Melanoma Peptide MART-1(27−35) Analogues with Enhanced Binding Capacity to the Human Class I Histocompatibility Molecule HLA-A2 by Introduction of a β-Amino Acid Residue: Implications for Recognition by Tumor-Infiltrating Lymphocytes

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The design of heteroclytic antigens with high MHC binding capacity is of particular interest to overcome the weak immunogenicity of peptide epitopes derived from tissue antigens expressed by tumors. In the present study, double-substituted peptide analogues of the tumor-associated antigen MART-1(27−35) incorporating a substitution at a primary anchor residue and a β-amino acid residue at different positions in the sequence were synthesized and evaluated for binding to the human histocompatibility class I molecule HLA-A2 and for recognition by tumor-infiltrating lymphocytes. Interestingly, by combining a Leu for Ala substitution at P2 (which alone is deleterious for antigenic activity) with a β-amino acid substitution at a putative TCR contact residue, recognition by tumor-infiltrating lymphocytes was partially restored. The analogue [Leu^{28},β-HIle^{30}]MART-1(27−35) displays both a higher affinity to HLA-A2 and a more prolonged complex stability compared to [Leu^{28}]MART-1(27−35). Overall, these results suggest that double-substitution strategies and β-amino acid replacements at putative TCR contact residues might prove useful for the design of epitope mimics with high MHC binding capacity.

Introduction

Metastatic melanoma is the major cause of death in patients with skin cancer. In recent years, the identification of a number of melanoma-associated antigens (MAA) recognized by CD8$^+$ CTL from cancer patients has raised new prospects in the development of melanoma vaccines. Identified MAAs include (i) antigens derived from tumor-specific mutations, (ii) overexpressed or inappropriately expressed antigens (such as the product of genes *MAGE*, *BAGE*, or *GAGE* which are silent in normal tissues except male germline cells but are expressed in a significant proportion of tumors), and (iii) tissue-specific differentiation antigens (such as melan-A/MART-1 (hereafter referred to as MART-1), tyrosinase, gp100, TRP1, and TRP2 proteins which are expressed by normal melanocytes as well as by melanoma cells).[1]

MART-1 is expressed in about 88% of primary and metastatic melanoma.[2] The MART-1 gene product is recognized by tumor-infiltrating lymphocytes (TILs) from most HLA-A2 melanoma patients.[3] Recently, screening of peptides derived from MART-1 led to the identification of two immunodominant overlapping peptides, MART-1(27−35) and MART-1(26−35), that could elicit tumor-reactive CTL upon in vitro stimulation of peripheral blood mononuclear cells (PBMC) and TILs from HLA-A2 melanoma patients.[4] Taken together, these results and first in vivo experiments[5] suggest that MART-1-derived peptides either alone or in combination with other tumor antigens might represent attractive candidates for the development of a melanoma vaccine in HLA-A2 patients.

However, despite encouraging results, early vaccination trials with peptides derived from gp100, MART-1, tyrosinase, MAGE-1, and MAGE-3 have experienced difficulty in inducing CTL immunity. Peptide epitopes derived from differentiation antigens including MART-1 generally display only weak to moderate binding affinity for HLA-A2 due to the absence of an optimal residue at the second anchor position (P2). Since differentiation antigens are nonmutated normal "self-proteins", the lack of immunodominant peptides with high affinity probably results from negative selection or tolerance induction of the specific T cell repertoire during thymocyte differentiation. In the case of viral antigens, several studies have established that immunogenicity correlates with MHC class I binding affinity and also with complex stability.[6] Hence, optimization of HLA-A2 binding by amino acid substitution at major anchor positions has been proposed as a potential strategy to enhance the immunogenicity of tumor peptide epitopes. Improved induction of melanoma-specific CTL in vitro has been reported for gp100 epitopes modified at the P2 anchor position.[7] Surprisingly, in the case of MART-1(27−35), we and others found that substitution of Ala^{28} at P2 by Leu or Met, although it considerably improved

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Table 1. Sequence and Analytical Data of MART-1(27−35), [Leu[28]]MART-1(27−35), Double-Substituted MART-1(27−35) Analogues **1**−**9**, and Single-Substituted MART-1(27−35) Analogues **10** and **11**

peptide	compound	t_R (min) HPLC	t_R (min) CZE	purity (%) HPLC	purity (%) CZE	MS
MART-1(27−35)	H-Ala-Ala-Gly-Ile-Gly-Ile-Leu-Thr-Val-OH					
[Leu[28]]MART-1(27−35)	H-Ala-Leu-Gly-Ile-Gly-Ile-Leu-Thr-Val-OH	14.00	23.55	96	>98	856.9
1	H-*β*-HAla-Leu-Gly-Ile-Gly-Ile-Leu-Thr-Val-OH	14.47	24.23	>96	93	871.3
2	H-Ala-*β*-Hleu-Gly-Ile-Gly-Ile-Leu-Thr-Val-OH	14.16	24.12	>95	92	870.9
3	H-Ala-Leu-*β*-HGly-Ile-Gly-Ile-Leu-Thr-Val-OH	14.07	23.81	99	97	871.0
4	H-Ala-Leu-Gly-*β*-HIle-Gly-Ile-Leu-Thr-Val-OH	14.20	23.73	98	98	871.4
5	H-Ala-Leu-Gly-Ile-*β*-HGly-Ile-Leu-Thr-Val-OH	14.14	23.52	96	93	871.3
6	H-Ala-Leu-Gly-Ile-Gly-*β*-HIle-Leu-Thr-Val-OH	14.24	23.10	98	>98	871.7
7	H-Ala-Leu-Gly-Ile-Gly-Ile-*β*-HLeu-Thr-Val-OH	13.72	23.03	98	83	871.3
8	H-Ala-Leu-Gly-Ile-Gly-Ile-Leu-*β*-HThr-Val-OH	13.56	23.53	>96	95	871.5
9	H-Ala-Leu-Gly-Ile-Gly-Ile-Leu-Thr-*β*-HVal-OH	14.14	22.07	97	>98	871.4
10	H-Ala-Ala-Gly-*β*-HIle-Gly-Ile-Leu-Thr-Val-OH	12.50	22.87	99	>94	829.5
11	H-Ala-Ala-Gly-Ile-Gly-Ile-Leu-*β*-HThr-Val-OH	11.92	22.84	98	97	829.1

binding to HLA-A2, resulted in dramatic reduction of the antigenic activity of the peptide.[8]

Although MART-1(27−35) and [Leu[28]]MART-1(27−35) differ only at a major HLA-A2 anchor position, molecular modeling studies of HLA-A2/MART-1(27−35) and HLA-A2/[Leu[28]]MART-1(27−35) complexes suggested some differences in the surface accessible to the TCR, which could account for the deleterious effect observed. On the basis of these results, the aim of the present study was to design and synthesize analogues of [Leu[28]]MART-1(27−35) that could restore at least partially the recognition by TILs while still retaining high HLA-A2 binding capacity. We envisioned that single *β*-amino acid insertion in the sequence of [Leu[28]]-MART-1(27−35), because of the additional rotational freedom, could prevent unwanted conformational changes associated with the Leu for Ala substitution at P2 while still preserving side chain functionalities of the peptide. Herein, we prepared a series of [Leu[28]]MART-1(27−35)-derived peptides containing a *β*-amino acid substitution at various positions in the sequence. The capacity of these double-substituted MART-1(27−35) analogues to bind to HLA-A2 and to be recognized by TILs is described.

Results and Discussion

Chemistry. MART-1(27−35) analogues **1**−**11** (Table 1) were synthesized by solid-phase methodology using a Fmoc/*tert*-butyl strategy on an automated peptide synthesizer.

N-Fmoc-protected β^3-amino acids were prepared by Arndt−Eistert homologation of the commercially available *N*-Fmoc-protected α-amino acids as described previously.[9] Peptides **1**−**11** were synthesized on Wang or *o*-chlorotrityl chloride[10] resins (60-*μ*mol scale) using conventional solid-phase peptide synthesis procedures. *N*-Fmoc-protected amino acids were activated using BOP/HOBt/DIEA in DMF, and all coupling reactions were performed twice for 10 min. The purity of the crude peptides, obtained after cleavage from the resin and lyophilization, was between 78% and 96% as checked by RP-HPLC. The analogues were purified by C_{18} RP-HPLC and lyophilized. All peptides were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), and their homogeneity was assessed by C_{18} RP-HPLC and CZE (purity of all peptides determined to be >90%). Experimental ^{1}H NMR data for compound **4** in DMSO-d_6 are given in the Supporting Information.

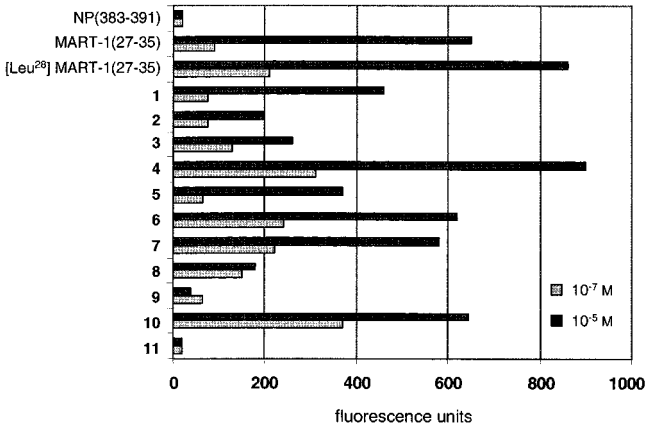

Figure 1. Binding of peptides to purified HLA-A2 molecules. Results are given for peptide concentrations of 10^{-5} and 10^{-7} M and are expressed as fluorescence arbitrary units measured at 355/460 nm. Peptides used were MART-1(27−35), [Leu[28]]-MART 27−35, and analogues **1**−**11**. Negative peptide was NP 383-391 from nucleoprotein (NP) of influenza virus associating with HLA-B27.

HLA-A2/Peptide Binding and Stability of Complexes. Double-substituted analogues **1**−**9**, MART-1(27−35), and [Leu[28]]MART-1(27−35) were tested for their capacity to bind to HLA-A2 molecules[11] (Figure 1). HLA-A2/peptide complexes were detected after incubation at 4 °C.

Although, both N- and C-termini were not permissive to amino acid homologation (the methylene insertion at P1, P2, P8, and P9 probably results in a strong perturbation of the highly conserved hydrogen bond network which involves in part main chain atoms of these residues), we found that each central P3−P7 residue with the exception of P5 could be replaced by the corresponding *β*-amino acid residue without affecting the ability of the peptide to bind to HLA-A2 molecules. Compound **4** was more potent than [Leu[28]]MART-1(27−35) in binding to HLA-A2 at 10^{-5} and 10^{-7} M. Interestingly, the benefit of the *β*-amino substitution at P4 was also observed for the single-substituted analogue **10** which at 10^{-7} M was found to be more potent than MART-1(27−35). The flexibility of the central region of the bound peptide that can account for the tolerance to methylene insertion observed in our study is well-documented. Although, the optimal length of natural HLA-A2 peptide ligands is 9 residues, HLA-A2 molecules can accommodate peptides varying in length from 8 to 11 residues. In the case of longer peptides, the

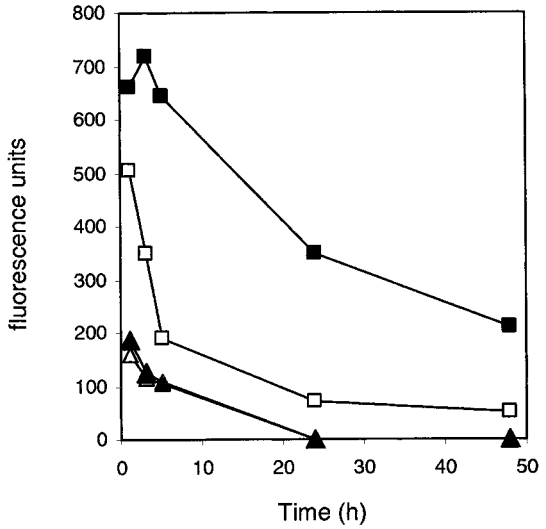

Figure 2. Stability of in vitro formed HLA-A2/peptide complexes. Detection of complex stability was assessed after formation of a maximal number of complexes at 4 °C with 10^{-5} M positive peptide, as specified in the direct assay (Experimental Section), unbound peptides being eliminated. Further incubation varying from 1 to 48 h at 37 °C was performed, and remaining complexes were detected in microplates. Peptides used were MART-1(27−35) (△), [Leu^{28}]MART-1(27−35) (□), analogue **4** (■), and analogue **10** (▲).

central part of the peptide zigzags[12] or bulges out[13] of the cleft. The permeability of the P3−P7 region to chemical modification is further illustrated by current strategies in the design of MHC blocking agents which are based on the replacement of the central residues by nonpeptidic spacers.[14−16]

Since it has been proposed in viral systems that immunogenicity correlates better with stability of HLA-A2/peptide complexes,[6b] we also evaluated the stability at 37 °C of the formed complexes. The greater potency of analogue **4** over [Leu^{28}]MART-1(27−35) was confirmed in the stabilization assay (Figure 2). HLA-A2 complexes formed with compound **4** were stable over a 24-h period (about 50% of remaining complexes), while those formed with [Leu^{28}]MART-1(27−35) dissociated almost completely in the same time (about 15% remaining complexes). Conversely, HLA-A2 complexes formed with MART-1(27−35) and compound **10** show no significative difference in stability, thus suggesting that in the absence of an optimal anchor residue at P2, the β-amino substitution alone at P4 does not contributes significantely to stabilization of the complexes.

Recognition by TILs. CTL clones (expressing different TCR Vβ segments) derived from human TILs, restricted by the HLA-A2 molecule, and specific to the MART-1(27−35) peptide were tested for their ability to recognize [Leu^{28}]MART-1(27−35) and the various substituted MART-1(27−35) analogues in a ^{51}Cr release cytolytic assay using T2 cells as target cells (Figure 3).

As expected, none of the three clones could induce lysis of target cells pulsed with [Leu^{28}]MART-1(27−35). Although the reactivity of TILs specific for MART-1(27−35) has been shown to be partially degenerate[17] (numerous peptides derived from a variety of endogeneous and foreign proteins and showing sequence similarity to MART-1(27−35) were found to be recognized by TILs and anti-MART-1 CTLs), it appears to be extremely sensitive to the modification at P2. Deleterious effect

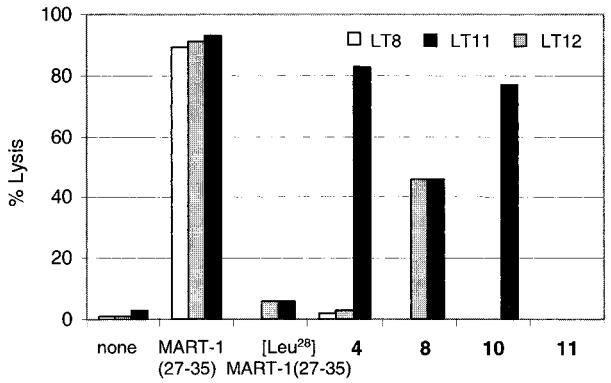

Figure 3. Recognition of MART-1(27−35), [Leu^{28}]MART-1(27−35), and analogues **4**, **8**, **10**, **11** by TILs. The cytolytic activity of TILs (LT8, LT11, LT12) was analyzed on T2 cells pulsed with peptides MART-1(27−35), [Leu^{28}]MART-1(27−35), and analogues in a 4 h ^{51}Cr release assay.

on CTL recognition as a consequence of changes at the peptide anchor residue has previously been described in another system.[18] In the case of the [Leu^{28}]MART-1(27−35) analogue, conformational changes in the region accessible to the TCR suggested by molecular modeling of the HLA-A2/peptide complex, as well as fast dissociation of the complex, may account for the loss of antigenic activity observed. Attempts to restore activity in the cytolytic assay by using our double-substitution strategy failed in the case of HLA-A2 binding peptides **3** and **5**−**7** (data not shown). However, the double-substituted MART-1(27−35) analogue **4** with a methylene insertion at P4 which we identified as a good HLA-A2 binder completely restored the recognition and lysis activity of one of the clones (LT12). The effect of peptide concentration on cell lysis was measured (Supporting Information). Half-maximal lysis of T2 cells by clone LT12 was observed at a concentration of 1.1×10^{-9} M for MART-1(27−35) vs 7.0×10^{-8} M for **4** (∼64-fold decrease in recognition). A similar pattern of recognition was observed in the case of monosubstituted analogue **10** (Figure 3).

Compound **8**, which was found to bind only weakly to HLA-A2 in the assembly test, was also able to restore partial lysis activity for two of the clones (LT11 and LT12). Not surprisingly, target cells sensitized with the corresponding single β-amino-substituted analogue **11**, which was found not to bind to HLA-A2 (see Figure 1), were not able to induce CTL lysis.

Conclusion

Among various strategies proposed to overcome the weak immunogenicity of peptides derived from tissue differentiation antigens expressed by tumors including MART-1, immunization with heteroclytic antigens with high MHC binding capacity is of particular interest. Such altered peptide ligands are usually designed by single or double susbtitutions at MHC primary or secondary anchor residues. Here, we show, in the case of the MART-1(27−35) CTL epitope that combining an Ala for Leu substitution at P2, which alone is deleterious for antigenic activity (although it improves binding to HLA-A2), with a β-amino acid susbstitution at a putative TCR contact residue (P4, P8) can partially restore recognition by TILs. In addition, the resulting analogue [Leu^{28},β-HIle^{30}]MART-1(27−35) displays both

a stronger binding to HLA-A2 and a more prolonged stability of formed complexes than the corresponding monosubstituted analogue [Leu[28]]MART-1(27−35). Compounds **4** and **8** thus represent potential candidates for the generation of CTL responses that could cross-react with the original poorly immunogenic antigen MART-1(27−35). Such heteroclytic analogues could be particularly useful to bypass the tolerance that can result from immunization with a self-antigen such as MART-1(27−35). It is worth mentioning that the β-amino acid insertion might contribute to increased resistance to enzymatic degradation and therefore might lead to improved in vivo activity. This is particularly relevant in view of the very short half-life of MART-1(27−35) in human plasma.[19] Hence, the immunogenicity of **4** will be tested in a transgenic mouse model expressing HLA-A2 to evaluate the potential benefit of increased peptide stability in human plasma. Furthermore, optimization of this double-substitution strategy by introduction of peptide bond isosteres in the central region that could compensate for the negative effect of the substitution at P2 is in progress in our laboratories.

Experimental Section

Chemistry. Optical rotations were measured at 25 °C on a Perkin-Elmer 241 polarimeter (Saint Quentin Yvelines, France). Column chromatographic separations were performed with silica gel 60, 230−400 mesh ASTM (Merck, Darmstadt, Germany). Analytical HPLC was run on a Beckman instrument (Gagny, France) with a Nucleosil C18 column 5 μm (4.6 × 150 mm) using a linear gradient of (A) 0.1% TFA and (B) acetonitrile containing 0.08% TFA, at a flow rate of 1.2 mL/min with UV detection at 214 nm. Preparative reverse-phase HPLC was performed using a Perkin-Elmer apparatus on an Aquapore ODS-20 μm column (100 × 10) by elution with a linear gradient of (A) aqueous 0.06% TFA and (B) 80% acetonitrile/20% (A), at a flow rate of 6 mL/min with UV detection at 220 nm. Mass spectra were obtained by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) on a Protein TOF apparatus (Bruker Spectrospin, Bremen, Germany). Amino acids and derivatives were from Neosystem (Strasbourg, France). All reagents and solvents were of analytical grade. *N*-Fmoc-β^3-amino acids bearing the side chains of Ala, Val, Leu and Ile were synthesized as described starting from the corresponding *N*-Fmoc-protected α-amino acids and gave spectroscopic data in good agreement with those previously reported.[9]

(3*S*,4*R*)-4-(*tert*-Butoxy)-1-diazo-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}pentan-2-one (Fmoc-(3*S*,4*R*)-Thr(*t*-Bu)-CHN$_2$) (12). To a cold −20 °C solution of Boc-Thr(*t*-Bu)-OH (15.39 g, 40 mmol) in THF under Ar were sucessively added *i*-BuOCOCl (5.71 mL, 44 mmol) and NMM (4.81 mL, 44 mmol). After 20 min of stirring at −20 °C the resulting white suspension was filtered rapidly and a solution of CH_2N_2 in Et_2O was added to the filtrate. Stirring was continued for 3h as the mixture was allowed to warm to room temperature. Excess CH_2N_2 was destroyed by vigorous stirring. The mixture was diluted with Et_2O and washed with saturated $NaHCO_3$ solution, water, 1 M $KHSO_4$ and saturated NaCl solution. The organic phase was dried over $MgSO_4$ and concentrated in vacuo. Flash chromatography (EtOAc/hexane 1:5) yielded **12** (14.58 g, 84%) as a yellow oil: HPLC t_R 16.51 min (linear gradient, 30−100% B, 20 min); ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.92 (d, J = 7.2 Hz, 3 H), 1.22 (s, 9 H), 4.03−4.26 (m, 3 H), 4.38−4.56 (m, 2 H), 5.60 (s, 1 H), 5.84 (d, J = 6.8 Hz, 1 H), 7.23−7.44 (m, 5 H), 7.62 (d, J = 7.2 Hz, 2 H), 7.75 (d, J = 7.0 Hz, 2 H); ^{13}C NMR (50 MHz, CD_3CN) δ 18.9, 28.4 (CH_3), 47.4, 54.3, 63.6 (CH), 66.8 (CH_2), 67.2 (CH), 74.8 (C), 120.1, 125.1, 127.1, 127.8 (CH), 141.4, 143.8, 156.1, 192.5 (C).

(3*R*,4*R*)-4-(*tert*-Butoxy)-3-{[(9*H*-fluoren-9-ylmethoxy)-carbonyl]amino}pentanoic Acid (Fmoc-(*R*,*R*)-β-HThr(*t*-Bu)-OH) (13). To a cold −20 °C solution of **12** (14.38 g, 34 mmol) in THF containing 10% H_2O was added under exclusion of light a solution of silver trifluoroacetate (826 mg, 3.74 mmol) in NMM (9.3 mL, 85 mmol) and the resulting mixture was allowed to warm to room temperature in 4 h in the dark. It was diluted with saturated $NaHCO_3$ solution and extracted with Et_2O. The aqueous phase was acidified to pH 2−3 with 1 M $KHSO_4$ at 0 °C and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. Flash chromatography (EtOAc/hexane 3:5) yielded **13** (8.24 g, 59%) as a solid foam: $[\alpha]^{25}_D$ + 9.6 (*c* 1.04, $CHCl_3$); HPLC t_R 14.59 min (linear gradient, 30−100% B, 20 min); ^{1}H NMR (200 MHz, CD_3OD) δ 1.02 (d, J = 6.3 Hz, 3 H), 1.18 (s, 9 H), 2.34 (dd, J = 9.1 Hz, J = 15.9 Hz, 1 H), 2.62 (dd, J = 5.05 Hz, J = 15.9 Hz, 1 H), 3.66−3.84 (m, 1 H), 3.97−4.05 (m, 1 H), 4.16 (t, J = 6.5 Hz, 1 H), 4.33 (d, J = 7.3 Hz, 2 H), 7.23−7.39 (m, 5 H), 7.62 (d, J = 7.2 Hz, 2 H), 7.75 (d, J = 6.7 Hz, 2 H); ^{13}C NMR (50 MHz, CD_3CN) δ 18.5, 28.9 (CH_3), 35.4 (CH_2), 48.6, 54.3 (CH), 67.7 (CH_2), 68.9 (CH), 75.2 (C), 121.0, 126.3, 128.2, 128.8 (CH), 142.7, 145.2, 145.5, 158.4, 175.6 (C); MS (MALDI-TOF) m/z 451 $[M + K]^+$, 434 $[M + Na]^+$.

Peptide Synthesis. Peptides **1**−**11** were synthesized in Fmoc chemistry by the stepwise solid-phase methodology. Assembly of the protected peptide chains was carried out on a 60-μmol scale starting from either Fmoc-Val-Wang resin (peptides **1**−**8, 10, 11**) or *o*-chlorotrityl chloride resin (peptide **9**). The Fmoc group was removed using 20% piperidine in DMF (1 × 5 min, 1 × 15 min) under nitrogen bubbling. The resin was then filtered and washed with DMF (6 × 3 min). For each coupling step, a solution of the Fmoc-α- or -β-amino acid (5 equiv), BOP (5 equiv), and HOBt (5 equiv) in DMF and DIEA were added successively to the resin, and the suspension was mixed for 10 min. A double coupling was performed systematically. Monitoring of the coupling reaction was generally performed with the Kaiser ninhydrin test or with 2,4,6-trinitrobenzene sulfonic acid (TNBS). After the removal of the last Fmoc protecting group, the resin was washed with CH_2Cl_2, Et_2O and dried under nitrogen. Side chain deprotection and cleavage of the peptide from the resin was performed by treatment with King's reagent (TFA/phenol/water/thioanisole/1,2-ethanedithiol 82.5:5:5:2.5) for 150 min at 20 °C. After precipitation in cold ether and centrifugation, the peptide was solubilized and lyophilized. The crude peptides were finally purified by HPLC (linear gradient, 5−65% B, 30 min) and lyophilized. Analytical data are reported in Table 1.

Detection of Peptide/HLA Interactions. Direct test: HLA molecules were purified from B-EBV cell lines by affinity columns, as previously described[20] and stored frozen at −80 °C. 50 μg diluted in 500 μL of PBS plus 0.1% NP40 was mixed with 100 μL of 1% bovine serum albumin (BSA) and denatured in the presence of 12.5 mM NaOH pH 11.7 plus 1.5 M urea (total volume 800 μL) for 1 h at 4 °C. The heavy chain and β2-microglobulin (β2m) were separated from endogenous peptides on a G25 column (PD10, Pharmacia, Orsay, France) equilibrated with PBS containing 0.05% Tween 20, 10 mg/mL BSA, 1 mM PMSF, 10 μg/mL trypsin inhibitor, 3 mM sodium azide (in PBS−Tween). HLA molecules diluted in a volume of 2.5 mL were added 2 μg/mL exogenous β2m (Sigma, St. Louis, MO) and 6 mM CHAPS (Sigma, St. Louis, MO). Aliquots of 1 μg of HLA were incubated with exogenous peptides at concentrations varying from 10^{-4} to 10^{-8} M, in Eppendorf microtubes for 2 h at room temperature then 24 h at 4 °C. Peptide from the influenza nucleoprotein NP 383-391 (SRYWAIRTR) was used as negative control. Each aliquot were further dispatched in 2 wells of microtiter plates (100 μL/well, Maxisorp Nunc) coated with anti-HLA monoclonal antibody (mAb) (coating with 10 μg/mL in PBS) and incubated for 20h at 4 °C. Correctly folded HLA/complexes were revealed with anti-β2m mAb M28 coupled to alkaline phosphatase and 4-methylumbelliferyl phosphate (MUP, Sigma, St. Louis, MO). Fluorescence was measured at 355/460 nm (Victor, Wallac, Evry, France).

Stability of peptide/HLA complexes: HLA denaturation and renaturation with 10^{-5} M exogenous peptide were per-

formed as specified above. After an overnight incubation at 4 °C, unbound peptides were eliminated by centrifugation on NANOSEP 10K (Pall Filtron, Northborough, MA). Samples were diluted in PBS-Tw, and aliquoted in Eppendorf microtubes for further incubations (1, 3, 5, 24 and 48 h) at 37 °C. An aliquot (time 0) was immediately tested for having the maximal number of formed complexes. The two incubations with anti-HLA mAb were performed in microplates at 37 °C for 1 h. Final detection was performed as described above.

T2 Cell Line. T2 is a variant of the cell line T1 produced by fusion of the lymphoma cell CEM and the B lymphoblastoid cell line 721.174. It expresses low amounts of HLA-A2 and no HLA-B or -C molecules at the cell surface because a lack of peptide transporters results in an accumulation of HLA heavy chain in the endoplasmic reticulum.[21] HLA-A2 molecules can be stabilized at the cell surface by the addition of exogenous peptides associating with them.

TILs. Human T cell clone-infiltrating melanomas, restricted by the HLA A2.1 molecule and specific to the MART-1(27−35) peptide, namely LT12 (Vβ2), LT11 (Vβ9), LT8 (Vβ5), were kindly provided by F. Farace (Institut Gustave Roussy, Villejuif, France). Cells were grown in complete medium. RPMI 10% human AB serum containing 50 IU/mL penicillin, 50 μg/mL streptomycin, nonessential amino acids, 10 mM hepes, 1 mM pyruvate, glutamine, 100 U/ml IL-2 (Boehringer) and TCGF 3%. The mutant T2 cell line was used as target cell in cytolytic assays. Cells were grown in complete medium: RPMI 10% FCS containing 50 IU/mL penicillin, 50 μg/mL streptomycin, nonessential amino acids, 10 mM hepes, 1 mM pyruvate, glutamine.

Cytolytic Activity Assay. Cytolytic activity was detected by a standard 4-h ^{51}Cr assay. The T2 target cells labeled with 100 μCi of sodium chromate (^{51}Cr, 100 mCi/mL, DuPont-NEN Research Products, Boston, MA) were incubated for 1 h with 1 μg/mL peptide, washed twice with 0.9% NaCl medium containing 5% FCS and dispatched at 3000 cells/well. Spontaneous release never exceeded 25% of the maximum ^{51}Cr uptake. The percent specific lysis was determined as: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

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Supporting Information Available: ^{1}H NMR data of analogue **4**, recognition of MART-1(27−35) and analogue **4** by clone LT12 at different concentrations, and molecular models of MART-1(27−35), [Leu^{28}]MART-1(27−35), and analogue **4** complexed toHLA/A2. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Boon, T.; Coulie, P. G.; Van den Eynde, B. Tumor antigens recognized by T cells. *Immunol. Today* **1997**, *18*, 267−268.

(2) Hofbauer, G. F.; Kamarashev, J.; Geertsen, R.; Boni, R.; Dummer, R. Melan A/MART-1 immunoreactivity in formalin-fixed paraffin-embedded primary and metastatic melanoma: frequency and distribution. *Melanoma Res.* **1998**, *8*, 337−343.

(3) (a) Kawakami, Y.; Eliyahu, S.; Delgado, C. H.; Robbins, P. F.; Rivoltini, L.; Topalian, S. L.; Miki, T.; Rosenberg, S. A. Cloning the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 3515−3519. (b) Coulie, P. G.; Brichard, V.; Van Pel, A.; Wolfel, T.; Schneider J.; Traversari C.; Mattei, S.; De Plaen, E.; Lurquin, C.; Szikora, J. P.; Renauld, J. C.; Boon, T. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* **1994**, 180, 35−42.

(4) (a) Kawakami, Y.; Eliyahu, S.; Sakaguchi, K.; Robbins, P. F.; Rivoltini, L.; Yannelli, J. R.; Appella, E.; Rosenberg, S. A. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J. Exp. Med.* **1994**, *180*, 347−352. (b) Rivoltini, L.; Kawakami, Y.; Sakaguchi, K.; Southwood, S.; Sette, A.; Robbins, P. F.; Marincola, F. M.; Salgaller, M. L.; Yannelli, J. R.; Appella, E.; Rosenberg, S. A. Induction of tumor-reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by in vitro stimulation with an immunodominant peptide of the human melanoma antigen MART-1. *J. Immunol.* **1995**, *154*, 2257−2265.

(5) Jaeger, E.; Bernhard, H.; Romero, P.; Ringhoffer, M.; Arand, M.; Karbach, J. Ilsemann C, Hagedorn M, Knuth A. Generation of cytotoxic T-cell responses with synthetic melanoma-associated peptides in vivo: implications for tumor vaccines with melanoma-associated antigens. *Int. J. Cancer* **1996**, *66*, 162−169.

(6) (a) Sette, A.; Vitiello, A.; Reherman, B.; Fowler, P.; Nayersina, R.; Kast, W. M.; Melief C. J.; Oseroff, C.; Yuan, L.; Ruppert, J.; sidney, J. del Guercio, M. F.; Southwood, S.; Kubo, R. T.; Chesnut, R. W.; Grey, H. M.; Chisari, F. V. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J. Immunol.* **1994** *153*, 5586−5592. (b) van der Burg, S. H.; Visseren, M. J.; Brandt, R. M.; Kast, W. M.; Melief, C. J. Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. *J. Immunol.* **1996** *156*, 3308−3314.

(7) (a) Parkhurst, M. R.; Salgaller, M. L.; Southwood, S.; Robbins, P. F.; Sette, A.; Rosenberg, S. A.; Kawakami, Y. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. *J. Immunol.* **1996** *157*, 2539−2548. (b) Rosenberg, S. A.; Yang, J. C.; Schwartzentruber, D. J.; Hwu, P.; Marincola, F. M.; Topalian, S. L.; Restifo, N. P.; Dudley, M. E.; Schwarz, S. L.; Spiess, P. J.; Wunderlich, J. R.; Parkhurst, M. R.; Kawakami, Y.; Seipp, C. A.; Einhorn, J. H.; White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med.* **1998**, *4*, 321−327.

(8) Valmori, D.; Fonteneau, J. F.; Lizana, C. M.; Gervois, N.; Liénard, D.; Rimoldi, D.; Jongeneel, V.; Jotereau, F.; Cerottini, J. C.; Romero, P. Enhanced Generation of Specific Tumor-Reactive CTL in vitro selected Melan-A/MART-1 immunodominant peptide analogues. *J. Immunol.* **1998**, *160*, 1750−1758.

(9) (a) Podlech, J.; Seebach, D. On the preparation of β-amino acids from α-amino acids using the Arndt-Eistert reaction: scope, limitations and stereoselectivity. Application to carbohydrate peptidation. Stereoselective a-alkylations of some β-amino acids. *Liebigs Ann.* **1995**, 1217−1228. (b) Guichard, G.; Abele, S.; Seebach, D. Preparation of *N*-Fmoc-protected β^2- and β^3-amino acids and their use as building blocks for the solid-phase synthesis of β-peptides. *Helv. Chim. Acta* **1998**, *52*, 187−206.

(10) Barlos, K.; Gatos, D.; Kallitsis, J.; Papaphotiu, G.; Sotiriu, P.; Wenging, Y.; Schäfer, W. Darstellung geschützter peptid-fragmente unter einsatz substituierter triphenylmethyl-harze. *Tetrahedron Lett.* **1989**, *30*, 394−396.

(11) Connan, F.; Hlavac, F.; Hoebeke, J.; Guillet, J.-G.; Choppin, J. A simple assay for detection of peptides promoting the assembly of HLA class I molecules. *Eur. J. Immunol.* **1994**, *24*, 777−780.

(12) Guo, H. C.; Jardetzky, T. S.; Garrett, T. P.; Lane, W. S.; Strominger, J. L.; Wiley, D. C. Different length peptides bind to HLA-Aw68 similarly at their ends but bulge out in the middle. *Nature* **1992**, *360*, 364−366.

(13) Madden, D. R.; Garboczi, D. N.; Wiley: D. C. The antigenic identity of peptide-MHC complexes: A comparison of the conformations of five viral peptides presented by HLA-A2. *Cell* **1993**, *75*, 693−708.

(14) Weiss, G. A.; Collins, E. J.; Garboczi, D. N.; Wiley: D. C.; Schreiber, S. L. A tricyclic ring system replaces the variable regions of peptides presented by three alleles of human MHC class I molecules. *Chem. Biol.* **1995**, *2*, 401−407.

(15) Poenaru, S.; Lamas, J. R.; Folkers, G.; Lopez de Castro, J. A.; Seebach, D.; Rognan, D. Nonapeptide analogues containing (R)-3-hydroxybutanoate and beta-homoalanine oligomers: synthesis and binding affinity to a class I major histocompatibility complex protein. *J. Med. Chem.* **1999**, *42*, 2318−2331.

(16) Bianco, A.; Brock, C.; Zabel, C.; Walk, T.; Walden, P.; Jung, G. New synthetic non-peptide ligands for classical major histocompatibility complex class I molecules. *J. Biol. Chem.* **1998**, 273, 28759−28765.

(17) Loftus D. J.; Castelli, C.; Clay, T. M.; Squarcina, P.; Marincola, F. M.; Nishimura, M. I.; Parmiani, G.; Appella, E.; Rivoltini, L. Identification of epitope mimics recognized by CTL reactive to the melanoma/melanocyte-derived peptide MART-1(27−35). *J. Exp. Med.* **1996**, *184*, 647−657.

(18) Chen, W.; McCluskey, J.; Rodda, S.; Carbone, F. R. Changes at peptide residues buried in the major histocompatibility complex (MHC) class I binding cleft influence T cell recognition: a possible role for indirect conformational alterations in the MHC class I or bound peptide in determining T cell recognition. *J. Exp. Med.* **1993**, *177*, 869−873.

(19) Brinckerhoff, L. H.; Kalashnikov, V. V.; Thompson, L. W.; Yamshchikov, G. V.; Pierce, R. A.; Galavotti, H. S.; Engelhard, V. H.; Slingluff, C. L. Jr. Terminal modifications inhibit proteolytic degradation of an immunogenic MART-1(27−35) peptide: implications for peptide vaccines. *Int. J. Cancer* **1999**, *83*, 326−334.
(20) Choppin, J.; Martinon, F.; Gomard, E.; Bahraoui, E.; Connan, F.; Bouillot, M.; Levy, J. P. Analysis of physical interactions between peptides and HLA molecules and application to the detection of human immunodeficiency virus 1 antigenic peptides. *J. Exp. Med.* **1990**, *172*, 889−899.
(21) Salter, R. D.; Cresswell, P. Impaired assembly and transport of HLA-A and −B antigens in a mutant TxB cell hybrid. *EMBO J.* **1986**, *5*, 943−949.

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