLA-E. Fig. 1 shows binding of mAb 3D12 to HLA-E and different HLA-Ia alleles at different dilutions (expressed in Log_2 scale). Very similar to MEM-E/02 (Ravindranath et al., 2010a), *mAb* 3D12 bound to several HLA-Ia antigens. Based on the binding at different dilutions, the affinity of *mAb* 3D12 for HLA-Ia molecules can be ranked as follows: Cw*1701 > Cw*0403 > B*2708 = B*4006 > B*6701 = Cw*0702 = CW*1802 = B*1301 = B*1302 = B*3801 = B*4403 = B*5201 = B* 5703 = Cw*1203. mAb 3D12 reacts with B*2708. It is noteworthy because the antibody was developed by immunizing HLA-B-27+ transgenic mice with recombinant HLA-E. Possibly the specificity of B-27 allele of the transgenic mice is different from B*2708. MEM-E/02 also recognized most of these HLA-B and C alleles (Ravindranath et al., 2010a). Interestingly, both anti-HLA-E mAbs failed to recognize most of the HLA-A alleles.

mAb 3D12 is also inhibited by shared peptide sequence (^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142}) (Fig. 2). The inhibition is much stronger than that observed with MEM-E/02. Unlike MEM-E/02, the binding of mAb 3D12 was also inhibited dosimetrically (in a triplicate analyses) by ^{126}LNEDLRSWTA^{135} (MFI was 555 ± 18, at a peptide concentration of 0.44 μg/well, *data not shown*).

The α helical conformations for polypeptide chains are known to be thermodynamically stable of the regular secondary structures (http://www.friedli.com/herbs/phytochem/Proteins:html#secondary; Horovitz et al., 1992; Serrano et al., 1992; Chakrabartty and Baldwin, 1995). The inhibitory peptide sequences are located either at the terminal region (^{139}AAQI^{142}) or at the outside (^{115}QFAYDGKDY^{123} and ^{137}DT^{138}) of the α2 Helix. Since the location of the epitopes is outside the α2 Helix, we envisage that the binding of the mAb could be thermal sensitive. To assess the assumption, we have treated the microbeads, coated with the HLA class Ia alleles recognized by mAb 3D12, in a neutral buffer at 37 °C for 10 min and then mAb binding was tested. Fig. 3 shows about 30% decrease in the binding indicative of the thermal susceptibility of the binding domain, supporting the view that the thermal sensitivity of mAb-binding could be due to the location of the epitopes outside the α2 Helix.

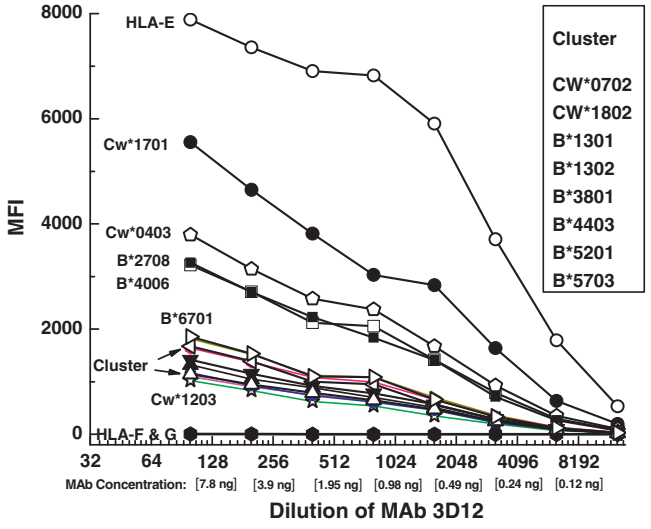

Fig. 1. Titration of binding of murine mAb 3D12 with HLA-E (heavy chain only). 1 μl of D312 (supplied as 200 ml of 0.5 mg/ml) was diluted 1/400 and serially diluted. 20 μl of diluted antibody is exposed to beads coated with HLA-E, HLA-F and HLA-G (bead volume for each HLA is 2 μg) and 20 μl of diluted antibody is exposed to beads coated with HLA-A, HLA-B and HLA-Cw (total bead volume of all beads: 3 μg). The binding of mAb to beads is determined by a PE-conjugated secondary antibody using a Luminex platform. IgG1 isotype and PBS were run as controls simultaneously. Negative and positive control beads were tested simultaneously as described earlier (Ravindranath et al., 2010a).

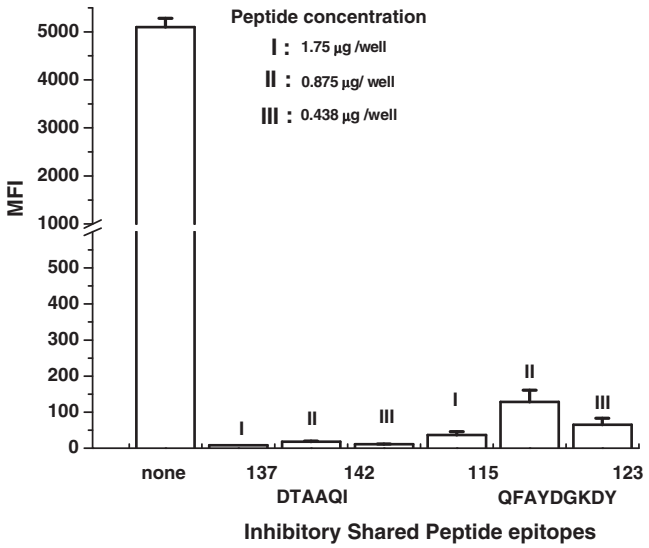

Fig. 2. mAb 3D12 is inhibited by shared peptide sequence as is mAb MEM-E/02. The inhibition is much stronger than that of MEM-E/02. Unlike MEM-E/02, the binding of mAb 3D12 was also inhibited dosimetrically but less efficiently by ^{126}LNEDLRSWTA^{135} (MFI with a peptide concentration of 0.44 µg/well was 555 ± 18, data not shown).

3.2. Linear epitope prediction results

The HLA-E protein sequence (Table 2) was submitted to different web tools (Chou and Fasman beta turn, Karplus and Schulz flexibility, Kolaskar and Tongaonkar antigenicity, Parker Hydrophilicity). Results are summarized in Table 3. While HLA-E specific sequences (^{65}RSARDTA^{71} and ^{143}SEQKSNDASE^{152}) proved to be highly antigenic, as evident from the various scores (for β turn (Chou & Fasman model), flexibility (Karplus & Schulz model), antigenicity (Kolasker & Tongaonker model) and hydrophobicity (Parker model) among HLA-E shared sequences (about 15), the equally high antigenicity and hydrophobicity scores were obtained for the discontinuous sequences, ^{117}AYDGKDY^{123} and ^{137}DTAAQIS^{143} (Table 3), the sequences that inhibited the binding of MEM-E02 to HLA-E. The scores were uniformly higher for ^{137}DTAAQIS^{143} than for ^{137}DTAAQI^{142}, suggesting that S^{142} may enhance immunogenicity. The numbers of HLA-Ia alleles that carry ^{137}DTAAQI^{142} are far more than those that carry ^{137}DTAAQIS^{143} (see Table 3). While ^{137}DTAAQIS^{143} is also found in HLA-G, -B and -C, the sequence is altered in HLA-A (^{137}DMAAQIS^{143}) and -F (^{137}DTAVQIS^{143}). The substitution of M^{138} in HLA-A and V^{140} in HLA-F, might have caused stearic hinderance to mAb binding to HLA-A and -F, respectively. Similarly, the scores are frequently higher for ^{117}AYDGKDY^{123} than for ^{115}QFAYDGKDY^{123}, but the incidence of ^{117}AYDGKDY^{123} is much higher than its longer sequence version. The antigenicity assessed based on the scores is much lower for other shared peptide epitopes.

3.3. ElliPro: discontinus epitope prediction of mAb E/02

ElliPro web tool evaluates the discontinuous epitopes from the 3D structure and amino acid sequences of HLA-E. After input of the HLA-E protein sequence (shown in Table 2) in box provided in the program, the 3D structure of HLA-E (e.g. 3BZF, the protein database ID (PDB ID) was entered and submitted for both linear and discontinuous sequence analyses. The scores of linear sequence analyses and the results of discontinuous sequences identified by ElliPro are presented in Table 3. The linear sequence data confirmed the data reported above. Based on the scores, the potent immunogenic epitopes can be ranked as follows: ^{143}SEQKSNDASE^{152} > ^{65}RSARDTA^{71} > ^{117}AYDGKDY^{123} > ^{137}DTAAQIS^{143}. The first two sequences are specific for HLA-E and the last two sequences are shared by HLA-B, -C, -F & -G. That both anti-HLA-E mAbs recognize the discontinuous sequences of ^{117}AYDGKDY^{123} and ^{137}DTAAQIS^{143} is supported by the ElliPro analyses (Table 4). Of the five discontinuous residues identified by ElliPro web-tool, two carry ^{117}AYDGKDY^{123} and ^{137}DTAAQIS^{143},

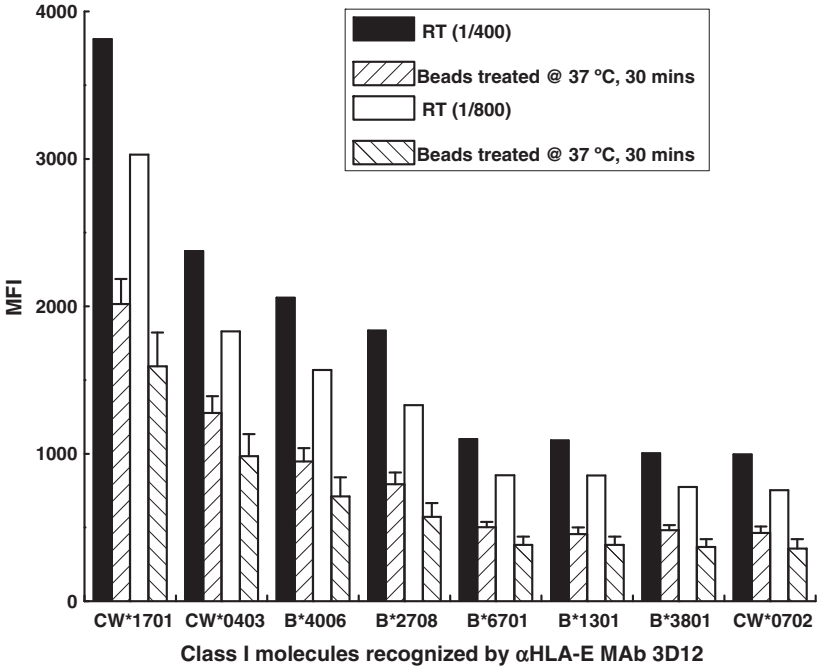

Fig. 3. Thermal alterations in the class Ia molecules coated on to beads as assessed by the binding of mAb 3D12. These HLA class Ia molecules are recognized by MAb 3D12. The binding of mAb 3D12 declines when the molecules are treated at higher temperature suggesting that the epitopes thermolabile and are located at thermal sensitive domain of the molecules. The α-helix conformations for polypeptide chains are generally the most thermodynamically stable of the regular secondary structures (http://www.friedli.com/herbs/phytochem/Proteins:html#secondary).

Table 2
Different web tools (methods) used for assessing accessibility by the αHLA-E monoclonal antibodies to various shared and unique peptide sequences of HLA-E and the outcome as assessed by the scores. The scores marked in bold refer to high scores observed for the peptide epitopes. The epitope criteria used by different methods are indicated (β-turn. Flexibility, antigenicity, hydrophilicity and ellipsoid projection). The highest scores are observed for HLA-E specific epitopes (^{65}RSARDTA^{71} or ^{143}SEQKSNDASE^{152}) and shared peptide sequences that inhibited the binding of αHLA-E mAbs to HLA-E, HLA-B and HLA-C (^{117}AYDGKDY^{123} and ^{137}DTAAQIS^{143}).

HLA-E peptide sequences	HLA alleles						Specificity	Method 1	Method 2	Method 3	Method 4	Method 5
	Classical class Ia			Non-classical class Ib				Chou& FasmanBeta turn prediction	Karphis& Schulz flexibility prediction	Kolaskar & Tangaonkar anligencity	Parker HydropMliciry prediction	ElliPra Linear sequence analysis
[Total number of amino acids]	A	B	Cw	F	G							
^{47}PRAPWMEQE^{55} [91]	1	0	0	0	0		A*3306	0.993	0.969	0.948	0.586/1.143/1.657	4.476
^{58}EYWDRETR^{65} [8]	5	0	0	0	0		A restricted	0.993	1.024	0.915	3.301/2.786	**5.234**
^{108}RSARDTA^{114} [7]	0	0	0	0	0		E restricted	**1.011**	**1.038**	0.952	**4.901**	3.733
^{117}AGSHTLQW^{97} [8]	1	10	48	0	0		Polyspecific	1.019	0.989	1.033	2.629/0.901	**4.648**
^{108}RPLRGYE^{114} [7]	24	0	0	0	0		A restricted	0.933	0.996	0.996	0.229	0.267
^{105}QFAYDGKDY^{123} [9]	1	104	75	0	0		Polyspecific	**1.059**	0.993	**1.001**	**2.629/3.201**	3.453
^{117}AYDGKDY^{123} [7]	491	831	271	21	30		Polyspecific	**1.204**	**1.061**	**0.989**	**4.243**	3.042
^{126}LNEDLRSWTA^{135} [10]	239	219	261	21	30		Polyspecific	1.046	1.039	0.983	2.443/2.329	2.263
^{137}DTAAQI^{142} [6]	0	824	248	0	30		Polyspecific	**0.813**	0.978	**1.065**	1.957	**4.421**
^{157}DTAAQIS^{143} [7]	0	52	4	0	30		Polyspecific	**0546**	0.97	**1.012**	**3.414**	**5.077**
^{143}SEQKSNDASE^{152} [10]	0	0	0	0	0		E restricted	**1.231**	1.222	0.923	**7.071/6443/6.257/6.514**	**6.71**
^{157}RAYLED^{162} [6]	0	1	0	0	0		B*8201	0.929	0.969	0.996	2.601	0.968
^{163}TCVEWL^{168} [6]	282	206	200	0	30		Polyspecific	0.841	0.929	1.115	−0.914	1.061
^{183}EPPKTHVT^{190} [8]	0	0	19	0	0		C restricted	1.029	1.042	1.044	3.043	3.842

confirming the suggestion that both these peptides are responsible for binding of mAb E/02.

Both anti-HLA-E mAb s (3D12 & E/02) rarely bind to HLA-A. Therefore, the ElliPro webtool is employed to find out whether the two discontinuous sequences are exposed or not. The results of the analyses of A*1101 and A*2402 (Table 4) reveal that the epitopes ^{117}AYDGKDY^{123} and ^{137}DTAAQIS^{143} in HLA-A molecules are not exposed.

4. Discussion

Both anti-HLA-E murine mAbs, MEM-E/02 (Menier et al., 2003; Lo Monaco et al., 2008, 2010) and 3D12 (Lee et al., 1998) failed to recognize HLA-F or HLA-G but specifically bound to HLA-E, as was reported earlier in literature. Both antibodies recognized in an identical manner several HLA-B and HLA-C alleles, though the rank of alleles, based on MFI intensity differed between the two mAbs. Strikingly, both mAbs failed to react with HLA-A alleles. The number of HLA-Ia antigens recognized by the antibodies may vary with dilution of antibody and the volume of beads added onto the wells (Fig. 3F in Ravindranath et al., 2010a). The antibody binding is not an artifact but varies in relation to antibody concentration and was dilution dependent (Fig. 1). The biological significance of HLA-Ia reactivity of both the mAbs is evident from the observations made on the HLA-Ia reactive natural antibodies seen in non-alloimmunized healthy individuals (Ravindranath et al., 2010c) and to HLA-Ia reactive non-donor specific antibodies observed in allograft recipients (manuscript in preparation).

The binding of E/02 and other mAbs (E/06, E/07, E/08) to both HLA-E and HLA-Ia molecules was inhibited by ^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142} (Ravindranath et al., 2010a). The same is precisely true for mAb 3D12. A striking difference is that the peptide sequence ^{126}LNEDRSWTA^{135}, that enhanced the binding of mAb MEM-E/02, inhibited the binding of 3D12 to HLA-E. The differential behavior of the two mAbs could be due to surface differences in the amino acid residues of the binding region of the Fab portion. The binding domains are also thermal sensitive (Fig. 3) and therefore easily susceptible to thermal denaturation.

Failure of binding of both anti-HLA-E mAbs to HLA-F and HLA-A could be due to inaccessibility of the epitopes due to substitution of the amino acid residues of the epitope (DTAAQI) or due to stearic hinderance (to recognize the epitope) caused by neighboring amino acids. Therefore, we have compared the region of the discontinuous epitopes for substitution of amino acids in the epitopes in HLA-F, HLA-G and HLA-A, to ascertain whether such substitution of amino acids cause stearic hinderance to antibody binding to the epitope. We also anticipated that certain amino acids might cause surface accessibility of the epitopes. Fig. 4 dissects the probable cause for epitope recognition and non-recognition in the amino acid sequences in the vicinity of the discontinuous epitopes (^{115}QFAYDGKDY^{123} and ^{137}DTAAQI^{142}).

The first row of Fig. 4 documents amino acids before and after the discontinuous epitopes in HLA-E. The discontinuous epitopes are located from 117 to 123 and from 137 to 142. First, the amino acid substitutions in the epitope sequences are examined in HLA-F and HLA-G. In HLA-F, alanine (A) in position 139 is substituted by valine (V) (double bar). The difference between the two amino acids is the presence of an extended methyl group (CH3) in valine. No such alterations were observed in HLA-G. Interestingly in HLA-A, to which both anti-HLA-E mAbs failed to bind, showed methionine (M) substitution of threonine (T) at position 138 (double bar). This is consistent for most of the HLA-A alleles. M differs from T in the presence of sulphur and in the absence of –OH groups. These substitutions in ^{137}DTAAQI^{142} may be one of the factors that impede binding of the mAbs to HLA-A.

Table 3
Prediction of ElliPro analysis: anti-HLA-E mAbs recognize the discontinuous epitopes. The epitopes of shared sequences are shown in bold and HLA-E specific unshared sequences are underlined. Note Y118, D119, G120, Y123 and D137, T138, A139, A140, Q141, I142, S143.

Predicted discontinous epitope(s) in **HLA-E** by ElliPro web tool	
1 GSHSLKYFHT SVSRPGRGEP RFISVGYVDD TQFVRFDNDA ASPRMVPRAP WMEQEGSEYW	
61 DRETRSARDT AQIFRVNLRT LRGYYNQSEA GSHTLQWMHG CELGPDRRFL RGYEQF*AYDG*	
121 *KDY*LTLNEDL RSWTAV*DTAA QI*SEQKSNDA SEAEHQRAYL EDTCVEWLHK YLEKGKETLL	
181 HLEPPKTHVT HHPISDHEAT LRCWALGFYP AEITLTWQQD GEGHTQDTEL VETRPAGDGT	
241 FQKWAAVVVP SGEEQRYTCH VQHEGLPEPV TLRWKP	
Residues	**Nos:**
S11, V12, S13, R14, P15, G16, R17, G18, E19, P20, R21	11
D37, N38, D39, A40, A41, S42, P43, R44, M45	9
E53, Q54, E55, G56, S57, E58, Y59, W60, D61, R62, T64, R65, S66, A67, R68, D69, T70, A71, Q72, I73, F74, R75, V76, N77, L78, R79, T80, L81, R82, G83, Y84, Y85, N86, Q87, S88, E89, A90, G91,S92, H93	40
Y118, **D119, G120, Y123**,	4
S132, V136, **D137, T138, A139, A140, Q141, I142, S143,** E144, Q145, K146, S147, N148, D149, A150, S151, E152,	18

In addition, unique amino acid substitutions are also observed in both HLA-G and HLA-F. At position 144, an acidic amino acid (glutamic acid, E) is substituted by a basic amino acid (Lysine, K) in HLA-G. Furthermore, at position 147 serine (S) was substituted with cystine (C). These two changes might have affected the exposure of the epitope and thus creating stearic hinderance to the antibody. Similarly, positions 146, 147 and 159 of HLA-F are much different from that of HLA-E. Substitution of lysine with phenylalanine (F) (position 146), serine (S) with tyrosine (Y) (position 147) and alanine (A) with glutamic acid (E) (position 150) and substitution of glutamic acid (E) at the position 114 and phenylalanine (F) at position 116 with histidine (H) in HLA-F might explain the failure of the antibody to recognize the discontinuous epitopes. It appears that H (see shaded box) at position 114 may interfere with binding of both anti-HLA-E mAbs to HLA-F and HLA-A alleles. Fig. 4 also shows several clear boxes in all other HLA-Ia and HLA-Ia alleles, which indicate that at these positions the amino acids are different between HLA-E and these alleles. Several amino acids are identical in the boxes between the alleles recognized by either mAbs or either one of the mAbs or in the alleles not recognized by mAbs, which signifies total irrelevance of these amino acids in epitope recognition. HLA-A shows several unique substitutions characteristic of non-binding alleles, in addition to M substitution of T at position 137, as observed in HLA-F, at the position 114 the acid amino acid, glutamic acid (E) is substituted with basic amino acids such as histidine (H) or arginine (R) or glutamine (Q). This acid-base shift may account for surface inaccessibility of the epitopes by the mAbs. mAb 3D12 is able to differentiate B*2708 from HLA-F and HLA-A alleles in spite of the presence of H[114]. It is speculated that aspartic acid (D) at position 116 might have enabled recognition of the epitope.

Webtool # 1 (for linear sequences) gave high scores for the two peptides ^{117}AYDGKDY^{123} and ^{137}DTAAQIS^{143} (Table 3). ElliPro web tool implements a residue-clustering algorithm and allows the prediction and visualization of antibody epitopes in a given structure of protein (HLA-E). ElliPro analyses (for discontinuous sequences) of HLA-E validated ^{117}AYDGKDY^{123} and ^{137}DTAAQIS^{143} as conformational and discontinuous epitope for both antibodies. Indeed these two peptides are the potential inhibitors. We wanted to find out whether the modified sequence of ^{137}DTAAQIS^{143} present in HLA-A is available for antibody recognition. In fact, both ^{137}DMAAQIS^{143} and ^{126}LNEDRSWTA^{135} were to be exposed in A1101 but remained cryptic in A2402 (Table 4). Obviously, M substitution for T did not enabled binding of both anti-HLA-E mAbs. Thus, this web tools provide insight into why the two anti-HLA-E mAbs did not bind to HLA-A, -F and -G.

In conclusion, both anti-HLA-E mAbs, 3D12 and MEM-E/02, recognize HLA-E, HLA-B and HLA-C alleles but not HLA-F, HLA-G and HLA-A alleles. Both anti-HLA-E mAbs are not truly HLA-E specific antibody, since such antibodies are expected to bind only

Table 4
ElliPro analysis predicts that anti-HLA-E mAbs cannot recognize the epitopes in HLA-A*1101 or A*2402. The data suggests why anti-HLA-E mAbs are not binding to A-Locus even when their epitopes are present. DTAAQI is altered to DMAAQI. Note that in A*1101 as well as in A*2402, the sequences carrying Y118, D119, G120, Y123 or T138, are missing ElliPro analysis suggesting that the peptide sequences ^{117}AYDGKDY^{123} and ^{137}DTAAQIS^{143} are not recognized during the webtool analysis.

A. Predicted discontinous epitope(s) on PDB ID 1Q94: structures Of HLA-A1101 in complexed with immunodominant nonamer and decamer HIV-1 epitopes
HLA – A1101 amino acid sequence
gshsmryfft svsrpgrgep rfiavgyvdd tqfvrfdsda asqkmeprap wieqegpeyw dqetrnmkah sqtdranlgt lrgyynqsed gshtiqimyg cdvgpdgrfl rgyrqd**aydg kdy**ialnedl rswtaa**dmaa qi**tkrkweav haaeqrrvyl egrcvdglrr ylengketlq rtdppkthmt hhpisdheat lrcwalgfyp aeitltwqrd gedqtqdtel vetrpagdgt fqkwaavvvp sgeeqrytch vqheglpkpl tlrw

(1) Score 0.766, residue 43: S13, R14, P15, G16, R17, G18, E19, P20, R75,A76, G79, T80, L81, R82, G83,Y84, Y85, N86, Q87, S88, E89, D90, G91, S92, H93, D119, T134, A135, A136, **D137, M138, A139, A140, Q141, I142**, T143, K144, R145, K146, E148, A149, V150, H151
(2) Score 0.619, residue 11: N127, E128, D129, L130, R131, S132, A153, E154, R157, V158, E161

B. Predicted discontinuous epitope(s) on PDB ID 2BCK: crystal structure of HLAa-A2402 complexed with a telomerase peptide
(1) Score 0.766, residue 142: G1, S2, S38, D39, A40, A41, S42, Q43, R44, M45, E46, P47, R48, A49, P50, W51, I52, E53, Q54, E55, G56, P57, E58, Y59, W60, D61, E62, E63, T64, G65, V103, G104, S105, D106, G107, R108, F109, L110, L130, L215, T216, W217, Q218, R219, D220, G221, E222, D223, Q224, T225, Q226, D227, T228, E229, V231, W244,A245, A246, V247, V248, V249, P250, S251, G252, E253, E254, R256, Y257, T258, C259, H260, V261, Q262, H263, E264, G265, L266, P267, K268, P269, L270, T271, L272, R273, W274, E275, P276, G277, S278, G279, G280, G281, L282, N283, D284, I285, F286
(2) Score 0.728, residue 146: G1, S2, S38, D39, A40, A41, S42, Q43, R44, M45, E46, P47, R48, A49, P50, W51, I52, E53, Q54, E55, G56, P57, E58, Y59, W60, D61, E62, E63, T64, V103, G104, S105, D106, G107, R108, F109, L110, L130, D129, R131, R157, A158, E161, G162, T163, V165, D166, G167, L168, R169, R170, Y171, L172, E173, N174, G175, K176, E177, T178, L179, Q180, R181, T182, D183, P184, P185, T187, H188, M189, T190, H191, H192, P193, I194, S195, D196, H197, E198, A199, T200, L201, R202, C203, W204, Y209.
I213, L215, T216, W217, Q218, R219, D220, G221, E222, D223, Q224, T225, Q226, D227, T228, E229, V231, W244, A245, A246, V247, V248, V249, P250, S251, G252, E253, E254, R256, Y257, T258, C259, H260, V261, Q262, H263, E264, G265, L266, P267, K268, P269, L270, T271, L272, R273, W274, E275, P276, G277, S278, G279, G280, G281, L282, N283, D284, I285, F286

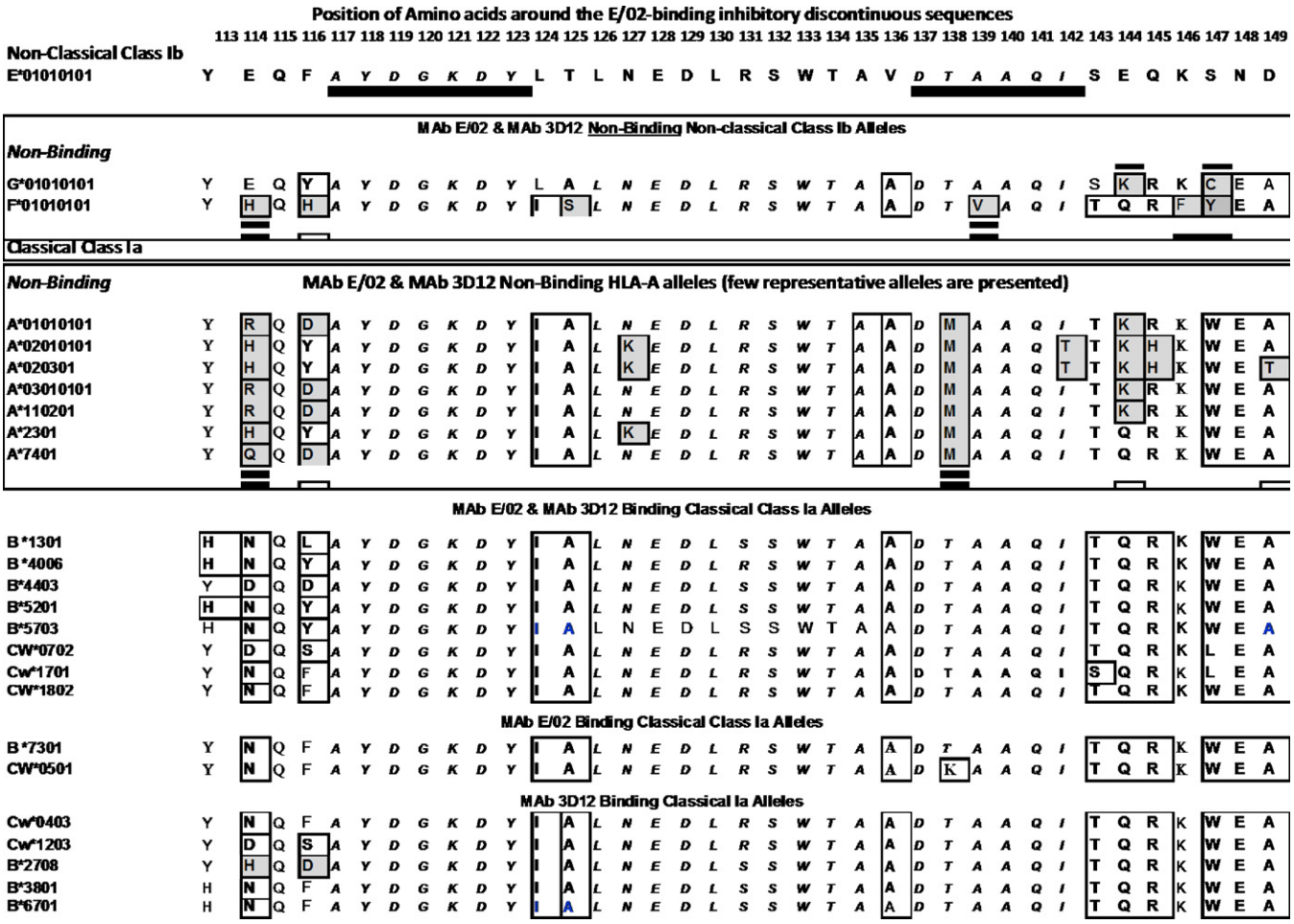

Fig. 4. mAbs 3D12 & E/02 fail to recognize HLA-A, HLA-F and HLA-G but binds to HLA-E, HLA-B & HLA-C. mAb binding discontinuous epitopes are located in the outer edge of α2 Helix. ^{115}QFAYDGKDY^{123} and ^{126}LNEDRSWTA^{135} are not in the region of α2 Helix. Former is potentially inhibitory. ^{137}DTAAQI^{142}. Is on the edge of α2 Helix. The boxes indicate the amino acids at different locations that are different from that found in HLA-E. There are box types. (1) Clear box (without shade) which simply indicate that the amino acids at the specific locations are different from that found in HLA-E. (2) Shaded box signifying amino acids that may affect recognition of the discontinuous epitopes by the antibodies. There are three kinds: (a) Alterations in the peptide sequence of the epitope such as methionine in position 138 (indicated by double bars) and valine at position 139 in HLA-F (indicated by double bars) may affect the binding of the antibody directly. For example both the antibodies do not bind to HLA-A, possibly because of methionine at position # 138. (b) Other shaded boxes underlined by single bar are amino acids that may influence and induce alterations in the expression of the discontinuous epitopes. Though they are not directly involved in direct binding, these unique amino acids such Histidine replacing glutamic acid at position 114 may affect orientation of the discontinuous epitopes and impair antibody recognition. Some such amino acids maybe located at a distance as shown by empty bars. (c) These are some of the amino acids that may affect the orientation and exposure of the epitopes and affect antibody binding. mAbs E/02 and 3D12 do not bind to HLA-F and HLA-G due primary alteration at position # 139 (V) at HLA-F and secondary alteration at positions # 114 (H) # 146 (F), #147 (Y) & #150 (E) in HLA-F and at positions # 144 (K) and # 147 (C) in HLA-G. Failure of both antibodies to bind to HLA-A could be due to primary alteration at position #138 (M) and at secondary alteration at position # 114 (H/R/Q) and due to tertiary alterations indicated by empty box. An exception is also seen. mAb 3D12 binds to B*2708 which has also histidine at the position # 114 but recognized by 3D12. May be other amino acids negate the interference of histidine in B*2708 allele. Secondary and teritiary alterations may significantly affect the orientation of critical epitope.

to peptide sequences restricted to HLA-E, such as ^{65}RSARDTA^{71} or ^{143}SEQKSNDASE^{152}. As both anti-HLA-E mAbs are inhibited by ^{117}AYDGKDY^{123} and ^{137}DTAAQIS^{143}, these discontinuous sequences may represent the conformational and discontinuous epitopes of these mAbs. The web-tools that recognize both linear and discontinuous epitopes validated the finding that both sequences could be the epitopes for mAb 3D12 and MEM-E/02. Obviously, the two peptides may be the most immunogenic epitopes in mice as well as in humans, as reported in a recent report (Ravindranath et al., 2010c). The web-tools favor the contention that the sequences unique for HLA-E (^{65}RSARDTA^{71} or ^{143}SEQKSNDASE^{152}) can also have potential immunogenicity. Such an antibody that recognizes the unique HLA-E specific peptides would be highly invaluable for immunochemical localization of HLA-E molecules in immune cells, inflammatory tissues and tumor cells.

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References

Bianchini, M., Levy, E., Zucchini, C., Pinski, V., Macagno, C., De Sanctis, P., Valvassori, L., Carinci, P., Mordoh, J., 2006. Comparative study of gene expression by cDNA microarray in human colorectal cancer tissues and normal mucosa. Int. J. Oncol. 29, 83–94.

Chakrabartty, A., Baldwin, R.L., 1995. Stability of alpha helices. Adv. Protein Chem. 46, 141–176.

Chou, P.Y., Fasman, G.D., 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. Relat. Areas Mol. Biol. 47, 45–148.

Coupel, S., Moreau, A., Hamidou, M., Horejsi, V., Soulillou, J.P., Charreau, B., 2007. Expression and release of soluble HLA-E is an immunoregulatory feature of endothelial cell activation. Blood 109, 2806–2814.

Derre, L., Corvaisier, M., Charreau, B., Moreau, A., Godefroy, E., Moreau-Aubry, A., Jotereau, F., Gervois, N., 2006. Expression and release of HLA-E by melanoma cells and melanocytes: potential impact on the response of cytotoxic effector cells. J. Immunol. 177, 3100–3107.

Horovitz, A., Matthews, J.M., Fersht, A.R., 1992. Alpha-helix stability in proteins II. Factors that influence stability at an internal position. J. Mol. Biol. 227, 560–568.

Karplus, P.A., Schulz, G.E., 1985. Prediction of chain flexibility in proteins – a tool for the selection of peptide antigens. Naturwissenschaft 72, 212–213.

Kolaskar, A.S., Tongaonkar, P.C., 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 276, 172–174.

Kren, L., Muckova, K., Lzicarova, E., Sova, M., Vybihal, V., Svoboda, T., Fadrus, P., Smrcka, M., Slaby, O., Lakomy, R., Vanhara, P., Krenova, Z., Michalek, J., 2010. Production of immune-modulatory nonclassical molecules HLA-G and HLA-E by tumor infiltrating amoeboid microglia/macrophages in glioblastomas: a role in innate immunity? J. Neuroimmunol. 220, 131–135.

Lee, N., Goodlett, D.R., Ishitani, A., Marquardt, H., Geraghty, D.E., 1998. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA Class I signal sequences. J. Immunol. 160, 4951–4960.

Lin, Y.J., Wan, L., Wu, J.Y., Sheu, J.J., Lin, C.W., Lan, Y.C., Lai, C.H., Hung, C.H., Tsai, Y., Tsai, C.H., Lin.F T.H., Lin, J.G., Hsueh, K.C., Huang, Y.M., Chang, J.S., Tsai, F.J., 2009. HLA-E gene polymorphism associated with susceptibility to Kawasaki disease and formation of coronary artery aneurysms. Arthritis Rheum. 60, 604–610.

Lo Monaco, E., Sibilio, L., Melucci, E., Tremante, E., Suchànek, M., Horejsi, V., Martayan, A., Giacomini, P., 2008. HLA-E: strong association with beta2-microglobulin and surface expression in the absence of HLA class I signal sequence-derived peptides. J. Immunol. 181, 5442–5450.

Lo Monaco, E., Tremante, E., Cifaldi, L., Fruci, D., Giacomini, P., 2010. HLA-E and the origin of immunogenic self-HLA epitopes. Mol. Immunol. 47, 1660–1662.

Malmberg, K.J., Levitsky, V., Norell, H., de Matos, C.T., Carlsten, M., Schedvins, K., Rabbani, H., Moretta, A., Söderström, K., Levitskaya, J., Kiessling, R., 2002. IFN gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD 94/NKG2A-dependent mechanism. J. Clin. Invest. 110, 1515–1523.

Menier, C., Saez, B., Horejsi, V., Martinozzi, S., Krawice-Radanne, I., Bruel, S., Le Danff, C., Reboul, M., Hilgert, I., Tableau, M., Larrad, M.L., Pla, M., Carosella, D., Rouas-Freiss, N., 2003. Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules. Hum. Immunol 64, 315–326.

Parker, J.M., Guo, D., Hodges, R.S., 1986. New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. Biochemistry 25, 5425–5432.

Ponomarenko, J., Bui, H.-H., Li., W., Fusseder, N., Bourne, P.E., Sette, A., Peters, B., 2008. ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinformatics 9, 514–521.

Ravindranath, M.H., Taniguchi, M., Chen, C.W., Ozawa, M., Kaneku, H., El-Awar, N., Cai, J., Terasaki, P.I., 2010a. HLA-E monoclonal antibodies recognize shared peptide sequences on classical HLA class Ia: relevance to human natural HLA antibodies. Mol. Immunol. 47, 1121–1131.

Ravindranath, M.H., Kaneku, H., El-Awar, N., 2010b. HLA-E monoclonal antibody MEM-E/02 binds to discontinuous but shared peptide sequences on HLA B & C heavy chains not treated by acid. Mol. Immunol. 47., 1663–1664.

Ravindranath, M.H., Kaneku, H., El-Awar, N., Morales-Buenrostro, L.E., Terasaki, P.I., 2010c. Antibodies to HLA-E in nonalloimmunized males: pattern of HLA-Ia reactivity of anti-HLA-E-positive sera. J. Immunol. 185, 1935–1948.

Serrano, L., Sancho, J., Hirshberg, M., Fersht, A.R., 1992. Alpha-helix stability in proteins I. Empirical correlations concerning substitution of side-chains at the N and C-caps and the replacement of alanine by glycine or serine at solvent-exposed surfaces. J. Mol. Biol. 227, 544–559.

Thornton, J.M., Edwards, M.S., Taylor, W.R., Barlow, D.J., 1986. Location of 'continuous' antigenic determinants in the protruding regions of proteins. EMBO J. 5, 409–413.

Wei, X.H., Orr, H.T., 1990. Differential expression of HLAE HLA-F, and HLA-G transcripts in human tissue. Hum. Immunol. 29, 31–142.