A general strategy to enhance immunogenicity of low-affinity HLA-A2.1-associated peptides: implication in the identification of cryptic tumor epitopes

Sophie Tourdot[1], Antonio Scardino[1], Eleanna Saloustrou[3], David A. Gross[1], Steve Pascolo[2], Pavlos Cordopatis[3], François A. Lemonnier[2] and Kostas Kosmatopoulos[1]

[1] INSERM 487, Villejuif, France
[2] Unité d'Immunité Cellulaire Antivirale, Department SIDA-Retrovirus, Institut Pasteur, Paris, France
[3] Department of Pharmacy, University of Patras, Patras, Greece

Low-affinity MHC class I-associated cryptic epitopes derived from self proteins overexpressed in a wide variety of human tumors or derived from antigens of viruses exhibiting a high mutation rate, could be interesting candidates for tumor and virus immunotherapy, respectively. However, identification of low-affinity MHC-associated epitopes comes up against their poor immunogenicity. Here we describe an approach that enhances immunogenicity of nonimmunogenic low-affinity HLA-A2.1-binding peptides. It consists of modifying their sequence by introducing a tyrosine in the first position (P1Y). P1Y substitution enhances affinity of HLA-A2.1-associated peptides without altering their antigenic specificity. In fact, P1Y variants of ten nonimmunogenic low-affinity peptides exhibited a 2.3- to 55-fold higher binding affinity and/or stabilized the HLA-A2.1 for at least 2 h more than the corresponding native peptides. More importantly, P1Y variants efficiently triggered *in vivo* native peptide-specific CTL which also recognized the corresponding naturally processed epitope. The possibility for generating CTL against any low-affinity HLA-A2.1-associated peptide provides us with the necessary tool for the identification of cryptic tumor and virus epitopes which could be used for peptide-based immunotherapy.

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1 Introduction

Peptide-based tumor and virus vaccination has been proven efficient in protecting against virus infection and tumor growth in many mouse experimental models and more recently in humans [1–4]. The efficacy of this therapeutic approach depends on two parameters. First, the capacity of peptides to mobilize their specific CTL repertoire and second, the quality of this repertoire in terms of avidity and CTL precursor (CTLp) frequency. Immunodominant high-affinity MHC-associated epitopes of known virus and tumor antigens fulfil these conditions. However, there are two situations where using immunodominant peptides for vaccination may be ineffective: tumors overexpressing antigens that are also expressed in normal tissues including the thymus, and infection with virus exhibiting a high mutation rate.

Peptides presented by MHC class I molecules and derived from self proteins that are expressed at elevated levels by cells from a wide variety of human malignancies like p53 and telomerase, provide in theory, potential targets for a broad-spectrum, CTL-based immunotherapy of cancer. However, since they are self antigens their specific CTL repertoire must be tolerized, as recently demonstrated for the p53 protein [5, 6]. This tolerance principally concerns the high-affinity MHC-associated immunodominant epitopes but not cryptic epitopes with low MHC affinity [7]. Therefore, the antitumor CTL repertoire that remains available for recruitment by peptide-based vaccination *in vivo* must be specific for the low-affinity cryptic tumor epitopes, which could be considered as the best candidates for vaccination.

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The first two authors have equally contributed to this work.

Abbreviations: **RA:** Relative affinity **DC_{50}:** Dissociation complex (time required for the loss of 50% of the HLA-A2.1/peptide complexes)

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In the case of viruses exhibiting a high mutation rate, like HIV, selection pressure imposed by naturally developed antiviral CTL response favors the survival of virus variants with mutations in their immunodominant, high MHC affinity sequences [8–10]. These variants are no longer recognized by immunodominant epitope-specific CTL. Cryptic epitopes with low MHC affinity are not subjected to similar selection pressure and could, therefore, represent useful targets for eliminating wild-type viruses and their variants.

A major barrier to the use of low-affinity epitopes for immunotherapy is the difficulty of their identification due to their poor immunogenicity. In fact, a correlation between immunogenicity and MHC-binding affinity and/or stability of MHC/peptide complexes for class I epitopes has been demonstrated [11–13]. Enhancement of immunogenicity of low MHC affinity epitopes is, therefore, a necessary condition for their identification and can be achieved by the improvement of their binding affinity and MHC/peptide stability as we have previously shown [14].

In this work we describe a general approach to enhancing affinity and, consequently, immunogenicity of nonimmunogenic peptides with low affinity for HLA-A2.1. It comprises modifying peptide sequence by replacing the amino acid at position 1 with a tyrosine (Y). P1Y substitution enhances affinity of HLA-A2.1-associated peptides without altering their antigenic specificity. More importantly, P1Y variants of nonimmunogenic peptides can efficiently recruit the native peptide-specific CTL repertoire *in vivo*. The possibility for generating CTL against low-affinity peptides allows the identification of cryptic tumor and virus epitopes, which could be used for immunotherapy.

2 Results

2.1 Relationship between affinity, HLA-A2.1/peptide complex stability and immunogenicity of tumor and virus peptides

Thirty-two peptides derived from known virus and tumor antigens were included in this study. The majority of them (30 of 32) had the HLA-A2.1-specific primary anchor and strong residues (L/M/V/I/A at P2 and c-terminal P). On the basis of their capacity to bind and to stabilize the HLA-A2.1 molecule, the thirty-two peptides were classified into three different groups (Table 1). The first group is constituted by 18 peptides having relative affinity (RA) ≤ 10 and DC_{50} (dissociation complex; defined as the time required for the loss of 50% of the HLA-A2.1/peptide complexes stabilized at t=0) >2 h. All these peptides triggered a CTL response in a high percentage (60–93%) of HHD mice. The second group includes four peptides with RA >10 and DC_{50} >2 h. Two of them were nonimmunogenic and two peptides induced a response in a low percentage of HHD mice (8% and 17%). Ten peptides with a DC_{50} <2 h and variable RA belong to the third group. They were nonimmunogenic even if they had high RA. The following conclusions can be drawn from these results: (i) secondary anchor motifs strongly influence HLA-A2.1 binding since peptides having the optimal HLA-A2.1 primary anchor residues exhibit a very large spectrum of affinities; (ii) binding affinity does not always correlate with the capacity to stabilize the HLA-A2.1 molecule. Mart-1 32 and HER-2/neu 851 peptides were weak binders but they formed stable peptide/HLA-A2.1 complexes. In contrast, HBVpol 28, HBVpol 594, HER-2/neu 650 and HER-2/neu 466 peptides were strong binders but they formed unstable HLA-A2.1/peptide complexes. (iii) Immunogenicity of peptides depends primarily on their capacity to stabilize the HLA-A2.1 molecule. Peptides with a DC_{50} <2 h were never immunogenic even if they had a high binding affinity. However, peptide-induced HLA-A2.1 stability is not sufficient to ensure immunogenicity. In fact, peptides with a DC_{50} >2 h may be nonimmunogenic (Mart-1 32 and HER-2/neu 661) or very weakly immunogenic (Tyrosinase 1 and HER-2/neu 851) if they exhibit a low binding affinity.

We deduced from these observations that enhancement of both binding affinity and HLA-A2.1/peptide complex stability is necessary for nonimmunogenic peptides to generate a strong CTL response.

2.2 Binding and CTL recognition impact of amino acid substitutions in the P3-P8/9 peptide segment

The simplest way to increase both binding affinity and HLA-A2.1/peptide complex stability should be to introduce MHC class I binding favorable amino acids in primary anchor positions as previously reported [15–17]. However, this approach is limited to a small number of peptides since the majority of MHC class I epitopes have the MHC allele-specific primary anchor motifs [18]. Amino acids in secondary anchor positions influence MHC class I binding ([19] and Table 1). Therefore, to increase affinity of peptides having the optimal primary anchor motifs, the identification of deleterious secondary anchor residues and their substitution with favorable amino acids is required. These modifications in the peptide sequence should preserve the conformation of the peptide segment that interacts with the TCR, ensuring the antigenic specificity. We evaluated the role of each

Table 1. HLA-A2.1 affinity, stabilization capacity and immunogenicity of peptides

Peptides	Sequences	RA	DC50 (hrs)	% responding mice[a), b)]
		Group I		
HIVgag76	SLYNTVATL	1,0	8	70
FluM58	GIGLFVFTL	0.2	8	67
HBVpol 575	FLLSLGIHL	2.5	8	75
HBVpol 765	LLGCAANWIL	2.0	4	ND
Mart-1 27	AAGIGILTV	2.2	3	80
gp100 177	AMLGTHTMEV	0.5	6	60
gp100 178	MLGTHTMEV	0.3	7	67
gp100 154	KTWGQYWQV	2.3	7	78
gp100 570	SLADTNSLAV	1.1	5	67
gp100 209	TDQVPFSV	1.3	4	67
gp100 476	VLYRYGSFSV	10.0	6	80
gp100 457	LLDGTATLRL	1.6	3	67
HER-2/neu 799	QLMPYGCLL	1.0	7	75
HER-2/neu 369	KIFGSLAFL	2.3	4	93
HER-2/neu 789	CLTSTVQLV	1.6	7	67
HER-2/neu 48	HLYQGCQW	1.7	8	83
HER-2/neu 773	VMAGVGSPYV	1.7	6	67
HER-2/neu 5	ALCRWGLL	2.3	8	83
		Group II		
Tyrosinase 1	MLLAVLYCL	>60.0	3	17
Mart-1 32	ILTVILGVL	21.1	4	0
HER-2/neu851	VLVKSPNHV	24.0	4	8
HER-2/neu 661	ILLVVVLGV	>60.0	3	0
		Group III		
HBpol28	LLDDEAGPL	5.3	<2	0
HBVpol 594	PLEEELPRL	$>$ 4.2	<2	0
HBVpol 985	NLQSLTNLL	43.3	<2	0
Tyrosinase 224	KLTGDENFTI	>50.0	<2	0
Tyrosinase 207	FLPWHRLFLL	>50.0	2	0
HER-2/neu 650	PLTSIISAV	1.4	<2	0
HER-2/neu 466	ALIHHNTHL	4.8	2	0
HER-2/neu 402	TLEEITGYL	19.0	<2	0
HER-2/neu 391	PLQPEQLQV	>70.0	2	0
HER-2/neu 971	ELVSEFSRM	>70.0	2	0

a) Mice were considered responding when specific cytotoxicity towards peptide-pulsed targets was >15% above cytotoxicity towards unpulsed targets.

b) Each peptide was tested in more than six mice.

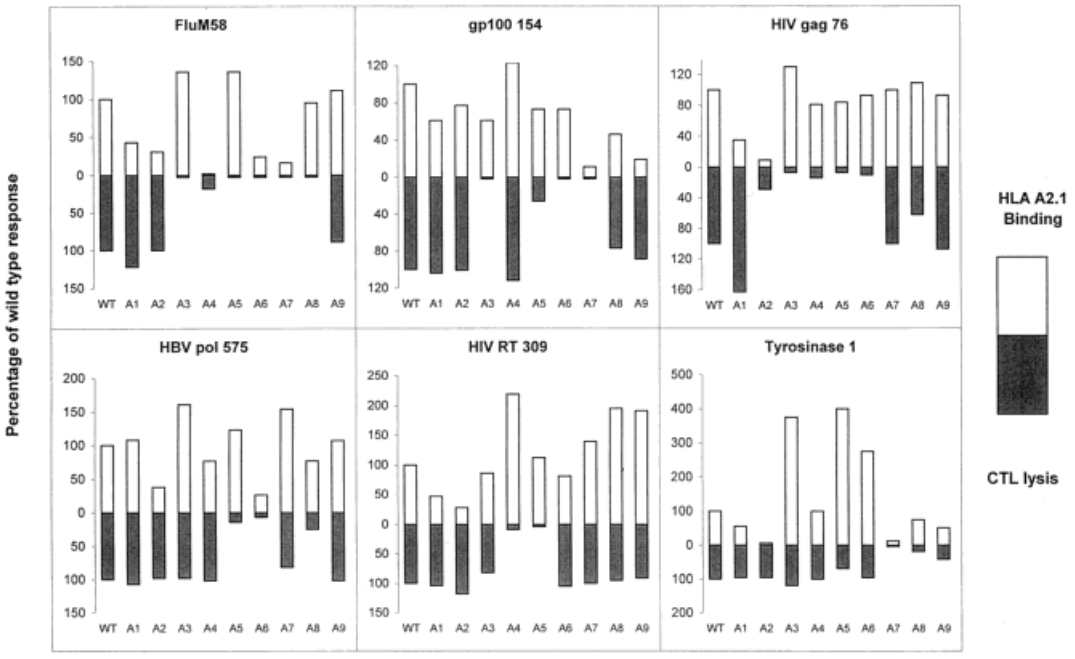

Fig. 1. Role of each peptide amino acid in the HLA-A2.1/peptide and TCR/peptide interactions. A variants for the six indicated peptides were tested for their capacity to bind to the HLA-A2.1 molecule and to be recognized by the wild-type peptide-specific CTL. Results for each variant are expressed as % of results obtained by the corresponding wild-type peptide (WT). Cytotoxicity was measured at an E/T ratio of 50/1.

amino acid in the peptide/HLA-A2.1 and peptide/TCR interactions by testing A variants of six HLA-A2.1-restricted peptides (Flu M58, gp100 154, HIVgag 76, HBVpol 575, HIVrt 309 and Tyrosinase 1) for their capacity to bind to the HLA-A2.1 molecule and to be recognized by CTL specific for the corresponding native peptide (Fig. 1). The following observations were made: (i) substitution by A in anchor P2 prevents peptide/HLA-A2.1 interaction. The unique exception is the gp100 154 peptide that lacks the optimal residue in P2. (ii) Residues contacting the TCR are always situated in the P3-P8/9 sequence. Their substitutions by A prevents CTL recognition but does not influence HLA-A2.1 binding. (iii) Secondary anchor residues with positive or negative effect on HLA-A2.1 binding do exist in the P3-P8/9. However, their location and nature differ from one peptide to another. (iv) Substitution by A of secondary anchor residues located in the neighborhood of residues that point up towards the TCR influences not only HLA-A2.1 binding but also CTL recognition, suggesting that these substitutions affect CTL recognition by modifying the orientation of the lateral chain of residues that contact the TCR. (v) Finally, substitution by A in P1 never prevents CTL recognition but reduces HLA-A2.1 binding for all peptides but one, HBVpol 575.

In the light of these observations we concluded that substitution of secondary anchor residues in the P3-P8/9 segment would not allow the design of a general strategy to enhance affinity without loss of antigenicity.

2.3 Binding impact of amino acid substitutions in P1

Our interest therefore focused on the residue in P1. This residue is a secondary anchor motif and does not contact the TCR (Fig. 1). Moreover, it has been suggested that the presence of aromatic amino acids (Y, F or W) in P1 enhances HLA-A2.1 binding affinity [19]. Thus we postulated that substitutions of residues in P1 by one of the three aromatic amino acids could result in enhancement of both binding affinity and HLA-A2.1/peptide stability. To determine which amino acid to use for the P1 substitution, the affinity and stabilization capacity of P1Y, P1W and P1F variants of the weak HLA-A2.1 binders HBVpol 985, Tyrosinase 224, HER-2/neu 402, and HER-2/neu 391 were tested. As expected, P1Y, P1W and P1F variants very often exhibited a higher binding affinity and stabilized HLA-A2.1 more efficiently than the corresponding native peptides. However, these two effects were more constant and more pronounced for the P1Y than for the P1W and P1F variants (Table 2). On the basis of these data, P1Y variants for the 32 peptides included in this work were produced and tested for their affinity and their HLA-A2.1 stabilization capacity.

Table 2. Binding affinity and stabilization capacity of P1Y, P1W and P1F variants[a)]

	RA				DC_{50}			
	WT	P1Y	P1F	P1W	WT	P1Y	P1F	P1W
HBVpol 985	43	**3.1**	**10**	**3.0**	2	**6**	2	**3**
Tyrosinase 224	50	**9.7**	15	17	2	**4**	**3**	**3**
HER-2/neu 402	19	**3.6**	**3.9**	**4.2**	2	**6**	2	**3**
HER-2/neu 391	70	**1.2**	**3.0**	**2.8**	2	**6**	**3**	**6**

a) RA and DC_{50} values defining a high-affinity, immunogenic peptide are in bold (RA <10 and DC_{50} >2 h).

Results in Table 3 show that P1Y substitution enhances binding of almost all the low-affinity peptides and promotes HLA-A2.1 stabilization of all the poor stabilizers (groups II and III in Table 1). The RA of all the P1Y-modified low- and intermediate-affinity peptides except two (HER-2/neu 661Y1, Tyrosinase 1Y1) was <10 and the DC_{50} for all of them was >4 h. Increase of affinity measured by the ratio between RA of native and modified peptide ranged from >2.3 to >55.5, whereas increase of HLA-A2.1 stabilization, measured by the difference between DC_{50} of modified and native peptide ranged from 2 to >6 h. This effect of P1Y substitution was not limited to the low-affinity peptides but was also observed with the majority of the strong HLA-A2.1 binders (group I in Table 1). It is noteworthy that increase in affinity was independent of the nature of the residue in P1 of the native peptide, and it was observed even if the substituted residue was not a deleterious one (P, E, D).

These results demonstrate that P1Y substitution enhances binding and HLA-A2.1 stabilization capacity of almost all the HLA-A2.1-bound peptides. This effect is much more pronounced for the nonimmunogenic peptides of low HLA-A2.1 affinity.

2.4 Cross-recognition of native peptides and their P1Y variants by specific CTL

Enhancement of HLA-A2.1 affinity was the first condition for P1Y substitution of low-affinity peptides to restore their immunogenicity. The second one was that this substitution preserves their antigenic specificity. In this case, CTL generated in native peptide-vaccinated HHD mice should recognize both the native peptide and its P1Y variant with the same efficacy. Moreover, P1Y variants should be able to recruit the native peptide-specific CTL repertoire *in vivo*.

This is illustrated in Fig. 2. CTL induced in HHD mice primed with eight immunogenic peptides (HIVgag 76, HBVpol 575, gp100 154, gp100 457, gp100 476, gp100 570, gp100 177 and HER-2/neu 369) (Fig. 2A) and their P1Y variants (Fig. 2B) killed both native peptide- and P1Y variant-pulsed RMAS-HHD cells with the same efficacy. This demonstrates that P1Y substitution does not alter antigenic specificity and, therefore, P1Y variants can mobilize the native peptide-specific CTL repertoire.

The most important question to answer is whether P1Y variant-induced CTL recognize the naturally processed epitope. CTL generated in HIVgag 76Y1-, HER-2/neu 369Y1-, HER-2/neu 5Y1-, gp100 476Y1- and fluM 58Y1-primed mice were tested for their capacity to kill RMA-HHD cells infected with vac-HIVgag, vac-neu, vac-gp100 or flu PR8 viruses to endogenously express the corresponding antigen. Data in Fig. 3 show that, indeed, P1Y variant-specific CTL recognize the naturally processed epitope since they killed targets infected with the appropriate virus but not vac-wt-infected targets.

Taken together these data show that P1Y substitution satisfies the two criteria that are necessary for the induction of a CTL response against any low-affinity HLA-A2.1 peptide, irrespective of their sequence. First, it increases binding affinity and HLA-A2.1 stabilization capacity of HLA-A2.1-bound peptides and, second, it does not interfere with the peptide/TCR interaction, thus preserving their antigenic specificity.

2.5 P1Y variants induce a strong CTL response against nonimmunogenic peptides with low HLA-A2.1 affinity

To directly prove that P1Y substitution restores immunogenicity of any nonimmunogenic peptide with low HLA-A2.1 affinity, HHD mice were immunized with the P1Y variants of the low-affinity HER-2/neu 402, HER-2/neu 466, HER-2/neu 650, HER-2/neu 391, Tyrosinase 207, HBVpol 594, HBVpol 28 and HBVpol 985 peptides, and their spleen cells were restimulated *in vitro* with the cor-

Table 3: Enhancement of binding affinity and stabilization capacity by P1Y substitution[a)]

Peptides	RA	DC50 (hours)
	Group I	
HIVgag 76Y1	**0.3**	**8**
FluM 58Y1	**0.1**	**8**
HBVpol 575Y1	**0.9**	**8**
HBVpol 765Y1	**0.1**	**8**
Mart-1 27Y1	**1.1**	**3**
gp100 177Y1	**0.8**	**8**
gp100 178Y1	**0.4**	**8**
gp100 154Y1	**2.9**	**5**
gp100 570Y1	**0.3**	**8**
gp100 209Y1	**0.8**	**6**
gp100 476Y1	**2.4**	**8**
gp100 457Y1	**0.7**	**6**
HER-2/neu 369Y1	**0.6**	**8**
HER-2/neu 799Y1	**0.2**	**8**
HER-2/neu 789Y1	**0.8**	**7**
HER-2/neu 48Y1	**0.6**	**8**
HER-2/neu 773Y1	**0.8**	**8**
HER-2/neu 5Y1	**2.1**	**8**
	Group II	
Tyrosinase 1Y1	16.2	**5**
Mart-1 32Y1	**1.3**	**6**
HER-2/neu 851Y1	**8.0**	**6**
HER-2/neu 661Y1	40.0	**6**
	Group III	
HBVpol 28Y1	**0.4**	**4**
HBVpol 594Y1	**0.3**	**8**
HBVpol 985Y1	**3.2**	**8**
Tyrosinase 224Y1	**9.8**	**4**
Tyrosinase 207Y1	**7.5**	**6**
HER-2/neu 650Y1	**0.2**	**6**
HER-2/neu 466Y1	**1.4**	**6**
HER-2/neu 402Y1	**3.6**	**4**
HER-2/neu 391Y1	**1.3**	**8**
HER-2/neu 971Y1	**6.0**	**4**

a) RA and DC_{50} values defining a high-affinity, immunogenic peptide are in bold (RA <10 and DC_{50} >2 h).

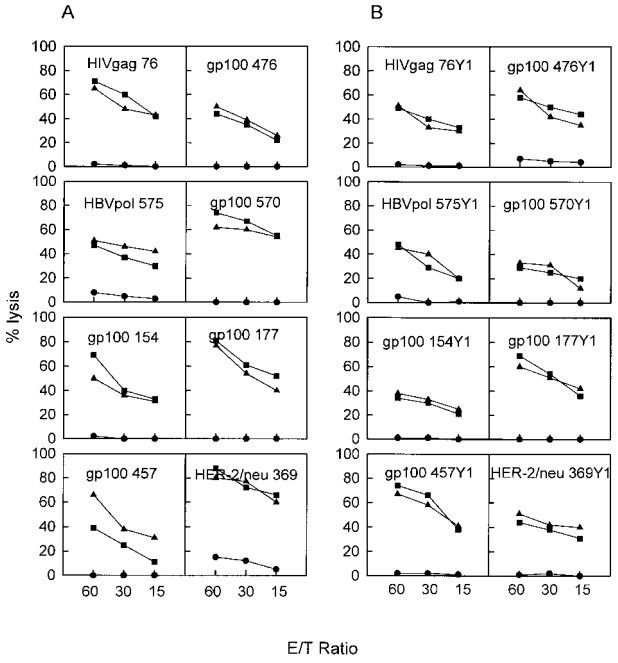

Fig. 2. Cross recognition of native peptides and their P1Y variants by specific CTL. CTL induced in HHD mice primed with the indicated native peptides (A) and their P1Y variants (B) were tested for their capacity to kill unloaded RMAS HHD targets (●) or RMAS HHD targets loaded with either the wild type peptides (■) or the corresponding P1Y variants (▲).

responding native peptide. As expected, HHD mice primed with P1Y variants generated native peptide-specific CTL (Fig. 4). The percentage of responding mice ranged between 33 and 77%. These data demonstrate that P1Y substitution is a general strategy for enhancing immunogenicity of low-affinity HLA-A2.1-associated peptides.

The possibility to induce a CTL response against low-affinity HLA-A2.1 peptides enables us to identify cryptic tumor or virus epitopes useful for specific immunotherapy. This is the case for the low-affinity HER-2/neu 650 peptide, which was not found to participate in the HER-2/neu-specific CTL response developed in HER-2/neu$^+$ tumor-bearing patients, either because it is a cryptic epitope or because it is not naturally processed by HER-2/neu$^+$ tumor cells. HER-2/neu-specific CTL were generated in HER-2/neu 650Y1-primed mice and tested for their capacity to kill vac-neu-infected RMA HHD cells. Results in Fig. 5 show that CTL killed vac-neu-infected but not vac-wt-infected targets, demonstrating that HER-2/neu 650 peptide is, in fact, a cryptic HER-2/neu epitope.

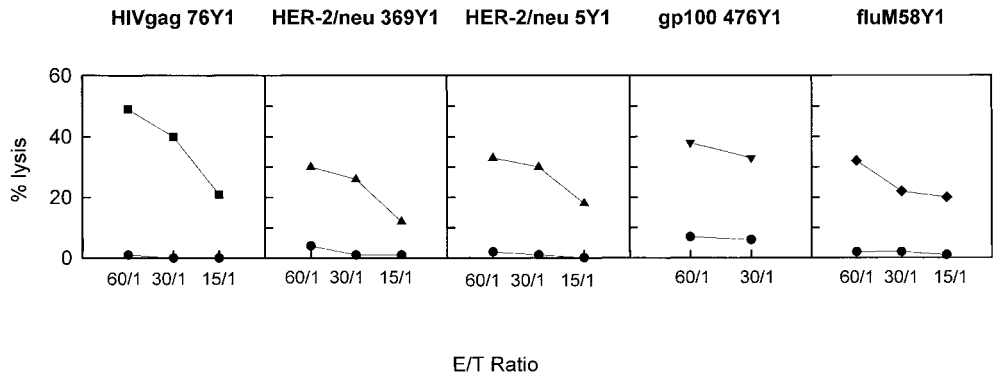

Fig. 3. Recognition of naturally processed epitopes by P1Y variant specific CTL. CTL generated in HHD mice primed with the indicated peptides were tested for their capacity to kill RMA-HHD cells infected with vac-wt (●), vac-HIVgag (■), vac-neu (▲), vac-gp100 (▼) and flu PR8 (◆) viruses.

3 Discussion

The aim of our work was to find an amino acid substitution that was common for different peptides and that enhances MHC affinity and immunogenicity of weak HLA-A2.1 binders, irrespective of their sequence. Generating CTL against low-affinity peptides would allow identification of cryptic tumor and virus epitopes. Data showed that P1Y substitution increases affinity and HLA-A2.1/peptide stability of almost all peptides with HLA-A2.1-specific primary anchor motifs. These effects were more pronounced for peptides that were naturally weak binders and weak stabilizers. More interestingly, P1Y substitution restored immunogenicity of low-affinity HLA-A2.1 peptides.

Our results confirmed the role of MHC affinity and MHC/peptide stability in the immunogenicity of MHC class I-bound peptides that has been established recently [11–14]. Peptides with high affinity (RA $\leq$10) and MHC stabilization capacity (DC_{50} >2 h) were always immunogenic, while peptides with low affinity (RA >10) and stabilization capacity (DC_{50} <2 h) were not. It is noteworthy that both efficient induction of MHC molecules and for-

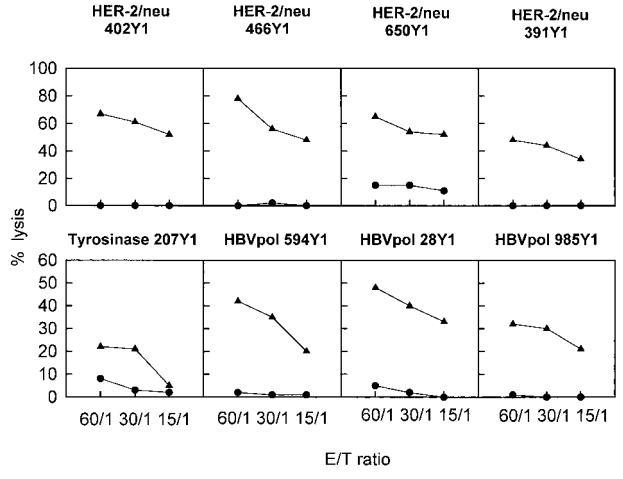

Fig. 4. Induction of low HLA-A2.1 affinity peptide specific CTL response by immunization with the P1Y variants. Spleen cells of P1Y variant primed HHD mice were *in vitro* stimulated with the corresponding wild type peptide and generated CTL were tested against unpulsed (●) or wild type peptide pulsed (▲) RMAS HHD targets.

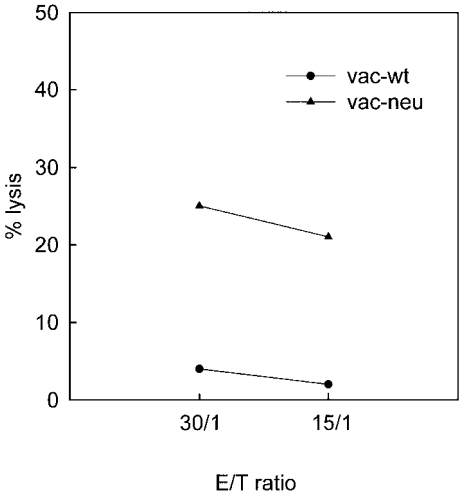

Fig. 5. HER-2/neu 650Y1 specific CTL recognize a naturally processed HER-2/neu epitope. CTL generated in HER-2/neu 650Y1 peptide primed HHD mice were tested for their capacity to kill RMA-HHD targets infected with vac-wt (●) and vac-neu (▲) virus.

mation of stable MHC/peptide complexes are necessary for a peptide to be immunogenic. Peptides with high binding affinity but weak stabilization capacity (HBVpol 28, HBVpol 594, HER-2/neu 650 and HER-2/neu 466) and inversely, peptides with strong stabilization capacity but low binding affinity (Tyrosinase 1, Mart-1 32, HER-2/neu 851 and HER-2/neu 661) are weakly or not immunogenic.

The most straightforward way to increase both HLA-A2.1-binding and stabilization capacity of peptides is to introduce MHC-binding favorable amino acids in primary anchor positions P2 and P9/10. This has successfully been achieved for three epitopes (gp100 154, gp100 209 and Mart-1 26) which do not have the optimal residue in P2 [4, 15–17, 20]. However, the majority of virus and tumor epitopes do have the MHC class I-specific primary anchor motifs and their affinity is positively or negatively controlled by residues in secondary anchor positions. Therefore, increase in their affinity would require the identification of MHC-binding unfavorable secondary anchor residues and their substitution with MHC-binding favorable amino acids. This is an easy procedure for peptides presented by the murine H-2D^b molecule since the architecture of the floor of the H-2D^b-binding cleft gives to all the H-2D^b-bound peptides the same conformation, orienting residues in P1–3 towards the MHC and residues in P6–8 towards the TCR [21]. Introduction of favorable amino acids in P1–3 increased affinity and H-2D^b/peptide stability of all weak H-2D^b binders [14]. This is not the case for HLA-A2.1-bound peptides because (i) HLA-A2.1 peptides adjust their conformation and the position of individual residue side chain in the P3–P8/9 segment in response to their sequence [22]. This is confirmed by our results showing that TCR- and HLA-A2.1-contacting residues are situated at different positions for each peptide. Therefore, there are not common MHC-contacting secondary residues for all the HLA-A2.1-bound peptides. (ii) The conformation of the central P3–P8/9 portion is flexible and, therefore, substitutions in secondary anchor positions should alter the orientation of the residue side chains that contact the TCR and ensure the antigenic specificity. This is strongly suggested by our results (Fig. 1) showing that A substitutions in some positions influence both HLA-A2.1 binding and TCR recognition. These positions are always neighboring to residues that contact the TCR. Therefore, substitutions of unfavorable secondary anchor residues in the central portion of peptide could create new antigenic specificities and CTL generated by such modified peptides would fail to recognize the corresponding native peptide. Thus, we concluded that it is not possible to find a common single secondary anchor residue substitution in the central P3–P8/9 portions of HLA-A2.1-bound peptides that would enhance their affinity without altering their antigenic specificity.

Crystallographic studies show that the main characteristic of residues in P1 is that they always conserve the same orientation (towards the MHC), whatever the overall peptide sequence is [22]. This is also strongly suggested by our results showing that A substitution in P1 reduces binding affinity of almost all peptides tested. P1 residue corresponds to the A pocket of the HLA-A2.1 which favorably accommodates aromatic amino acids [19]. Therefore, substitution of the P1 residue by an aromatic amino acid could be a common approach to enhancing affinity and immunogenicity of HLA-A2.1-bound peptide. In fact, P1Y substitution substantially enhanced affinity (more than twofold) and/or HLA-A2.1 stability (by more than 2 h) of 30 out of 32 peptides tested. The strongest positive effect of P1Y substitution in affinity and HLA-A2.1 stability concerned the weak binders and weak stabilizers.

P1Y substitution maintains the conformation of the central peptide portion that interacts with the TCR. In fact, P1Y variants were recognized by native peptide-specific CTL with the same avidity as the native peptide (data not shown). Moreover, they were able to recruit the native peptide-specific CTL repertoire *in vivo*. This was expected because the strong interaction of anchor amino acids at P2 with the B pocket of the HLA-A2.1 groove precludes any modification of the original peptide conformation in P1Y variants.

In conclusion, our results demonstrate that P1Y substitution fulfils the two conditions necessary for the restoration of immunogenicity of low HLA-A2.1 affinity peptides: it enhances affinity and conserves native peptide antigenicity. Confirmation is given by our results showing that P1Y variants of nonimmunogenic low HLA-A2.1 affinity peptides generate native peptide-specific CTL *in vivo*.

Increase in the HLA-A2.1 binding and/or HLA-A2.1/peptide complex stability by P1Y substitution has been previously described for one virus (HIVrt 309) and two tumor (gp100 209, and MART-1 26) peptides [15, 17, 20, 23]. In each case P1Y variants corresponded to immunogenic, high-affinity, native peptides and they were tested for their capacity to stimulate CTL lines *in vitro*.

Here, we demonstrate that P1Y substitution may be a common sequence modification for nonimmunogenic low HLA-A2.1 affinity peptides to enhance their affinity and consequently restore their immunogenicity. This approach, therefore, provides a tool for the identification of low-affinity cryptic tumor and virus epitopes. This is the case for HER-2/neu 650 peptide which was not previously described as a tumor epitope. Indeed, it was not recognized by HER-2/neu-specific TIL or TAL from pancreatic and metastatic breast cancer patients and it did

not induce CTL from HLA-A2.1 pancreatic cancer patient PBMC [24, 25]. However, this does not constitute definitive proof that this peptide is not a tumor epitope that is a potential target of antitumor CTL. Knowing the correlation between MHC/peptide stability and immunogenicity of MHC class I-restricted peptides, it would not be surprising that HER-2/neu 650 failed to trigger a CTL response *in vivo* or *in vitro* but could still be recognized by activated T cells. Indeed, killing of tumor cells by activated CTL requires a low level of HLA-A2.1/peptide expression that can be reached by the low-affinity peptides [26]. Our results showed that the HER-2/neu 650 peptide is a naturally processed cryptic epitope and that activated specific CTL can efficiently recognize and kill HER-2/neu-expressing tumor cells. Similar results were obtained for three other HLA-A2.1 low-affinity HER-2/neu (A.S., manuscript in preparation), two HLA-A2.1 low-affinity hTRT (D.A.G., manuscript in preparation) and two HLA-A2.1 low-affinity HIV (submitted for publication) peptides in both HHD mice and humans.

All these observations strongly suggest that low-affinity cryptic epitopes can be used in immunotherapy protocols. Their identification is now conceivable by the approach we describe here.

4 Materials and methods

4.1 Mice

HHD mice were previously described [27]. They are $\beta 2m^{-/-}$, $D^{b-/-}$ and express a HLA-A2.1 monochain composed of the $\alpha 1$ and $\alpha 2$ domains of HLA-A2.1 and the $\alpha 3$ and intracellular domains of D^b linked by its N terminus to the C terminus of the human $\beta 2m$ by a 15-amino acid peptide.

4.2 Cells

Human T2 cells are deficient for TAP transporters and, therefore, they express low amounts of HLA-A2.1 molecules. RMA-HHD and RMAS-HHD cells were obtained by transfection of murine RMA and their TAP-deficient variant RMAS cells with the HHD construct as previously described [27]. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS.

4.3 Peptides

The A variant peptides were produced according to the solid-phase method, on phenylacetamidomethyl resins. Synthetic protocols were based on the classical t-Boc/Bzl/Hf chemistry as previously described. Native peptides and their P1Y variants were synthesized by Synt:em (Nimes, France).

4.4 Viruses

VVTG1144 (vac-HIVgag) HIVgag-expressing vaccinia recombinant virus was provided by Dr. M. P. Kieny (Transgene, Strasbourg, France). VT39 (vac-neu) HER-2/neu-expressing recombinant and TBC-Wy (vac-wt) wild-type vaccinia viruses were provided by Dr. G. Mazzara (Therion Biologics, Cambridge, MA), vac-gp100 vaccinia virus was provided by Dr. N. P. Restifo (NIH, Bethesda) and flu PR8 virus was provided by Dr. J. C. Manuguerra (Institut Pasteur, Paris).

4.5 Generation of CTL

HHD mice were injected subcutaneously at the base of the tail with 100 μg peptide emulsified in IFA in the presence of 140 μg of the IA^b-restricted HBVcore antigen-derived T helper epitope (128–140; sequence TPPAYRPPNAPIL). After 11 days, spleen cells (5×10^7 cells in 10 ml) were stimulated *in vitro* with peptide (10 μM). At day 6 of culture, the bulk responder populations were tested for specific cytotoxicity.

4.6 Cytotoxic assay

RMAS-HHD and RMA-HHD cells were used as targets for cytotoxicity. For virus infection, RMA-HHD cells were incubated with vac-WT or vac recombinant viruses (10 PFU/cell) for 16 h or with flu PR8 (50 HAU) virus for 2 h. Targets were labeled with 150 μCi of ^{51}Cr for 90 min then washed three times and plated in 96-well round-bottom plates (10^4 cells/well in 100 μl of RPMI 1640 + 3% FCS). Uninfected RMA HHD or RMAS HHD cells were pulsed with 1 μg/ml of peptides at 37°C for 90 min. Then, 100 μl of various numbers of effectors were added to the wells and plates were incubated at 37°C for 4 h. After incubation, 100 μl of supernatant was collected and radioactivity was measured in a γ counter. Percentage of specific lysis was calculated by the formula: (experimental–spontaneous)/(maximal–spontaneous) ^{51}Cr release ×100. Spontaneous releases were $<$20% of maximal release induced by 3 N HCl in all experiments.

4.7 Flow cytometric analysis of HLA-A2.1 molecule induction

T2 cells (3×10^5 cells/ml) were incubated with various concentrations of peptides in serum-free RPMI 1640 medium supplemented with 100 ng/ml human $\beta 2m$ at 37°C for 16 h. They were then washed twice and stained with the HLA-A2.1 molecule-specific BB7.2 mAb. For each peptide concentration, HLA-A2.1-specific staining was calculated as the % of the staining obtained by 100 μM of the reference peptide (HIVpol 589; IVGAETFYV). The RA was the ratio of the concentration of each peptide to the concentration of the reference peptide that induce 20% of the HLA-A2.1 expression; the lower the RA, the stronger peptide binds to HLA-A2.1. The mean RA for each peptide was determined

from at least three independent experiments. In all experiments 20% of HLA-A2.1 expression using the reference peptide was obtained for 1–3 μM.

4.8 Measurement of the peptide/HLA-A2.1 complex stability

T2 cells (10^6/ml) were incubated overnight with 100 μM of each peptide in serum-free RPMI 1640 medium supplemented with 100 ng/ml β2m at 37°C. Thereafter, they were washed four times to remove free peptides, incubated with Brefeldin A (10 μg/ml) for 1 h to block cell surface expression of newly synthesized HLA-A2.1 molecules, washed and incubated at 37°C for 0, 2, 4, 6 or 8 h. Subsequently, cells were stained with the BB7.2 antibody to evaluate the HLA-A2.1 molecule expression. For each time point, peptide-induced HLA-A2.1 expression was evaluated by the formula: mean fluorescence of peptide preincubated T2 cells – mean fluorescence of T2 cells treated in similar conditions in the absence of peptide.

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Correspondence: Kostas Kosmatopoulos, INSERM 487, Institut Gustave Roussy, 39 rue Camille Desmoulins, F-94805 Villejuif, France
Fax: +33-1-42115288
e-mail: kostas@igr.fr