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Polarity of the P1 anchor residue determines peptide binding specificity between HLA-A*3101 and HLA-A*3303

Key words:
HLA-A*3101; HLA-A*3303; peptide; anchor

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Abstract: A previous pool sequence analysis showed that HLA-A*3101 and HLA-A*3303 binding peptides have the same anchor residues at P2 and the C-terminus, the only difference being that HLA-A*3303 binding peptides have two additional P2 anchor residues. Using a stabilization assay with RMA-S transfectants expressing HLA-A*3101 and human β_2-microglobulin, we tested the binding of 232 8- to 11-mer peptides carrying HLA-A*3303 anchor residues to HLA-A*3101. One hundred of these peptides (43.1%) bound to HLA-A*3101, confirming that these residues are also anchors for HLA-A*3101. Although aromatic hydrophobic P2 residues were previously shown to be stronger anchors than aliphatic hydrophobic P2 residues in HLA-A*3303 binding peptides, we detected no significant difference in HLA-A*3101 binding affinity between peptides carrying aromatic or aliphatic hydrophobic P2 residues. Statistical analysis previously showed a positive effect of negatively charged P1 residues and a negative effect of positively charged P1 residues for peptide binding to HLA-A*3303. In contrast such analysis demonstrated a positive effect of positively charged P1 residues and a negative effect of negatively charged P1 residues for peptide binding to HLA-A*3101. Analysis using mutated peptides confirmed these results. The present study therefore demonstrates that peptide binding specificity between HLA-A*3101 and HLA-A*3303 is determined by the polarity of the P1 anchor residue.

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HLA-A31 is a common HLA-A allele in Asia (1). Of the four reported HLA-A31 subtypes (A*3101, A*3102, A*3103 and A*3104) (2), HLA-A*3101 is the most common (1). Pool sequencing of natural ligands demonstrated that HLA-A*3101 and HLA-A*3303 binding peptides have the same C-terminal anchor (Arg) and similar P2 anchor residues: HLA-A*3101 ligands have four possible hydrophobic P2 anchor residues (Leu, Phe, Tyr or Val) while HLA-A*3303 ligands have six possible hydrophobic P2 anchor residues (Ala, Ile, Leu, Phe, Tyr or Val) (3). Our recent study of HLA-A*3303 peptide binding using 232 peptides containing HLA-A*3303 anchor residues confirmed these P2 and C-terminal anchor residues for HLA-A*3303 (4). These findings imply that HLA-A*3101 binding pep-

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tides should be capable of binding to HLA-A*3303. However, a previous study showed that natural HLA-A*3101 ligands are different from those of HLA-A*3303, suggesting that another position(s) may be critical for specific peptide binding to these HLA class I alleles. Our previous study demonstrated that negatively-charged residues at P1 also function as anchors for HLA-A*3303 binding (4), suggesting that P1 anchor residues may be responsible for the peptide binding specificity between HLA-A*3101 and HLA-A*3303.

In the present study, we investigated the role of anchor residues in peptide binding to HLA-A*3101 using an HLA-A*3101 stabilization assay with RMA-S transfectants expressing HLA-A*3101 and human β_2 microglobulin. Moreover, we attempted to clarify what determines peptide binding specificity between HLA-A*3101 and HLA-A*3303.

Material and methods

Cells

The HLA-A*3101 gene was transfected by electroporation into human β_2 microglobulin-transfected RMA-S (RMA-S-β_2 m) cells. Following selection with hygromycin B, resistant cells were isolated from separate wells. Surface expression of HLA-A*3101 molecules on transfected cells was confirmed by flow cytometry using the anti-HLA class I α3 domain monoclonal antibody (mAb) TP25.99 (5). RMA-S-A*3303 cells were previously generated (4). RMA-S-hβ_2m cells expressing HLA-A*3101 (RMA-S-A*3101) and RMA-S-A*3303 cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B.

Effect of P2 residues on the binding of 8- to 11-mer peptides to HLA-A*3101

P2 residues	NBP*/NTP*	MBR
L	24/66 (36.4%)	0.53
V	26/54 (48.1%)	0.74
F	8/17 (47.1%)	0.65
Y	14/22 (63.6%)	0.95
A	16/37 (43.2%)	0.70
I	12/36 (33.3%)	0.44
Total	100/232 (43.1%)	0.65

* NBP: number of binding peptides
* NTP: number of tested peptides

Table 1

Peptides

The amino acid sequences of Env, Gag, Pol and Nef from the human immunodeficiency virus type-I (HIV-1) SF2 strain were screened for HLA-A*3303 binding peptide motifs (3). Candidate peptides were synthesized using an automated multiple peptide synthesizer (Shimadzu Model PSSM-8) with the Fmoc strategy followed by cleavage as previously described (6). The peptides were shown to be homogenous by liquid secondary ionmass spectrometry and reverse-phase HPLC.

Stabilization assay

Peptide binding to HLA-A*3101and HLA-A*3303 was determined using the stabilization assay as described previously (7). Briefly, RMA-S-A*3101 or RMA-S-A*3303 cells were cultured at 26°C for 14~18 h. Cells (2×10^5) in 50 µl phosphate-buffered saline supplemented with 20% fetal calf serum (PBS-FCS) were incubated with a 50 µl solution of peptides (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, or 10^{-7} M) at 26°C for 1 h and then at 37°C for 3 h. After washing with PBS-FCS, the cells were incubated for 30 min on ice with an appropriate dilution of mAb TP25.99. After two washes with PBS-FCS, the cells were incubated for 30 min on ice with an appropriate dilution of fluorescein isothiocyanate conjugated sheep IgG anti-mouse Ig antibody (Silenus Laboratories, Hawthorn, Australia). The cells were washed three times with PBS-FCS and then the fluorescence intensity was measured using a FACS Calibur (Becton Dickinson, Mountain View, CA, USA). RMA-S-A*3101 and RMA-S-A*3303 cells cultured at 26°C and 37°C were stained with mAb TP25.99 under the same experimental conditions and used as controls.

Analysis of peptide binding to HLA-A*3101 and HLA-A*3303 molecules

Peptides which, when incubated with cells at a concentration of 10^{-3} M, gave more than 25% of the mean fluorescence intensity (MFI) of RMA-S-A*3101 or RMA-S-A*3303 cells cultured at 26°C were defined as binding peptides. This criterion was previously used for HLA-B*3501 (8, 9) and HLA-A*2402 (10) binding peptides. The relative MFI of RMA-S-A*3101 or RMA-S-A*3303 cells was calculated by subtracting the MFI value of peptide-unloaded RMA-S-A*3101 or RMA-S-A*3303 cells stained with mAb TP25.99 from the MFI value of peptide-loaded RMA-S-A*3101 or RMA-S-A*3303 cells stained with mAb TP25.99. The half-maximal binding level (BL_{50}) was calculated as the peptide concentration yielding the half-maximal MFI. Binding peptides were classified into three categories according to their BL_{50}: high binding ($BL_{50}<10^{-5}$ M), medium binding ($10^{-5}\leq BL_{50}<10^{-4}$) and low binding ($BL_{50}\geq10^{-4}$). High, me-

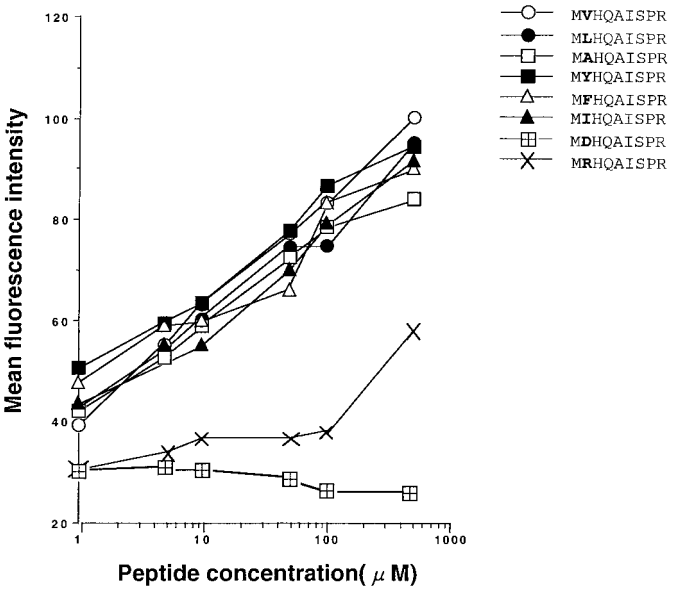

Fig. 1. **The effect of substitution at P2 on binding of the 9-mer peptide MVHQAISPR to HLA-A*3101.** The BL_{50} (M) of these peptides is: M**V**HQAISPR 2.5×10^{-6}, M**L**HQAISPR 2.4×10^{-6}, M**A**HQAISPR 4.1×10^{-6}, M**Y**HQAISPR 2.1×10^{-7}, M**F**HQAISPR 1.1×10^{-6}, M**I**HQAISPR 2.4×10^{-6}, M**D**HQAISPR no binding, M**R**HQAISPR 9.2×10^{-2}.

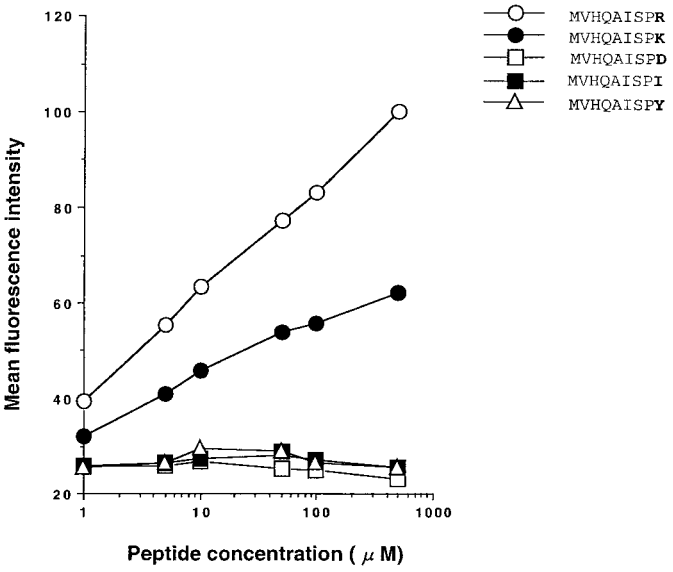

Fig. 2. **The effect of substitution at the C-terminus on binding of the 9-mer peptide MVHQAISPR to HLA-A*3101.** The BL_{50} (M) of these peptides is: MVHQAISP**R** 2.5×10^{-6}, MVHQAISP**K** 3.1×10^{-5}, MVHQAISP**D**: no binding, MVHQAISP**I**: no binding, and MVHQAISP**Y**: no binding.

dium, low and non-binding peptides were assigned values of 3, 2, 1 and 0, respectively. The mean binding rank (MBR) of a group of peptides was then calculated. For example, if in a group of 10 peptides, three were high binding, one was medium binding, one was low binding and five were non-binding, then the MBR would be 12/10=1.20. The MBR of each amino acid group and each peptide length was analyzed by the Mann-Whitney U-test (Stat View 4.02; Abacus Concepts, Berkeley, CA, USA).

Results and discussion

HLA-A*3303 binding peptides bind to HLA-A*3101

RMA-S transfectants expressing HLA-A*3101 were generated following transfection of the HLA-A*3101 gene into RMA-S-hβ_2m cells. Transfectant (RMA-S-A*3101) cells cultured at 26°C had a much higher HLA-A*3101 surface expression level than those cultured at 37°C. HLA-A*3101 surface expression on cells cultured at 26°C rapidly decreased following incubation at 37°C (data not shown). These transfectants are therefore suitable for use in stabilization assays using the same conditions as described previously (8, 10, 11). We therefore used this method to evaluate peptide binding to HLA-A*3101.

We tested the binding of 232 peptides (45 8-mer, 79 9-mer, 55 10-mer and 53 11-mer peptides) with HLA-A*3303 anchor residues at P2 (Ala, Ile, Leu, Val, Phe or Tyr) and the C-terminus (Arg) (4) to HLA-A*3101 molecules. Although Ala and Ile at P2 have not previously been shown to be anchors for HLA-A*3101 (4), peptides with these P2 residues were analyzed to clarify peptide binding specificity between HLA-A*3101 and HLA-A*3303. 100 of these peptides bound to HLA-A*3101 molecules (Table 1). Although the

Table 2

Residues forming the A-pocket of HLA-A*3101 and HLA-A*3303

	Residues										
Allele	5	7	59	62	63	66	99	159	163	167	171
HLA-A*3101	M	Y	Y	Q	E	N	Y	Y	T	W	Y
HLA-A*3303	M	Y	Y	R	N	N	Y	Y	T	W	Y

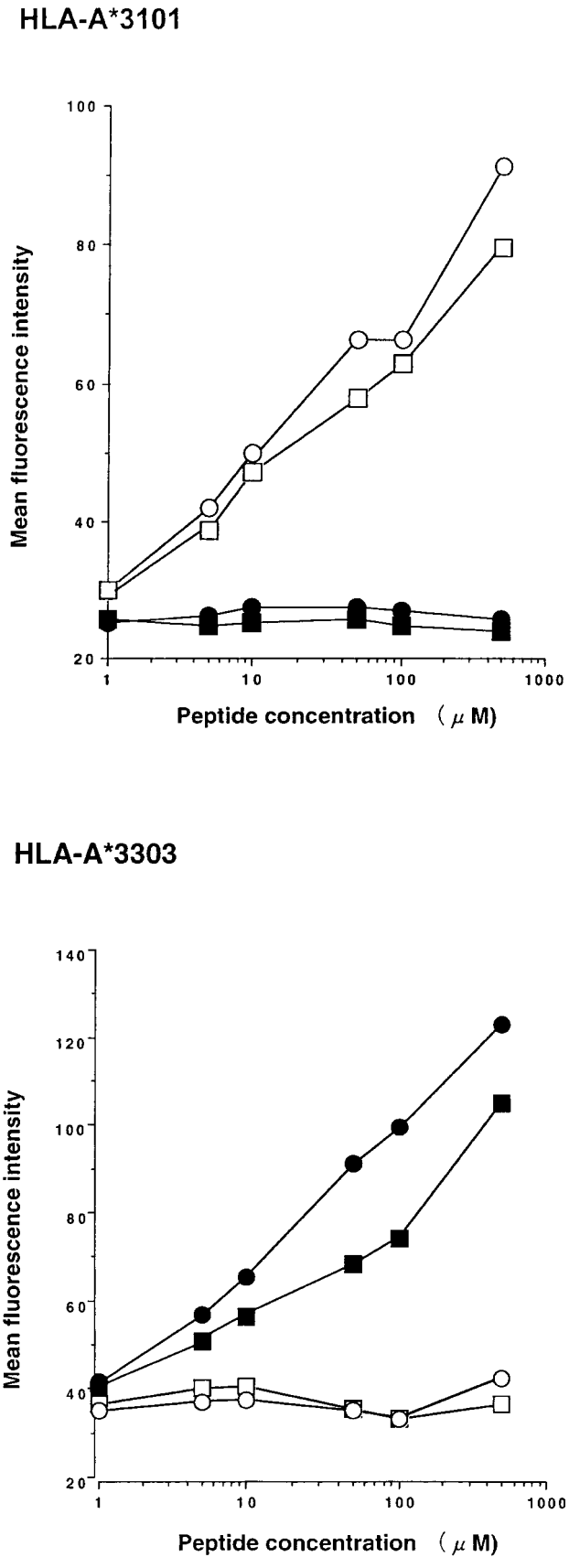

Fig. 3. **The effect of substitution at P1 on peptide binding to HLA-A*3101 and HLA-A*3303. A) Binding of the 9-mer peptide RANSPTRR and its mutants to HLA-A*3101.** The BL_{50} (M) of these peptides is: **R**ANSPTRR 1.4×10^{-5}, **K**ANSPTRR 2.3×10^{-5}, **E**ANSPTRR no binding, **D**ANSPTRR no binding. **B) Binding of the 8-mer peptide EVAQRAYR and its mutants to HLA-A*3303.** The BL_{50} (M) of these peptides is: **E**VAQRAYR 1.8×10^{-6}, **D**VAQRAYR 2.5×10^{-6}, **R**VAQRAYR no binding, **K**VAQRAYR no binding.

percentage of peptides which bound to HLA-A*3101 (43.1%) was slightly lower than that which bound to HLA-A*3303 (51.3%), these results indicate that HLA-A*3303 P2 and C-terminal anchor residues also function as anchors for HLA-A*3101.

HLA-A*3101 has the same hydrophobic P2 anchor residues as HLA-A*3303 but shows no preference between aromatic or aliphatic residues

The binding of peptides with Ala or Ile at P2 to HLA-A*3101 was not significantly lower than that of peptides containing the four previously identified HLA-A*3101 P2 anchor residues (Table 1). Analysis using P2 mutants of a high affinity binding peptide (MVHQAISPR) showed that HLA-A*3101 binding was almost identical between peptides with Ala or Ile at P2 and peptides with Leu, Val, Phe or Tyr at P2 (Fig. 1). These results together with those above demonstrate that, in addition to Leu, Phe, Tyr and Val, Ala and Ile are also P2 anchors for HLA-A*3101.

HLA-A*3303 binds more strongly to peptides with aromatic hydrophobic P2 residues (Phe or Tyr) than to peptides with aliphatic hydrophobic P2 residues (Ala, Ile, Leu or Val) (4). However, statistical analysis showed no significant difference in HLA-A*3101 affinity between peptides with the two types of hydrophobic P2 residues. Furthermore, P2 mutants with aromatic or aliphatic hydrophobic P2 residues showed no significant difference in HLA-A*3101 binding (Fig. 1). HLA-A*3101 and HLA-A*3303 differ by only two residues (62 and 63), which face the A-pocket and bind to P1 residues (12). However, the observation that HLA-A*3101 and HLA-A*3303 exhibit a difference in P2 residue binding suggests that residues 62 and 63 are also involved in formation of the B-pocket and hence P2 residue binding. In support of this, previous X-ray crystallographic analysis showed that residues 62 and 63 of HLA-B27 bind to the P2 residue of binding peptides (13).

HLA-A*3101 has the same C-terminal anchor residues as HLA-A*3303

A previous pool sequence analysis of HLA-A*3101 and HLA-A*3303 ligands showed that Arg at P9 is an anchor residue for

these alleles (3). Our recent analysis using an HLA-A*3303 stabilization assay revealed that in addition to Arg, which is strong C-terminal anchor residue, Lys is weak C-terminal anchor residue (4). To confirm the role of these positively charged C-terminal residues in HLA-A*3101 peptide binding, binding of four mutant peptides with Lys, Asp, Ile or Tyr at P9 was compared to that of original peptide MVHQAISPR. Only peptides with the positively charged residues Lys and Arg at P9 bound to HLA-A*3101 (Fig. 2). The affinity of the peptide with Lys at P9 was weaker than that with Arg. Thus, as for HLA-A*3303 binding peptides, both positively charged residues are C-terminal anchors for HLA-A*3101 with Arg being a much stronger anchor residue than Lys. This can be explained by the fact that in these two HLA class I alleles, the residues forming the F-pocket are the same (residues 70, 74, 76, 77, 80, 81, 95, 97, 114, 116, 142, 143 and 147).

Effect of P1 residues on binding to HLA-A*3101

Amid acids	Number of peptides	MBR	Significance
Y F W H	17	0.41	
L V I M	51	0.84	
S T C	38	0.66	
P	17	0.24	
G A	40	0.65	
Q N	15	0.67	
R K	30	1.37	$P<0.001$
D E	24	0.04	$P<0.05$

Table 3

HLA-A*3101 and HLA-A*3303 binding specificity is conferred by the P1 anchor residue

Of the 144 peptides which could bind to either or both HLA-A*3101 and HLA-A*3303, 25 bound to only HLA-A*3101 and 43 bound to only HLA-A*3303 (Table 4). This suggests that there is peptide binding specificity between these two HLA class I alleles. As mentioned above, HLA-A*3101 and HLA-A*3303 differ at residues 62 and 63 (Table 2). An X-ray crystallographic study showed that these two residues face the A-pocket (12). Glu at residue 63 of HLA-A*3101 forms an A-pocket with a positively-charged environment suitable for binding to negatively-charged P1 residues, while Arg at residue 62 of HLA-A*3303 forms a negatively-charged A-pocket suitable for binding to positively charged residues at P1. This suggests that the P1 residue is critical for peptide binding specificity between HLA-A*3101 and HLA-A*3303.

In support of a role for P1 anchors, our previous statistical analysis showed a positive effect of negatively-charged P1 residues for peptide binding to HLA-A*3303 (4). To define the influence of P1 residues on peptide binding to HLA-A*3101, we performed a similar analysis of P1 residues for the 232 peptides analyzed in the above stabilization assay. The MBR of individual amino acids or groups of amino acids was tested for statistical significance in a Mann-Whitney U-test. A positive and negative effect was found for positively- and negatively-charged residues at P1, respectively (Table 3), providing further support for the role of P1 residues in peptide binding specificity.

To directly examine the effect of charged residues at P1 on peptide binding to HLA-A*3101, we synthesized P1 mutants of a peptide (RANSPTRR) which showed high binding to HLA-A*3101. Two peptides containing the positively-charged residues Arg and Lys at P1 effectively bound to HLA-A*3101, while two peptides containing the negatively-charged residues Glu and Asp at P1 failed to bind (Fig. 3A). The same experiment was performed with P1 mutants of a peptide (EVAQRAYR) which showed high binding to HLA-A*3303. In contrast to HLA-A*3101 binding peptides, peptides with negatively charged residues at P1 effectively bound to HLA-A*3303 while peptides with positively charged residues at P1 failed to bind (Fig. 3B). These results support the statistical analyses and indicate that P1 residue polarity determines peptide binding specificity for HLA-A*3101 and HLA-A*3303.

Cross-binding of peptides to HLA-A*3101 and HLA-A*3303

	Number of peptides binding to both or either HLA-A*3101 and/or HLA-A*3303			
Amino acid at P1	A*3101	A*3303	Both	Total
Y F W H	0	8	5	13
L V I M	6	6	25	37
S T C	1	6	14	21
P	0	0	3	3
G A	3	2	11	16
Q N	0	4	6	10
R K	15	0	10	25
D E	0	18	1	19
Total	25	44	75	144

Table 4

Cross-binding of peptides to HLA-A*3101 and HLA-A*3303

Of the 232 peptides analyzed in this and our previous study (4), 76 bound to both HLA-A*3101 and HLA-A*3303 (Table 4). These findings suggest the possibility that epitope peptides with residues

other than Glu and Asp at P1 are presented to T cells by both HLA class I alleles. In support of this, a CTL epitope (LLGPGPYR) derived from tyrosinase-related protein 2 is presented by both HLA-A31 and HLA-A33 (14). We are currently investigating whether newly identified HLA-A*3303-restricted epitopes can be presented by HLA-A*3101.

10 of 40 peptides (40%) with positively-charged P1 residues bound to both HLA class I alleles while only one of 18 peptides (5.6%) with negatively-charged P1 residues bound to both HLA class I molecules (Table 4). However, peptides with positively-charged P1 residues which bound to both HLA class I molecules showed a much stronger affinity for HLA-A*3101 than HLA-A*3303 (data not shown). This difference in peptide affinity between HLA-A*3101 and HLA-A*3303 may affect presentation of epitope peptides by these HLA class I alleles. Analysis of epitope peptides with charged P1 residues using specific CTL clones should clarify this.

In the present study, we confirmed P2 and the C-terminal anchors for HLA-A*3101 and showed an important role for the P1 residue in peptide binding to HLA-A*3101. Furthermore, we demonstrated that the polarity of the P1 residue determines peptide binding specificity between HLA-A*3101 and HLA-A*3303.

References

1. Clayton J, Lonjou C, White W. Allele and haplotype frequencies for HLA loci in various ethnic groups. In: Charron D, ed. *HLA. Genetic diversity of HLA. Functional and medical implications.* Vol I. Paris: EDK, 1997: 665–820.
2. Bodmer JG, March SGE, Albert ED et al. Nomenclature for factors of the HLA system. *Tissue Antigens* 1999: **53**: 407–46.
3. Falk K, Rötzschke O, Takiguchi M et al. Peptides motifs of HLA-A1, -A11, -A31 and -A33 molecules. *Immunogenetics* 1994: **40**: 238–41.
4. Takiguchi M, Matsuda T, Tomiyama H, Miwa K. Analysis of three HLA-A*3303 binding peptide anchors using an HLA-A*3303 stabilization assay. *Tissue Antigens* 2000: **55**: 296–302.
5. Tanabe M, Sekimata M, Ferrone S, Takiguchi M. Structural and functional analysis of monomorphic determinants recognized by monoclonal antibodies reacting with the HLA class I α3 domain. *J Immunol* 1992: **148**: 3202–9.
6. Nokihara K, Yamamoto R, Hazama M, Wakizawa O, Nakamura S. Design and applications of a novel simultaneous multiple solid-phase peptide synthesizer. *Intercept Andover* 1992: 455.
7. Takamiya Y, Schönbach C, Nokihara K et al. HLA-B*3501-peptide interactions: role of anchor residues of peptides in their binding to HLA-B*3501 molecules. *Int Immunol* 1994: **6**: 255–61.
8. Schönbach C, Ibe M, Shiga H et al. Fine tuning of peptide binding to HLA-B*3501 molecules by nonanchor residues. *J Immunol* 1995: **154**: 5951–8.
9. Schönbach C, Miwa K, Ibe M, Shiga H, Nokihara K, Takiguchi M. Refined peptide HLA-B*3501 binding motif reveals differences in 9-mer to 11-mer peptide binding. *Immunogenetics* 1996: **45**: 121–9.
10. Ibe M, Ikeda Moore Y, Miwa K, Kaneko Y, Yokota S, Takiguchi M. Role of strong anchor residues in the effective binding of 10-mer and 11-mer peptides to HLA-A*2402 molecules. *Immunogenetics* 1996: **44**: 233–41.
11. Yamada N, Ishikawa Y, Dumrese T et al. Role of anchor residues in peptide binding to three HLA-A26 molecules. *Tissue Antigens* 1999: **54**: 325–32.
12. Maenaka K, Maenaka T, Tomiyama H, Takiguchi M, Stuart DI, Jones EY. Nonstandard peptide binding revealed by crystal structures of HLA-B*5101 complexed with HIV immunodominant epitopes. *J Immunol* 2000: **165**: 3260–7.
13. Madden DR, Gorga JC, Strominger JL, Wiley DC. The three-dimensional structure of HLA-B27 at 2.1 Å resolution suggests a general mechanism for tight peptide binding to MHC. *Cell* 1992: **70**: 1035–48.
14. Wang RF, Johnston SL, Southwood S, Sette A, Rosenberg SA. Recognition of an antigenic peptide derived from tyrosinase-related protein-2 by CTL in the context of HLA-A31 and -A33. *J Immunol* 1998: **160**: 890–7.