Identification of Noncanonical Melanoma-Associated T Cell Epitopes for Cancer Immunotherapy[1]

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The identification of tumor-associated T cell epitopes has contributed significantly to the understanding of the interrelationship of tumor and immune system and is instrumental in the development of therapeutic vaccines for the treatment of cancer. Most of the known epitopes have been identified with prediction algorithms that compute the potential capacity of a peptide to bind to HLA class I molecules. However, naturally expressed T cell epitopes need not necessarily be strong HLA binders. To overcome this limitation of the available prediction algorithms we established a strategy for the identification of T cell epitopes that include suboptimal HLA binders. To this end, an artificial neural network was developed that predicts HLA-binding peptides in protein sequences by taking the entire sequence context into consideration rather than computing the sum of the contribution of the individual amino acids. Using this algorithm, we predicted seven HLA A*0201-restricted potential T cell epitopes from known melanoma-associated Ags that do not conform to the canonical anchor motif for this HLA molecule. All seven epitopes were validated as T cell epitopes and three as naturally processed by melanoma tumor cells. T cells for four of the new epitopes were found at elevated frequencies in the peripheral blood of melanoma patients. Modification of the peptides to the canonical sequence motifs led to improved HLA binding and to improved capacity to stimulate T cells. ***The Journal of Immunology,*** **2005, 174: 6716–6724.**

The identification of tumor-associated Ags (TAA)[5] and tumor-associated T cell epitopes (TATE) has led to new therapeutic vaccination strategies for the treatment of cancer (1–3) and is the basis for monitoring tumor-specific immune responses in patients (4). Since the mid-1990s, cancer vaccines of various designs such as synthetic peptides administered alone or together with IFAs or IL-2, or loaded onto autologous dendritic cells were tested in clinical trials (5–9). Although some trials have shown promising clinical responses, these were usually seen in no more than 20% of the patients. This low efficacy of therapeutic vaccination against cancer may, among other possible reasons, be caused by anergy, low functional avidity, or low frequency of the tumor-specific T cells (10, 11), or by a selection of Ag-loss variants of the tumor cells (12). It is now generally accepted that antitumor vaccines should include many different T cell epitopes to reduce the risk of immune evasion, and to address and activate as many tumor-specific T cells as possible. Consequently, the search for new TATE is greatly intensified. The most widely applied strategy for the identification of T cell epitopes, dubbed "reverse immunology," uses bioinformatic tools to predict peptides from the sequences of TAA that have the capacity to bind to the HLA molecules of interest. The predicted epitopes then need to be synthesized, tested in T cell assays, and validated as naturally processed and presented by the tumor cells (13, 14). The bioinformatics is based on HLA allele-specific sequence motifs that correspond to the amino acids that anchor the peptides in the peptide-binding grooves of the HLA molecules (15–17). The predictions use algorithms that are trained with the frequencies of the different amino acids at the different sequence positions of an epitope (18) or with binding data of known epitopes (19). As an example, HLA-A*0201, the most frequent HLA class I allomorph in Caucasian populations, binds preferentially nonapeptides with the aliphatic amino acids leucine, isoleucine, valine, or methionine at positions 2 and 9. The majority of the T cell epitopes known to date have been identified with such bioinformatic approaches. Notwithstanding, the available algorithms are very restrictive in that they select epitopes for their HLA-binding capacity and base these predictions predominantly on the individual input of the anchor amino acids. However, T cell epitopes need not be good HLA-binding peptides, as continuous production by the Ag-processing machinery may ensure sufficient representation at the cell surface for T cell stimulation. Moreover, it is expected and has been shown that, in HLA-restricted T cell epitopes, the entire sequence context and not only the amino acid occupancy at the individual sequence positions determines the HLA-binding and T cell-stimulating capacity of the peptides (20, 21). To overcome the limitations of the available bioinformatic tools, we developed a prediction algorithm that uses

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[5] Abbreviations used in this paper: TAA, tumor-associated Ag; ANN, artificial neural network; ICC, intracellular cytokine; TATE, tumor-associated T cell epitope; TRP-2, tyrosinase-related protein 2; RT, room temperature; BIMAS, BioInformatics and Molecular Analysis Section.

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multilayer artificial neural networks (ANN) that, by their parallel data processing, compute the HLA-binding properties of peptides based on the entire sequence context and, thus, can account for the interdependences of the individual amino acids in an epitope. To test this algorithm we predicted HLA A*0201-restricted epitopes of the melanoma-associated TAAs gp100, MAGE-A1, MAGE-A2, p53, and tyrosinase-related protein 2 (TRP-2), and chose for further analyses seven of the predicted peptides that do not conform to the HLA allele-specific epitope sequence motifs for this HLA allomorph.

Materials and Methods

Bioinformatics

Fully connected three-layered, feed-forward networks were used for feature extraction by supervised learning. The neural networks were implemented as described (22, 23). In the hidden and in the output layer, sigmoid transfer functions were used. All networks were trained using an evolutionary strategy to optimize the free parameter (24). The positive data set included 139 HLA A*0201-binding peptide sequences from the SYFPEITHI (18) and in-house databases; the negative data set included 114 nonapeptides of the human p53 protein sequence serially overlapping by 8 aa. Thereby, false negatives were tolerated in the training and test data sets. A total of 227 of these 253 peptide sequences were used as training data; 26 were reserved as independent test data. The prediction accuracy was calculated from the mean square error (25) as 0.0066 for the training and 0.042 for the test data. A detailed description of the bioinformatics will be reported elsewhere. The predictions of T cell epitopes were done for all possible nonapeptides of MAGE-A1 and -A2, p53, gp100, and TRP-2. Of the predicted epitopes, seven that do not conform to the HLA A*0201-specific epitope sequence motif were selected for further analysis. In addition to the original peptides, variants were designed with the canonical leucine in sequence positions 2 and 9. To minimize problems with unfavorable conformers or in synthesis and handling, peptides containing proline, glycine, methionine, cysteine, or series of four or more aliphatic amino acids were excluded. The peptides were custom-synthesized by EMC Microcollections.

Cells

The TAP-deficient T2 cells were obtained from P. Cresswell (Yale University School of Medicine, New Haven, CT) (26). The melanoma cell line SK-mel 24, SK-mel 28, SK-mel 37, and Malme-3M were purchased from American Type Culture Collection; the melanoma cell lines LRD-mel, AEO-mel, HRR-mel, and IEL-mel were established in our laboratory from melanoma metastases. The cells were cultured in DMEM (Invitrogen Life Technologies) with 10% FCS (Sigma-Aldrich) at 37°C in 8% CO_2. PBMC of healthy donors and melanoma patients were isolated from peripheral blood by density centrifugation using Ficoll Paque (Pharmacia). $CD8^+$ T cells were isolated from PBMC after staining with FITC-labeled anti $CD8^+$ mAb (DakoCytomation) by magnetoseparation (MACS; Miltenyi Biotec) using anti-FITC microbeads. For in vitro priming $CD8^+$ cells were stimulated with equal numbers of irradiated cells of the CD8-negative fraction of the PBMC pulsed with 10 μg of each peptide per milliliter of serum-free X-Vivo 15 (Cambrex) with 30 μM 2-ME (Invitrogen Life Technologies) at 37°C with 8% CO_2. From day 1 after initiation of the cultures on, 50 U/ml recombinant human IL-2 (Chiron) was added to the cultures and replenished every third day. The cultures were restimulated biweekly with peptide-pulsed CD8-depleted PBMC left from the initial cell preparation that had been stored at −80°C. The resulting cell lines were then analyzed for epitope-specific cells by ELISPOT assays. The clinical material was used with approval of the institutional ethics committee of the Charité and written informed consent of the patients.

Stabilization assay

TAP-deficient T2 cells were incubated for 16 h at 37°C in serum-free X-Vivo 15 medium with the indicated peptides at a concentration of 100 μg/ml. The resulting HLA-A*0201 expression at the surface of the cells was quantified by flow cytometry using the biotinylated conformation-dependent HLA A2-specific mAb BB7.2 and PE-streptavidin secondary reagents, and taken as measure for the degree of HLA stabilization.

ELISPOT

For ELISPOT analysis, 96-well polyvinylidene difluoride membrane plates (MAIP S45 10; Millipore) were coated with anti-human IFN-γ Abs (Pierce). A total of 1×10^5 irradiated autologous PBMC stimulator cells pulsed with 10 μg/ml peptide and $CD8^+$ T cells from the induction cultures were added per well. Negative controls were peptide-primed $CD8^+$ T cells only, primed $CD8^+$ T cells plus PBMC without peptide and PBMC alone. The positive controls were $CD8^+$ T cells stimulated with 3 μg/ml (Sigma-Aldrich). In the case of the melanoma patients, the ELISPOT analyses were done with the PBMC directly without in vitro priming. Here, negative controls were PBMC without peptide. After 16–20 h at 37°C, the cells were washed off and the IFN-γ was captured on the plates counterstained with biotinylated anti-human IFN-γ Abs (Pierce) and alkaline phosphatase-labeled streptavidin (Roche). IFN-γ-positive spots were visualized using 5-bromo-4-chloro-3-indolyl phosphate/NBT (Moss) and analyzed with a Bioreader 3000 (BioSys). The background value for each assay was calculated as the average of the controls. Responses counted as positive were at least twice the background. Responses above five times background were valued as strong responses.

Chromium release assay

The specificity of the T cells was analyzed with a chromium release assay as described elsewhere (27). Briefly, target cells were labeled with ^{51}Cr for 1 h at 37°C in X-Vivo 15 medium, then washed four times. A total of 5000 target cells were peptide-pulsed for 30 min at room temperature (RT) or left without peptide, and then plated with the $CD8^+$ effector T cells at the indicated ratios in 200 μl of medium in U-bottom microtiter wells. After 5 h at 37°C, 50 μl of the supernatant were harvested for determination of the release radiochromium. Percent-specific ^{51}Cr release was calculated as $100 \times$ ((experimental release − spontaneous release)/(maximum release − spontaneous release)). The T cells had been restimulated at least three times after the initial priming before the assays.

Intracellular cytokine (ICC) staining

ICC staining for epitope-specific $CD8^+$ T cells in the peripheral blood of melanoma patients was described elsewhere (27). Briefly, PBMC were used after cryopreservation and incubated for 30 min at RT and 30 min at 37°C in DMEM (Invitrogen Life Technologies) with 0.1% BSA (Sigma-Aldrich) with the peptides at a concentration of 10 μg/ml. Then, equal volumes of 20 μg of BFA per milliliter of DMEM with 20% FCS was added, and the incubation continued for 5 h. The cells were pelletted, resuspended in PBS/1 mM EDTA, and incubated for 10 min at 37°C. After centrifugation and resuspension, lysis buffer (BD Biosciences) was added, the cells incubated for 3 min at RT, then washed and incubated with permeabilization buffer (BD Biosciences) for 10 min at RT in the dark. For flow cytometry, the cells were stained with anti-CD8-allophycocyanin, anti-CD3-PerCP, anti-CD69-PE, and anti-IFN-γ-FITC (all BD Biosciences) and analyzed with a FACSCalibur flow-cytometer and CellQuest software (BD Biosciences).

Results

Prediction of noncanonical T cell epitopes

This study aimed at identifying with bioinformatic means HLA class I-restricted TATEs that bind only weakly to the corresponding HLA molecules and, therefore, are missed by other algorithms that are designed to predict peptides that bind strongly to HLA molecules and that conform to the HLA allele-specific epitope motifs as defined by the canonical amino acids listed in the SYFPEITHI database (Ref. 12; see introduction). To identify epitopes independent from these restrictive criteria we developed a strategy of in silico pattern recognition of HLA-binding T cell epitopes by ANN, which by their parallel mode of data processing can extract common features of complex amino acid sequence patterns (22, 23). Besides the specific design of the ANNs and the selection of input data sets, the predictions depend critically on the descriptors used to code the amino acids. We used the physicochemical-properties hydrophobicity, polarity, refractivity, and side chain volume as referenced (28–30). The ANNs were applied to MAGE-A1 and A2, gp100, p53, and TRP-2 to identify HLA A*0201-restricted epitopes. The output data included the known T cell epitopes $gp100_{178}$ MLGTHTMEV, $gp100_{619}$ RLMKQDFSV; TRP-2_{180} SVYDFFVWL, TRP-2_{288} SLDDYNHLV, TRP-2_{360} TLDSQVMSL; and MAGE-$A2_{112}$ KMVELVHFL that feature canonical anchors as well as $gp100_{154}$ KTWGQYWQV, $gp100_{280}$ YLEPGPVTA, $gp100_{209}$ ITDQVPFSV, $gp100_{639}$ RLPRIFCSC;

and MAGE-A1$_{278}$ KVLEYVIKV that deviate from these motifs (31–33). The seven newly predicted epitopes together with variants with the canonical leucine at the anchor positions 2 and 9 were studied for their capacity to bind to HLA-A*0201 and to stimulate specific T cells responses.

Table I lists the 7 predicted epitopes and the variants with leucines at positions 2 and 9, together with the scores calculated by our ANN. The algorithms had been trained with peptide sequences classified as either HLA-A*0201 ligands or as negative for HLA A*0201-binding. Correspondingly, the output defines the test sequences as potential epitopes or as negative without grading, meaning that any number in Table I column ANN is a positive output. Thus, all 7 peptides and their variants were classified as potential epitopes despite differences in the scoring. The 14 peptides were also scored with the algorithms from the BioInformatics and Molecular Analysis Section (BIMAS) of the Center for Information Technology, National Institutes of Health, which is based on kinetic parameter of peptide-binding to the HLA molecules and predicts the half-life times of the respective HLA peptide complexes (19). Here, only the peptide TRP-2$_{185}$ is identified as a potential epitope, none of the other 6 peptides are (Table I). In contrast, all of the variants peptides with the motif amino acid leucine in the anchor positions were scored highly. Consequently, TRP-2$_{185}$ had been identified earlier with BIMAS (34), but none of the other peptides. Also the SYFPEITHI algorithm, which scores peptides according to the degree to which they conform to the HLA allele-specific epitope motifs indicates improved HLA-binding properties of the modified peptides (18). The SYFPEITHI scoring system values the amino acids in specific sequence positions that are conserved among different epitopes, and sums up these individual values to assess the capacity of the peptide to bind to the HLA molecule. Canonical amino acids in the dominant anchor positions get a value of 10. Because there are two dominant anchor positions that define the HLA allele-specific epitope motifs, strong binders should have a score of at least 20. A value below 20 indicates that at least one anchor position carries a suboptimal amino acid. Therefore, 20 is usually taken as the cut-off for T cell epitope prediction. By this criterion, none of the 7 peptides selected by our approach would qualify. However, the modified peptides do so as they were purposely adapted to the canonic anchor motifs (Table I). For an experimental assessment of the capacity of the 14 peptides to bind to HLA-A*0201, we performed HLA stabilization assays with the TAP-deficient cell line T2. Because of the TAP defect these cells lack the proper peptides for binding to and stabilizing of the native conformation of the HLA class I molecules. As a consequence, the cell surface expression levels of HLA are low but can increase when the cells are incubated with suitable peptides. The levels of HLA expression detected after peptide-pulsing can then be used as measure for the strength of binding as illustrated for the influenza matrix protein peptide MP$_{58}$ in comparison with T2 cells without peptide (Fig. 1). Except for TRP-2$_{185}$, HLA-A*0201 expression levels after incubation with the original peptides were unaltered when compared with the background without peptide. For TRP-2$_{185}$ that already in its unmodified form scored highly in BIMAS and SYFPEITHI predictions, the HLA expression after incubation with the natural epitope was high above background but still topped by the introduction of leucine into the anchor positions. For MAGE-A1$_{237}$ and gp100$_{286}$, the modifications resulted in peptides that, in contrast to their natural counterparts, stabilized the HLA molecules indicating enhanced binding. Consistent with the predictions by the BIMAS algorithms, the modification of peptide p53$_{347}$ did not produce a peptide with good HLA-binding properties. These results prove that six of the seven peptides predicted by our algorithms are weak HLA binders and would not be predicted as T cell epitopes by the other algorithms.

T cell responses to predicted noncanonical epitopes

To test and validate the predicted weak HLA-binding melanoma-associated T cell epitopes we chose two approaches. First, PBMC of melanoma patients were analyzed directly ex vivo by intracellular IFN-γ cytokine staining and ELISPOT for CD8$^+$ T cell that specifically respond to the peptides. In this instance, elevated frequencies of the specific T cells would prove that the peptides are T cell epitopes and that they are relevant for antimelanoma immunity. Second, CD8$^+$ T cells from peripheral blood of healthy donors were primed in vitro with the peptides, and the responding T cell lines were tested for their epitope-specificity by ELISPOT. Hereby, we identify peptides that, in principle, can be T cell epitopes even if the corresponding T cells are not expanded in melanoma patients.

Table I. *Potential T cell epitopes predicted by the ANN algorithm presented with this report and variants of these peptides that were modified at positions 2 and 9 by introducing leucine, the known canonical anchor amino acid for HLA-A*0201*

Ag	Position	Modification[b]	Peptide	Sequence	ANN[c]	SYFPEITHI Scores[d]	BIMAS Scores[e]
MAGE-A1	237–245		MAGE-A1$_{237}$	KLLTQDLVQ	67	14	0.079
MAGE-A1	237–245	Q245L	MAGE-A2$_{244}^{mod}$	KLLTQDLVL	99	24	113.424
MAGE-A2	116–124		MAGE-A2$_{116}$	LVHFLLLKY	98	13	0.025
MAGE-A2	116–124	V117L, Y124L	MAGE-A2$_{116}^{mod}$	LLHFLLLKL	99	29	83.527
MAGE-A2	250–258		MAGE-A2$_{250}$	LVQENYLEY	98	11	0.068
MAGE-A2	250–258	V251L, Y285L	MAGE-A2$_{250}^{mod}$	LLQENYLEL	99	27	223.344
gp100	286–294		gp100$_{286}$	VTAQVVLQA	81	16	0.27
gp100	286–294	T287L, A294L	gp100$_{286}^{mod}$	VLAQVVLQL	99	28	83.527
p53	17–25		p53$_{17}$	ETFSDLWKL	54	18	1.42
p53	17–25	T18L	p53$_{17}^{mod}$	ELFSDLWKL	97	24	102.259
p53	347–355		p53$_{347}$	ALELKDAQA	99	18	0.318
p53	347–355	A355L	p53$_{347}^{mod}$	ALELKDAQL	99	24	1.367
TRP-2	185–193		TRP-2$_{185}$	FVWLHYYSV	98	19	348.534
TRP-2	185–193	V186L, V193L	TRP-2$_{185}^{mod}$	FLWLHYYSL	99	25	1223.425

[a] The predictions are compared to the output of the SYFPEITHY and the BIMAS algorithms for T cell epitope prediction.
[b] Peptides were modified at positions 2 and 9 by introducing leucine, the known canonical anchor amino acid for HLA-A*0201.
[c] Predictions by the ANN as described in this report.
[d] The score for peptide/HLA-A*0201 binding according to the SYFPEITHY algorithm ⟨www.syfpeithi.de/⟩.
[e] The predicted half-time of the peptide/HLA class I/β_2-microglobulin complexes in minutes according to the BIMAS algorithm ⟨http://bimas.dcrt.nih.gov/molbio/hla_bind/⟩.

Table II. *Frequencies of CD8$^+$ T cells with specificities for noncanonical epitopes predicted by ANN and their anchor position-optimized variant detected in melanoma patients by ELISPOT assays*[a]

Peptide	Sequence	SER	LTT	LLI	RAL	REN
MAGE-A2$_{116}$	LVEFLLLKY	*300*[b]	**1088**[c]	**474**	*23*	**477**
MAGE-A2$_{116}^{mod}$	LLHFLLLKL	**2300**	353[d]	222	*0*	127
MAGE-A1$_{237}$	KLLTQDLVQ	900	**529**	**533**	**293**	287
MAGE-A1$_{237}^{mod}$	KLLTQDLVL	800	412	400	*	**318**
MAGE-A2$_{250}$	LVQENYLEY	*200*	294	192	45	*64*
MAGE-A2$_{250}^{mod}$	LLQENYLEL	*300*	**765**	*59*	**181**	127
gp100$_{286}$	VTAQVVLQA	*0*	**824**	**533**	**226**	223
gp100$_{286}^{mod}$	VLAQVVLQL	*200*	**1235**	**993**	**451**	**637**
p53$_{17}$	ETFSDLWKL	500	*	370	*23*	*96*
p53$_{17}^{mod}$	ELFSDLWKL	*300*	*29*	*59*	45	*64*
p53$_{347}$	ALELKDAQA	*200*	*29*	237	*23*	*64*
p53$_{347}^{mod}$	ALELKDAQL	*300*	*88*	*59*	*0*	**319**
TRP-2$_{185}$	FVWLHYYSV	**1300**	**735**	**489**	45	**732**
TRP-2$_{185}^{mod}$	FLWLHYYSL	900	*88*	192	*0*	191
Background value		250	88	89	15	63

[a] The numbers denote spot-forming units per 1×10^6 CD8$^+$ cells.
[b] Italic figures show negative results with responses less than two times background value.
[c] Bold-face figures indicate strong responses five or more times background value.
[d] Standard figures indicated weak responses two or more times background value.
*, Not analyzable.

We analyzed six HLA A*0201-positive patients for T cells in their peripheral blood with specificities for the predicted epitopes. In every patient we could detect specific T cell responses with between two and seven of the seven epitopes addressed. In the case of patient IER, these analyses were done by intracellular IFN-γ staining to establish the phenotype of the responding cells. These cells were CD3$^+$CD8$^+$ and expressed also the activation marker CD69. Responses were seen against the epitopes MAGE-A2$_{116}$ and TRP-2$_{185}$ and to their anchor position-optimized variants MAGE-A2$_{116}^{mod}$ and TRP-2$_{185}^{mod}$ with frequencies among the peripheral blood CD3$^+$ CD8$^+$ T cells of 1.85, 1.93, 2.21, and 1.56%, respectively, vs a background of 0.15% (Fig. 2). The modified version of gp100$_{286}$ triggered a weak response of 0.31% but not its natural counterpart (Fig. 2). The other peptides induced no responses above the background measured without peptide. The PBMC of the other five melanoma patients (SER, LTT, LLI, RAL, and REN) were tested by ELISPOT for epitope-specific T cells (Table II). Taking these analyses together, all seven epitopes induced T cell responses. For the epitopes MAGE-A2$_{250,}$ p53$_{17}$, and

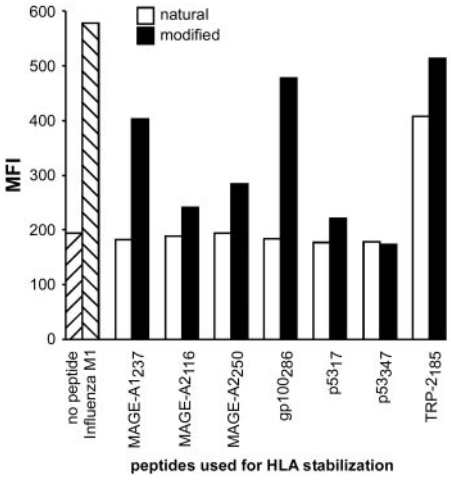

FIGURE 1. Lack of HLA stabilization by natural noncanonical T cell epitopes and strong stabilization by their anchor position-stabilized variant. T2 cells were incubated with 100 μg/ml of the indicated peptides for 16 h at 37°C. HLA expression by T2 cells was measured by flow cytometry using the HLA A2-specific mAb BB7.2. Mean fluorescence intensities (MFI) of the staining are shown. Negative controls were T2 cells without peptide and positive control T2 cells incubated with the influenza matrix-protein M1 epitope (47). ▨ and ▧, Negative and positive control results; □, the natural epitope; ■, the corresponding variant with optimized anchor positions. The data in the figure are representatives of three independent experiments with comparable results.

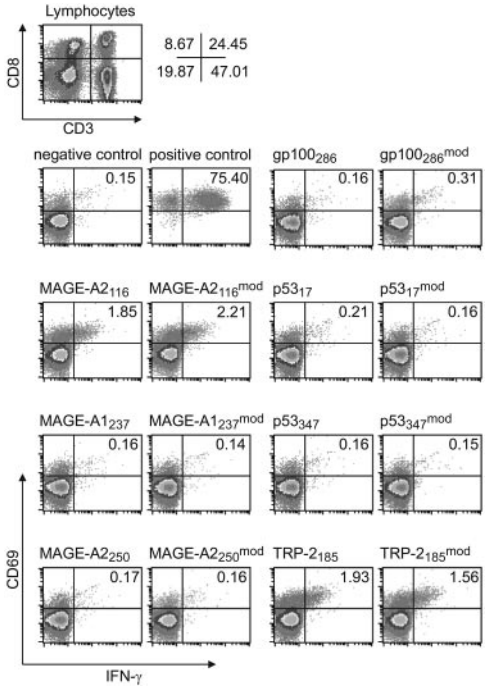

FIGURE 2. Enhanced frequencies of epitope-specific T cells in the peripheral blood of a melanoma patient. Bulk PBMC from melanoma patient IER were harvested and analyzed in an ICC staining without prior sensitization for the expression of CD3$^+$CD8$^+$ T cells that upon stimulation with the peptides express CD69 and IFN-γ. Cells were gated for CD3 and CD8 expression (*upper panel*) and analyzed for the expression of CD69 and IFN-γ. The numbers given are percent CD69$^+$IFN-γ$^+$ of CD3$^+$CD8$^+$ cells. Negative controls were cells without stimulation; positive controls were cells triggered with phorbol-12-myristate-13-acetate and ionomycin.

Table III. *Frequencies of CD8$^+$ T cells with specificities for the noncanonical epitopes predicted by ANN and their anchor position-optimized variant expanded from peripheral blood of healthy donors and detected by ELISPOT*

Peptide	Sequence	A[b]	B[b]	C[c]	C[d]	D[b]	E[b]
MAGE-A2$_{116}$	LVHFLLLKY	**343**[e]	**314**	342[f]	743	**2257**	**371**
MAGE-A2$_{116}^{mod}$	LLHFLLLKL	**3086**	**2086**	**1171**	**3228**	**1600**	**829**
MAGE-A1$_{237}$	KLLTQDLVQ	114	**571**	**486**	**1714**	**800**	229
MAGE-A1$_{237}^{mod}$	KLLTQDLVL	*29*[g]	**686**	*86*	**2543**	200	171
MAGE-A2$_{250}$	LVQENYLEY	86	*29*	*57*	*171*	*114*	*29*
MAGE-A2$_{250}^{mod}$	LLQENYLEL	**800**	57	**1943**	**943**	257	*86*
gp100$_{286}$	VTAQVVLQA	*29*	*0*	314	*142*	*57*	200
gp100$_{286}^{mod}$	VLAQVVLQL	86	86	**1029**	371	**1200**	*57*
p53$_{17}$	ETFSDLWKL	143	**800**	*114*	714	*142*	**314**
p53$_{17}^{mod}$	ELFSDLWKL	143	**714**	*86*	371	57	200
p53$_{347}$	ALELKDAQA	86	**171**	*114*	342	171	171
p53$_{347}^{mod}$	ALELKDAQL	*57*	**257**	*29*	*229*	*114*	*86*
TRP-2$_{185}$	FVWLHYYSV	**2914**	**1171**	**1089**	**2400**	**629**	**914**
TRP-2$_{185}^{mod}$	FLWLHYYSL	**2542**	**800**	314	628	**743**	**943**
Background value		38	19	86	152	86	57

[a] The numbers denote spot-forming units per 1×10^6 CD8$^+$ cells.
[b] After initial priming.
[c] After one restimulation.
[d] After two restimulations.
[e] Bold-face figures indicate strong responses five or more times background value.
[f] Standard figures indicated weak responses two or more times background value.
[g] Italic figures show negative results with responses less than two times background value.
∗, Not analyzable.

p53$_{347}$ these responses were relatively weak but still unequivocal. High frequency responses were seen against the epitopes MAGE-A2$_{116,}$ MAGE-A1$_{237}$, gp100$_{286}$, and TRP-2$_{185}$. All of the modified variants were recognized by the CD8$^+$ T cells of the patient. The frequencies of responding T cells detected by ICC staining and by ELISPOT assays cannot be compared directly. Although there is a correlation of the values obtained with ICC and ELISPOT assays, there is no consistent conversion factor, and the frequencies detected with ICC assays are typically higher than those obtained with ELISPOT assays (35). Thus, with two different T cell assays and in different patients, strong expansion of T cell with specificities for MAGE-A2$_{116}$ and TRP-2$_{185}$ were found. Therefore, these two epitopes may be seen as among the immunodominant epitopes of MAGE-A2 and TRP-2. The frequencies of T cells detected for the other epitopes were somewhat lower but still clearly detectable in the ELISPOT assays. In all, we saw relatively high levels of T cell responses against the natural epitopes MAGE-A2$_{116}$, MAGE-A1$_{237}$, gp100$_{286}$, and TRP-2$_{185}$. In the cases of the MAGE-A2$_{250}$ and p53$_{347}$, strong responses were only obtained with the modified version. Thus, these results prove that at least four of the seven predicted weak HLA-binding epitopes play an important role in anti-melanoma immunity in that T cells with the corresponding specificities were strongly expanded in the patients.

After in vitro priming and restimulation of CD8$^+$ T cells from the peripheral blood of five healthy HLA A*0201-positive donors we could detect by ELISPOT assays specific responses against all seven epitopes. MAGE-A2$_{116}$, MAGE-A1$_{237}$, p53$_{17}$, p53$_{347}$, and TRP-2$_{185}$ induced strong response (Table III). For MAGE-A2$_{250}$ and gp100$_{286}$ strong responses were seen only against the modified but not the natural epitopes. Two of the donors responded to six epitopes each, one against three and two against five epitopes. Taking the two sets of analyses with PBMC from melanoma patients and healthy donors together, all of the seven predicted noncanonical HLA-binding epitopes did induce specific T cell responses, six of them (MAGE-A2$_{116}$, MAGE-A1$_{237}$, gp100$_{286}$, p53$_{17}$, p53$_{347}$, and TRP-2$_{185}$) strong responses. Five of these (MAGE-A2$_{116}$, MAGE-A1$_{237}$, MAGE-A2$_{250}$, gp100$_{286}$, and

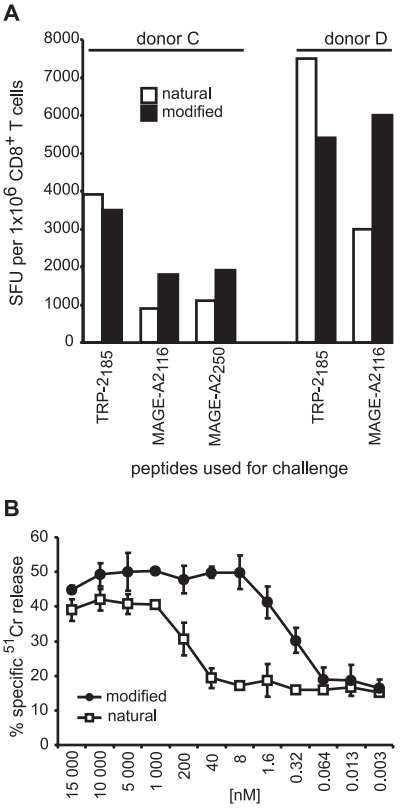

FIGURE 3. Recognition of the natural epitopes by T cells primed with the anchor position-optimized variants of these epitopes. *A*, CD8$^+$ T cells from donors C and D were primed with the variant peptides and analyzed by ELISPOT assays for IFN-γ secretion in response to natural epitopes (□) or the corresponding variant (■). The spot-forming units (SFU) are calculated for 1×10^6 CD8$^+$ T cells. *B*, CD8$^+$ T cells from donor D primed with MAGE-A2$_{116}^{mod}$ were tested in a cytolysis assay against serial dilutions of the natural epitope and its variant on TAP-deficient T2 cells. The E:T ratio was 30:1. The data are presented as the percentage of specific radiochromium release ± SD.

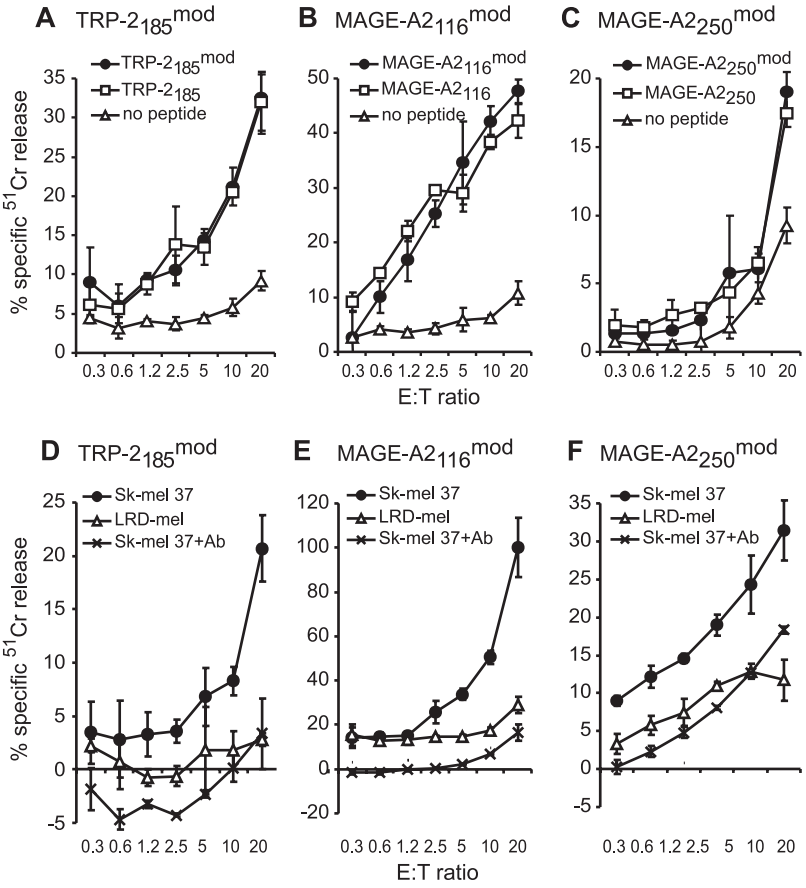

FIGURE 4. $CD8^+$ T cells primed with the anchor position-optimized epitopes recognize their natural counterpart. $CD8^+$ T cells from healthy donors primed with anchor position-optimized epitopes recognize T2 cells in the presence of the modified and the natural epitope (*A–C*) as well as endogenously processed and presented by melanoma tumor cells (*D–F*). The T cell responses were tested in triplicates by radiochromium release assays and are presented as the percentage of specific radiochromium release ± SD. *A* and *D*, The responses of T cells from donor D primed with TRP-2_{185}^{mod}; *B* and *E*, the responses of those primed with MAGE-$A2_{116}^{mod}$; *C* and *F*, the responses of T cells from donor C primed with MAGE-$A2_{250}^{mod}$. The target cells in *A–C* were T2 cells loaded with TRP-2_{185}^{mod} or TRP-2_{185} (*A*), MAGE-$A2_{116}^{mod}$ or MAGE-$A2_{116}$. (*B*), or MAGE-$A2_{250}^{mod}$ or MAGE-$A2_{250}$ (*C*). In all panels, ●, the response to the variant; □, the response to the natural epitope. △, The background response without peptide. *D–F*, The same T cell lines were tested against the melanoma cell lines Sk-mel 37, which express HLA-A*02, TRP-2, and MAGE-A2 (●) or LRD-mel, which expresses TRP-2 and MAGE-A2 but not HLA-A*02 (△). In addition, the cytolysis of SK-mel 37 was tested in the presence of the monoclonal HLA A2-specific mAb BB7.2 (×).

TRP-2_{185}) had already primed high frequencies of T cells in the patients. TRP-2_{185} had been identified before as an HLA A*0201-binding peptide but not yet as a T cell epitope (34). MAGE-$A2_{116}$ was reported earlier as an HLA A3-restricted epitope (36). We show in this study for the first time that TRP-2_{185} is indeed a T cell epitope and that MAGE-$A2_{116}$ is, in addition to HLA-A3, presented by HLA-A*0201. Six of the seven new HLA A*0201-restricted T cell epitopes were not identified by any of the other prediction algorithms for strong HLA-binding epitopes.

Comparison of the natural and the modified versions of the predicted epitopes

Several epitopes were recognized by the T cells in their natural as well as modified form. Therefore, we tested by ELISPOT whether T cells primed with the modified versions of the epitopes TRP-2_{185}, MAGE-$A2_{116}$, or MAGE-$A2_{250}$ also respond to the natural variant of these peptides. In all cases, the $CD8^+$ T cells primed with the modified epitope responded to both versions (Fig. 3*A*). In the case of TRP-2_{185}, the responses to the natural epitope were higher, whereas for the other two, the T cells responded better to the priming peptide than to their natural counterpart. Interestingly, $CD8^+$ T cells of donor C did not respond when primed, restimulated, and assayed with the unmodified peptide MAGE-$A2_{250}$ (Table III), suggesting that the modified epitope is more potent in priming T cells. Once primed, these T cells can mount good responses to the natural variant as well (Fig. 3*A*) although the peptide concentrations required may be higher as shown for cytolysis of MAGE-$A2_{116}$-pulsed T2 target cells by T cells primed with the corresponding anchor position-optimized variant of the epitope (Fig. 3*B*). The concentration of the natural epitope required for half-maximal cytolysis is ~150-times higher than what is required in the case of the modified peptide. Similar results were obtained for TRP-2_{185} (data not shown). In a more stringent setting, selected T cell lines raised against the modified versions of these three epitopes by repeated stimulation in vitro were tested in chromium release assays with both variants (Fig. 4, *A–C*). In the effector-to-target titration, the response curves against both epitope variants are congruent indicating that, as far detectable by these assays, all T cells primed with the modified epitopes responded to the natural counterpart as well. Thus, both sets of data indicate that the natural and the modified epitopes are recognized by the same T cells. Because the cytotoxicity assays were done with the T2 cells, which share only the HLA-A*0201 with the donors of the T cells, these results also prove that the newly identified epitopes are indeed presented by this HLA allomorph.

Processing and presentation of the noncanonical epitopes by melanoma cells

To confirm that the predicted epitopes are naturally processed and presented by the tumor cells, we tested the above cytolytic $CD8^+$ T cell lines from healthy donors that had been primed with the peptides TRP-2_{185}^{mod}, MAGE-$A2_{116}^{mod}$, or MAGE-$A2_{250}^{mod}$ for their capacity to lyse human melanoma cells that express the target Ags together with or without HLA-A*0201 (Fig. 4, *D–F*). The tumor cell lines used were SK-mel 37, which expressed TRP-2 and MAGE-A2 as well as HLA-A*0201, and LRD-mel, which expresses both Ags but not HLA-A*0201. The TRP-2-specific CTL lyse SK-mel 37 indicating that the natural counterpart of the priming peptide is presented by these cells. This lysis is completely inhibited with the HLA A2-specific mAb BB7.2. The HLA restriction of these CTL is confirmed by the fact that LRD-mel cells that lack HLA-A*0201 but express the Ag are not lysed. Likewise, CTL raised against MAGE-$A2_{116}^{mod}$ lysed Sk-mel 37 tumor cells but not the HLA A*0201-negative LRD-mel cells. Again this cell

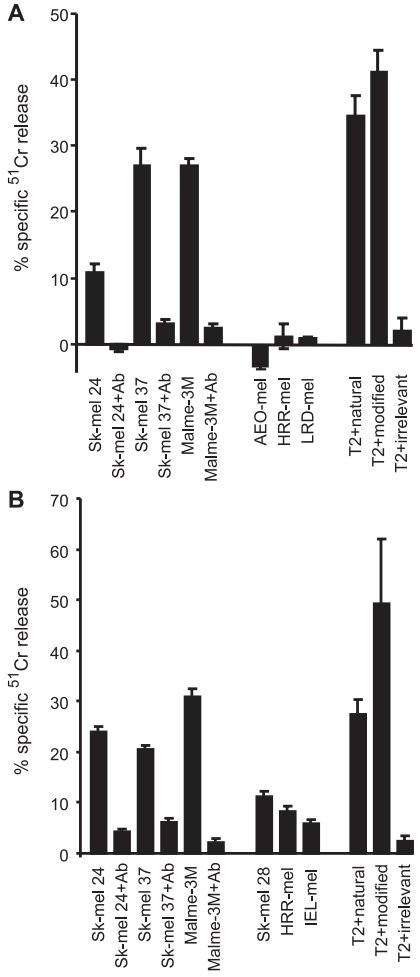

FIGURE 5. $CD8^+$ T cells primed with the modified peptides specifically recognize the endogenously processed and naturally presented epitopes. The T cell responses were tested in triplicates by radiochromium release assays at an E:T ratio of 30:1. The data are presented as the percentage of specific radiochromium release ± SD. *A*, The response of T cells from donor C primed with MAGE-$A2_{116}{}^{mod}$. The melanoma cell lines SK-mel 24 and SK-mel 37 are both MAGE-A2- and HLA-A*0201-positive. Malme-3M is also HLA-A*0201-positive but expresses MAGE-A3 and -A6. These two MAGE proteins contain a sequence identical to MAGE-$A2_{116}$. The cytolysis of SK-mel 24, SK-mel 37, and Malme-3M was tested in the absence and presence of the HLA A2-specific mAb BB7.2 (+Ab). The cell line AEO-mel is HLA-A*0201-positive but negative for all MAGE-A. The cell lines HRR-mel and LRD-mel express MAGE-A2 but not HLA-A*0201. T2 cells pulsed with MAGE-$A2_{116}$ or TRP-2_{185} as irrelevant peptide were used as controls for the specificity of the CTL. *B*, The response of T cells of patient REN that are specific for TRP-$2_{185}{}^{mod}$. The cell lines SK-mel 24, SK-mel 37, and Malme-3M are TRP2- and HLA-A*0201-positive. The cytolysis of these targets was tested in the absence or presence of the HLA A2-specific mAb BB7.2 (+Ab). The cell lines SK-mel 28, HRR-mel, and IEL-mel express TRP-2 but not HLA-A*0201. As before, T2 cells loaded with either the natural or modified form of TRP-2_{185} or MAGE-$A2_{116}$ as irrelevant peptide were used as controls.

lysis was inhibited by the HLA A2-specific Ab BB7.2. Also, MAGE-$A2_{250}{}^{mod}$-specific CTL lysed Sk-mel 37 but not or only weakly the LRD-mel cell line, and the lysis of Sk-mel 37 is inhibited with the HLA A2-specific mAb BB7.2. To extend these analyses, the MAGE $A2_{116}{}^{mod}$-primed CTL line was tested at a fixed E:T ratio against additional melanoma cell lines. Sk-mel 37, Sk-mel 24, and Malme-3M express MAGE-A2 and HLA-A*0201 and were lysed. This lysis was inhibited by the HLA A*0201-specific mAb BB7.2. In contrast, the cell lines HRR-mel and LRD-mel that express the Ag but not HLA-A*0201, and AEO-mel that expresses HLA-A*0201 but not the Ag, were not lysed (Fig. 5*A*). Also, a cell line with specificity for TRP-$2_{185}{}^{mod}$ generated from patient REN recognized naturally processed Ag presented by the melanoma cells Sk-mel 24, Sk-mel 37, and Malme-3M but not the TRP-2-positive, HLA-A*0201-negative cells SK-mel 28, HRR-mel, and IEL-mel (Fig. 5*B*). For both cell lines, specificity for their cognate epitope and its modified variant was demonstrated with peptide-pulsed T2 cells (Fig. 5). This series of experiments proves that, first, the three predicted epitopes TRP-2_{185}, MAGE-$A2_{116}$, or MAGE-$A2_{250}$ are indeed naturally generated and presented by the tumor cells; second, the recognition of the epitopes by the CTL is HLA A*0201-restricted; and third, again CTL primed with the modified epitopes recognize their natural counterpart.

Discussion

The identification of T cell epitopes has been immensely advanced by the introduction of prediction algorithms for the identification of potential HLA ligands in the sequences of TAAs (37–39). The vast majority of T cell epitopes known to date were identified by bioinformatic prediction followed by experimental validation (31). Despite this success, the available algorithms are based on two critical assumptions that restrict the output of the computation. First, it is assumed that T cell epitopes need to bind strongly to the HLA molecules for efficient induction of T cells. Second, the contributions of the individual amino acids in the epitopes sequences to the strength of binding to the HLA molecules are treated as being independent from the sequence context. However, experimental data are published that contradict both of these assumptions (20, 21, 40). With the work presented in this study, we attempted to develop and test bioinformatic algorithms that take the sequence context within the peptide into consideration and can predict epitopes that do not conform to the above binding requirements. All seven noncanonical epitopes predicted with the ANN are indeed T cell epitopes. Five can trigger strong T cell responses in healthy donors. For four of the epitopes, high frequencies could be detected in melanoma patients, which are comparable to the T cell responses found in patients against the known canonical epitopes of the same Ags. Therefore, the corresponding epitopes may be classified as among the immunodominant epitopes of these Ags. T cells with specificity for the new epitopes can recognize and lyse melanoma cells showing that they are generated and presented naturally. Six of the seven T cell epitopes would not have been predicted by the other algorithms because they either lack the canonical HLA allele-specific epitope motifs or the calculated half-life times of their complexes with the HLA molecule is too short. In fact, HLA stabilization experiments with the TAP-deficient cell line T2 proved that these peptides are poor HLA ligands. Despite this low binding capacity, these new epitopes are capable of efficiently stimulating T cells as shown for cells from healthy donors after in vitro priming as well as for T cells from cancer patients, which respond to the peptides directly ex vivo. These results suggest that probably many potent T cell epitopes have been missed by the current approaches to T cell epitope determination even in such thoroughly studied TAAs as MAGE and gp100. They also testify to the power of sequence context-sensitive algorithms for epitope prediction, which are bound to substantially extend our knowledge of tumor antigenicity.

Improved suboptimal T cell epitopes generated by introducing the canonical anchor amino acids into the sequences have been proposed for a few epitopes, one of which, the MART-1/Melan-A_{26} epitope (41) has already been tested in clinical therapeutic

vaccination trials with melanoma patients (42). However, this use of variant T cell epitopes with optimized HLA-binding properties has been controversial, because it was shown for some that T cells raised against the modified peptides do not efficiently recognize their natural counterparts as presented by the tumor cells and that fewer T cells in the peripheral blood of patients respond to natural than to the variant epitope (43, 44). However, in the examples presented with this report, all T cell lines induced with the optimized epitopes recognize their natural counterpart, also when presented naturally by the tumor cells. This outcome concords with the reports by several other investigators (45, 46). It obviously needs to be tested for every case whether anchor position-optimized epitopes can efficiently substitute for weak HLA binders and what modifications best guarantees efficient induction of antitumor T cell responses. Naturally presented weak HLA-binding epitopes may still induce T cells efficiently when their continuous production by the tumor cell translates into a sufficiently high steady-state representation at the cell surface. However, for use as vaccine Ag, it may be beneficial if the peptides bind fast and stably to the HLA molecules so to escape proteolytic degradation. The vaccination effect of suboptimal T cell epitopes may depend on improving their HLA-binding properties.

In summary, we demonstrate in this study that improved bioinformatics with ANN that predict potential T cell epitopes on the basis of considering the sequence context of the amino acids leads indeed to the identification of new epitopes that are not necessarily strong HLA binders, but nonetheless, are efficient inducers of T cell responses. These new tools will advance our understanding of tumor antigenicity and tumor immunity, enable us to monitor tumor-specific immune responses more comprehensively, and help to design improved cancer vaccines.

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Disclosures

The authors have no financial conflict of interest.

References

1. Parmiani, G., C. Castelli, P. Dalerba, R. Mortarini, L. Rivoltini, F. M. Marincola, and A. Anichini. 2002. Cancer immunotherapy with peptide-based vaccines: what have we achieved? where are we going? *J. Natl. Cancer Inst.* 94: 805–818.
2. Rosenberg, S. A. 2001. Progress in human tumour immunology and immunotherapy. *Nature* 411: 380–384.
3. Waldmann, T. A. 2003. Immunotherapy: past, present and future. *Nat. Med.* 9: 269–277.
4. Romero, P., J. C. Cerottini, and D. E. Speiser. 2004. Monitoring tumor antigen specific T-cell responses in cancer patients and phase I clinical trials of peptide-based vaccination. *Cancer Immunol. Immunother.* 53: 249–255.
5. Marchand, M., P. Weynants, E. Rankin, F. Arienti, F. Belli, G. Parmiani, N. Cascinelli, A. Bourlond, R. Vanwijck, Y. Humblet, et al. 1995. Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3. *Int. J. Cancer* 63: 883–885.
6. Jaeger, E., H. Bernhard, P. Romero, M. Ringhoffer, M. Arand, J. Karbach, C. Ilsemann, M. Hagedorn, and A. Knuth. 1996. Generation of cytotoxic T-cell responses with synthetic melanoma-associated peptides in vivo: implications for tumor vaccines with melanoma-associated antigens. *Int. J. Cancer* 66: 162–169.
7. Marshall, J. L., R. J. Hoyer, M. A. Toomey, K. Faraguna, P. Chang, E. Richmond, J. E. Pedicano, E. Gehan, R. A. Peck, P. Arlen, et al. 2000. Phase I study in advanced cancer patients of a diversified prime-and-boost vaccination protocol using recombinant vaccinia virus and recombinant nonreplicating avipox virus to elicit anti-carcinoembryonic antigen immune responses. *J. Clin. Oncol.* 18: 3964–3973.
8. Eder, J. P., P. W. Kantoff, K. Roper, G. X. Xu, G. J. Bubley, J. Boyden, L. Gritz, G. Mazzara, W. K. Oh, P. Arlen, et al. 2000. A phase I trial of a recombinant vaccinia virus expressing prostate-specific antigen in advanced prostate cancer. *Clin. Cancer Res.* 6: 1632–1638.
9. Rosenberg, S. A., J. C. Yang, D. J. Schwartzentruber, P. Hwu, F. M. Marincola, S. L. Topalian, N. P. Restifo, M. E. Dudley, S. L. Schwarz, P. J. Spiess, et al. 1998. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med.* 4: 321–327.
10. Kawakami, Y., and S. A. Rosenberg. 1996. T-cell recognition of self peptides as tumor rejection antigens. *Immunol. Res.* 15: 179–190.
11. Ohno, S. 1992. How cytotoxic T cells manage to discriminate nonself from self at the nonapeptide level. *Proc. Natl. Acad. Sci. USA* 89: 4643–4647.
12. Marincola, F. M., E. M. Jaffee, D. J. Hicklin, and S. Ferrone. 2000. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv. Immunol.* 74: 181–273.
13. Kawashima, I., S. J. Hudson, V. Tsai, S. Southwood, K. Takesako, E. Appella, A. Sette, and E. Celis. 1998. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. *Hum. Immunol.* 59: 1–14.
14. Celis, E., V. Tsai, C. Crimi, R. DeMars, P. A. Wentworth, R. W. Chesnut, H. M. Grey, A. Sette, and H. M. Serra. 1994. Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. *Proc. Natl. Acad. Sci. USA* 91: 2105–2109.
15. Pamer, E. G., J. T. Harty, and M. J. Bevan. 1991. Precise prediction of a dominant class I MHC-restricted epitope of *Listeria monocytogenes*. *Nature* 353: 852–855.
16. Rotzschke, O., K. Falk, S. Stevanovic, G. Jung, P. Walden, and H. G. Rammensee. 1991. Exact prediction of a natural T cell epitope. *Eur. J. Immunol.* 21: 2891–2894.
17. Falk, K., O. Rotzschke, S. Stevanovic, G. Jung, and H. G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351: 290–296.
18. Rammensee, H., J. Bachmann, N. P. Emmerich, O. A. Bachor, and S. Stevanovic. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50: 213–219.
19. Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J. Immunol.* 152: 163–175.
20. Gundlach, B. R., K. H. Wiesmuller, T. Junt, S. Kienle, G. Jung, and P. Walden. 1996. Specificity and degeneracy of minor histocompatibility antigen-specific MHC-restricted CTL. *J. Immunol.* 156: 3645–3651.
21. Udaka, K., K. H. Wiesmuller, S. Kienle, G. Jung, and P. Walden. 1995. Decrypting the structure of major histocompatibility complex class I-restricted cytotoxic T lymphocyte epitopes with complex peptide libraries. *J. Exp. Med.* 181: 2097–2108.
22. Schneider, G., and P. Wrede. 1993. Development of artificial neural filters for pattern recognition in protein sequences. *J. Mol. Evol.* 36: 586–595.
23. Schneider, G., and P. Wrede. 1994. The rational design of amino acid sequences by artificial neural networks and simulated molecular evolution: de novo design of an idealized leader peptidase cleavage site. *Biophys. J.* 66: 335–344.
24. Rechenberg, I. 1973. *Evolutionsstrategie-Optimierung Technischer Systeme nach Prinzipien der Biologischen Evolution.* Fromann-Holzboog, Stuttgart.
25. Matthews, B. W. 1975. Comparison of the predicted and observed secondary structure of T4 phage lysozyme. *Biochim. Biophys. Acta.* 405: 442–451.
26. Salter, R. D., D. N. Howell, and P. Cresswell. 1985. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics* 21: 235–246.
27. Sherev, T., K. H. Wiesmuller, and P. Walden. 2003. Mimotopes of tumor-associated T-cell epitopes for cancer vaccines determined with combinatorial peptide libraries. *Mol. Biotechnol.* 25: 53–61.
28. Engelman, D. M., T. A. Steitz, and A. Goldman. 1986. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biophys. Chem.* 15: 321–353.
29. Jones, D. D. 1975. Amino acid properties and side-chain orientation in proteins: a cross correlation appraoch. *J. Theor. Biol.* 50: 167–183.
30. Zamyatnin, A. A. 1972. Protein volume in solution. *Prog. Biophys. Mol. Biol.* 24: 107–123.
31. Novellino, L., C. Castelli, and G. Parmiani. 2005. A listing of human tumor antigens recognized by T cells: March 2004 update. *Cancer Immunol. Immunother.* 54: 187–207.
32. Pascolo, S., M. Schirle, B. Guckel, T. Dumrese, S. Stumm, S. Kayser, A. Moris, D. Wallwiener, H. G. Rammensee, and S. Stevanovic. 2001. A MAGE-A1 HLA-A A*0201 epitope identified by mass spectrometry. *Cancer Res.* 61: 4072–4077.
33. Sun, Y., M. Song, S. Stevanovic, C. Jankowiak, A. Paschen, H. G. Rammensee, and D. Schadendorf. 2000. Identification of a new HLA-A(*)0201-restricted T-cell epitope from the tyrosinase-related protein 2 (TRP2) melanoma antigen. *Int. J. Cancer* 87: 399–404.
34. Parkhurst, M. R., E. B. Fitzgerald, S. Southwood, A. Sette, S. A. Rosenberg, and Y. Kawakami. 1998. Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma antigen tyrosinase-related protein 2 (TRP2). *Cancer Res.* 58: 4895–4901.
35. Karlsson, A. C., J. N. Martin, S. R. Younger, B. M. Bredt, L. Epling, R. Ronquillo, A. Varma, S. G. Deeks, J. M. McCune, D. F. Nixon, and E. Sinclair. 2003. Comparison of the ELISPOT and cytokine flow cytometry assays for the enumeration of antigen-specific T cells. *J. Immunol. Methods* 283: 141–153.
36. Reynolds, S. R., E. Celis, A. Sette, R. Oratz, R. L. Shapiro, D. Johnston, M. Fotino, and J. C. Bystryn. 2000. Identification of HLA-A*03, A*11 and B*07-restricted melanoma-associated peptides that are immunogenic in vivo by vaccine-induced immune response (VIIR) analysis. *J. Immunol. Methods* 244: 59–67.
37. Flower, D. R. 2003. Towards in silico prediction of immunogenic epitopes. *Trends Immunol.* 24: 667–674.
38. Stevanovic, S. 2002. Identification of tumour-associated T-cell epitopes for vaccine development. *Nat. Rev. Cancer* 2: 514–520.

39. Saxova, P., S. Buus, S. Brunak, and C. Kesmir. 2003. Predicting proteasomal cleavage sites: a comparison of available methods. *Int. Immunol.* 15: 781–787.
40. Udaka, K., K. H. Wiesmuller, S. Kienle, G. Jung, and P. Walden. 1996. Self-MHC-restricted peptides recognized by an alloreactive T lymphocyte clone. *J. Immunol.* 157: 670–678.
41. Valmori, D., J. F. Fonteneau, C. M. Lizana, N. Gervois, D. Lienard, D. Rimoldi, V. Jongeneel, F. Jotereau, J. C. Cerottini, and P. Romero. 1998. Enhanced generation of specific tumor-reactive CTL in vitro by selected Melan-A/MART-1 immunodominant peptide analogues. *J. Immunol.* 160: 1750–1758.
42. Phan, G. Q., C. E. Touloukian, J. C. Yang, N. P. Restifo, R. M. Sherry, P. Hwu, S. L. Topalian, D. J. Schwartzentruber, C. A. Seipp, L. J. Freezer, et al. 2003. Immunization of patients with metastatic melanoma using both class I- and class II-restricted peptides from melanoma-associated antigens. *J. Immunother.* 26: 349–356.
43. Mandruzzato, S., E. Rossi, F. Bernardi, V. Tosello, B. Macino, G. Basso, V. Chiarion-Sileni, C. R. Rossi, C. Montesco, and P. Zanovello. 2002. Large and dissimilar repertoire of Melan-A/MART-1-specific CTL in metastatic lesions and blood of a melanoma patient. *J. Immunol.* 169: 4017–4024.
44. Rubio, V., T. B. Stuge, N. Singh, M. R. Betts, J. S. Weber, M. Roederer, and P. P. Lee. 2003. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. *Nat. Med.* 9: 1377–1382.
45. Tangri, S., G. Y. Ishioka, X. Huang, J. Sidney, S. Southwood, J. Fikes, and A. Sette. 2001. Structural features of peptide analogs of human histocompatibility leukocyte antigen class I epitopes that are more potent and immunogenic than wild-type peptide. *J. Exp. Med.* 194: 833–846.
46. Fong, L., Y. Hou, A. Rivas, C. Benike, A. Yuen, G. A. Fisher, M. M. Davis, and E. G. Engleman. 2001. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. *Proc. Natl. Acad. Sci. USA* 98: 8809–8814.
47. Gotch, F., J. Rothbard, K. Howland, A. Townsend, and A. McMichael. 1987. Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. *Nature* 326: 881–882.