A Novel Population of Human Melanoma-Specific CD8 T Cells Recognizes Melan-A^{MART-1} Immunodominant Nonapeptide but Not the Corresponding Decapeptide[1]

Laurent Derré,* Mathias Ferber,[†‡] Cédric Touvrey,* Estelle Devevre,* Vincent Zoete,[†] Antoine Leimgruber,[†‡§] Pedro Romero,* Olivier Michielin,[†‡§] Frédéric Lévy,[§] and Daniel E. Speiser[2]*

HLA-A2-restricted cytolytic T cells specific for the immunodominant human tumor Ag Melan-A^{MART-1} can kill most HLA-matched melanoma cells, through recognition of two naturally occurring antigenic variants, i.e., Melan-A nonamer AAGIGILTV and decamer EAAGIGILTV peptides. Several previous studies have suggested a high degree of TCR cross-reactivity to the two peptides. In this study, we describe for the first time that some T cell clones are exclusively nonamer specific, because they are not labeled by A2/decamer-tetramers and do not recognize the decamer when presented endogenously. Functional assays with peptides gave misleading results, possibly because decamers were cleaved by exopeptidases. Interestingly, nonapeptide-specific T cell clones were rarely Vα2.1 positive (only 1 of 19 clones), in contrast to the known strong bias for Vα2.1-positive TCRs found in decamer-specific clones (59 of 69 clones). Molecular modeling revealed that nonapeptide-specific TCRs formed unfavorable interactions with the decapeptide, whereas decapeptide-specific TCRs productively created a hydrogen bond between CDR1α and glutamic acid (E) of the decapeptide. Ex vivo analysis of T cells from melanoma metastases demonstrated that both nonamer and decamer-specific T cells were enriched to substantial frequencies in vivo, and representative clones showed efficient tumor cell recognition and killing. We conclude that the two peptides should be regarded as distinct epitopes when analyzing tumor immunity and developing immunotherapy against melanoma. *The Journal of Immunology,* **2007, 179: 7635–7645.**

Melan-A^{MART-1} is a tumor differentiation Ag which is expressed by melanocytes and malignant melanoma cells from tumors of >95% of patients. Melan-A-specific CD8 T cells are readily detectable in the majority of HLA-A*0201^{pos} normal individuals and in melanoma patients. During melanoma progression, Melan-A-specific CD8 T cells become activated and accumulate in metastatic lesions (1–3). Two natural peptide variants derived from Melan-A are presented by HLA-A*0201 and recognized by Melan-A-specific T cells: the nonapeptide AAGIGILTV (AA), and the decapeptide EAAGIGILTV (EAA), differing in glutamic acid (E) at peptide position one. Both peptides are naturally processed and presented by melanoma cells. The nonamer has been directly isolated from a melanoma cell line by acid elution (4). Evidence for decamer presentation by HLA-A*0201 is indirect and incomplete, essentially based on efficient recognition by CD8 T cell clones with preference for the decamer (5–7). Conclusive evidence exists for presentation of the same decapeptide by HLA-B*3501, where T cell clones derived from melanoma metastases exclusively recognize the decamer but not the nonamer (8).

Efficiency of recognition of human tumor cells by CTLs is often suboptimal, in part explaining lack of immune protection from tumor progression. For a full characterization of HLA-A*0201/Melan-A-specific T cells, further studies are necessary, e.g., to determine whether the nonapeptide and decapeptide are differentially expressed by melanoma cells and professional APCs, and to elucidate whether T cell recognition of the two peptides occurs differently, and by distinct T cells.

Several studies suggest that the majority of Melan-A-specific T cells preferentially recognize the decamer rather than the nonamer (5–7, 9). A large number of T cell clones generated from HLA-A*0201^{pos} healthy donors and patients demonstrated dominance of decamer-specific T cells, whereas T cells with preferred recognition of the nonapeptide were rare. Decamer vs nonamer specificity was usually determined by ^{51}Cr-release assays using target cells loaded with synthetic peptides. In most cases, the T cells were cross-reactive against both peptides, whereby the decapeptide was more efficiently recognized than the nonapeptide. Synthetic peptides, however, bear the problem that they may include contaminating peptides with other sequences, and that they can be degraded by peptidases during bioassays. Current peptide synthesis and separation techniques cannot prevent the presence of minute amounts of deletion peptides, which may elicit in vitro and in vivo T cell responses, owing to the high sensitivity of T cells.

In the present study, we determined the specificity of selected A*0201/Melan-A-specific T cell clones and of T cell populations

*Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, Lausanne, Switzerland; [†]Swiss Institute of Bioinformatics, Lausanne, Switzerland; [‡]Multidisciplinary Oncology Center, Lausanne University Hospital, Lausanne, Switzerland; and [§]Ludwig Institute for Cancer Research, Lausanne Branch, Epalinges, Switzerland

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[2] Address correspondence and reprint requests to Dr. Daniel E. Speiser, Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, Hôpital Orthopédique, Niveau 5 Est, Avenue Pierre-Decker 4, CH-1005 Lausanne, Switzerland. E-mail address: daniel.speiser@hospvd.ch

from melanoma patients analyzed ex vivo. We used peptides with the two natural sequences and modified peptides designed for defined positioning in the A*0201 peptide-binding pocket. The results show that T cells can efficiently distinguish between Melan-A nonamer and decamer, revealing the existence of two distinct epitopes with an unexpected lack of cross-reactivity by Melan-A-specific T cells.

Materials and Methods

Cells

The melanoma cell lines Me 275 and Me 290 (HLA-A2^{pos}/Melan-A^{pos}), and NA8-MEL (HLA-A2^{pos}/Melan-A^{neg}) were maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS, nonessential amino acids, and penicillin-streptomycin. Blood was withdrawn from healthy donors or stage III/IV melanoma patients, who also provided tumor infiltrating lymphocyte (TIL)[3]/tumor-infiltrated lymph node cells (TILNs) obtained through surgery. Patients had not received chemotherapy, immunotherapy, or irradiation. Ficoll-Paque-centrifuged PBMC were cryopreserved in RPMI 1640, 40% FCS, and 10% DMSO. Melan-A-specific T cell clones M77–80 and A42 were established by limiting dilution from TIL and mixed lymphocyte tumor cell cultures, respectively (10, 11). Clones LAU 337–6B7 and BC25-R3 were purified from patient PBL by ex vivo cell sorting (12) and from healthy donor PBMC stimulated by Melan-A$_{26-35}$ (A27L) peptides, respectively.

Stimulation of CD8 T cells was done with freshly isolated HLA-A*0201^{pos} PBMCs and cultivated at 0.2×10^6 cells/well (96 U-bottom plates) in the presence of 10 μM Melan-A peptides. IL-2 was added at day 2 at 1000 IU/ml. Media was changed every other day and CD8 T cells evaluated at day 10.

Peptides

Melan-A peptides used are listed in Table I. They were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer (Applied Biosystems) by using F-moc for transient N-terminal protection. Peptides were analyzed by mass spectrometry. The homogeneity of all peptides was >90%, as indicated by analytical HPLC (13). In functional assays, influenza matrix protein-derived peptide (GILGFVFTL) was used as negative control.

Fluorescent reagents and flow cytometry

HLA-A*0201/peptide tetrameric complexes were produced as described (2). Vα2-specific purified mAb was purchased from Beckman Coulter (clone RA8.4). All other Abs were purchased from BD Pharmingen. Samples were analyzed on a FACSCan or CANTO (BD Biosciences).

Tetramer-guided cell sorting and cloning at limiting dilution conditions

TIL, TILN, and stimulated PBMC were labeled with peptide-MHC (p-MHC) tetramers bearing two different fluorochromes. Briefly, CD8^{pos} T cells were positively enriched using anti-CD8-coated magnetic microbeads (Miltenyi Biotec), and washed in PBS 0.2% BSA, 5 mM EDTA (FACS buffer). Cells were incubated for 30 min at 4°C in FACS buffer with allophycocyanin-conjugated tetramers, then, after washing, with PE-conjugated tetramers, and finally, with FITC-conjugated CD8-specific mAb. Cells were immediately sorted with a FACSVantage cell sorter, and cocultured in limiting dilution conditions with 10^4 irradiated allogenic PBMC, 150 IU/ml rIL-2, and 1 μg/ml PHA-leukoagglutinin (PHA-L). Proliferating wells were screened at days 7–9. Subsequently, they were periodically restimulated with PHA, irradiated feeder cells and rIL-2. CD8 T cell clones were evaluated with tetramers for TCR specificity and subsequently analyzed as described in *Results*.

Assessment of Ag recognition by Melan-A-specific CD8 T cell clones

Ag-specific cytotoxicity was assessed using chromium-release assays. Target cells were labeled with ^{51}Cr for 1 h at 37°C and washed three times. Labeled target cells (1000 cells in 50 ml) were added to varying numbers of effector cells in V-bottom microwells. Where indicated, NA8-MEL cells labeled with ^{51}Cr were infected with recombinant vaccinia virus coding for Melan-A minigenes at a multiplicity of infection of 10, for 2 h before adding Melan-A-specific CD8 T cells at different L-T ratios. In peptide titration experiments, T2 target cells were incubated in presence of titrated peptide for 15 min at room temperature, before the addition of effector T cells. Chromium release was measured in supernatant after 4 h of incubation at 37°C. The percentage of specific lysis was calculated as following: $100 \times$ ((experimental − spontaneous release)/(total − spontaneous release)).

[3] Abbreviations used in this paper: TIL, tumor infiltrating lymphocyte; TILN, tumor-infiltrated lymph node cell; p-MHC, peptide-MHC.

Table I. *Natural and modified Melan-A peptides used in this study*

	Peptide	Abbreviation	Sequence[a]
Nonamer	MelanA$_{27-35}$	AA	AAGIGILTV
	MelanA$_{27-35}$(A28L)	AL	A**L**GIGILTV
	MelanA$_{27-35}$(A27L)	LA	**L**AGIGILTV
Decamer	MelanA$_{26-35}$	EAA	EAAGIGILTV
	MelanA$_{26-35}$(A27L)	ELA	E**L**AGIGILTV
	MelanA$_{26-35}$(E26A)	AAA	**A**AAGIGILTV
	MelanA$_{26-35}$(E26A, A27L)	ALA	**AL**AGIGILTV

[a] Modifications are indicated in bold letters.

Modeling the TCR-p-MHC complex

Homology models of the variable domain of the TCR bound to the decapeptide ELAGIGILTV presented by HLA-A*0201 were built using Modeller 6v2 (14), based on crystal structures of TCR and HLA-A2 MHC from the Protein Data Bank (www.rcsb.org/). From the available structures of TCR or TCR-p-MHC class I complexes, we selected the variable domains of nine TCR (Vα and Vβ) as templates for the homology modeling: 1fo0, 1g6r, 1kb5, 1kj2, 1lp9, 1mi5, 1nfd, 1oga, 2ckb. Lower resolution redundant structures were excluded. In addition, three unbound TCRVα (1b88, 1i9e, 1h5b) and two unbound TCRVβ (1bec, 1ktk) structures were added to the templates list. The TCRVα structure 1934.4 was also used (15). The three last templates structures were 1ao7 and 1bd2, as TCR bound to HLA-A2, and 1jf1, the structure of the ELAGIGILTV peptide presented by HLA-A*0201.

Once a homology model was obtained, each CDR loop was refined while the rest of the complex including the other CDR loops remained unchanged. The algorithm for the loop modeling consists in a cycle of molecular dynamics, combined with simulated annealing, optimizing the Modeller pseudo energy function. When possible, this function was improved by adding restraints specific to the nature of the CDR loop. These restraints were derived from the rules given by Al-Lazikani et al. (16): the identification of key amino acids defines limitations to the conformation space accessible to a CDR loop.

Binding free energy calculations

To estimate the role of the CDR1α residues on the TCR association to p-MHC, we used a binding free energy estimation and decomposition scheme using the molecular mechanics-generalized born surface area approach (17). We used previously this method on a TCR-p-MHC system and successfully reproduced the results of an experimental alanine scanning (18). The binding free energy was estimated according to the following equation: $\Delta G_{bind} = E_{vdW} + E_{elec} + \Delta E_{intra} + \Delta G_{elec,desolv} + \Delta G_{np,desolv} - T\Delta S$ (1), where E_{vdW} and E_{elec} are the van der Waals and electrostatic interaction energies between the TCR and the p-MHC. ΔE_{intra} is the variation of the internal energy of both partners upon complexation. Because we performed the calculations on a single model, we have $\Delta E_{intra} = 0$. $\Delta G_{elec,desolv}$ and $\Delta G_{np,desolv}$ are the electrostatic and nonpolar desolvation energies upon complexation, respectively. $\Delta G_{elec,desolv}$ is calculated according to the GB-MV2 generalized Born model (19, 20). $\Delta G_{np,desolv}$ is assumed to be proportional to the solvent accessible surface area that is buried upon complexation. We neglected the entropy term $T\Delta S$.

The use of equation (1) allows a straightforward decomposition of the binding free energy into backbone and side chain contributions for each residue. A detailed description of the approach can be found in Ref. 18. The binding free energy contributions have been calculated for all residues. All calculations were performed using the CHARMM package (version c31b1) (21) and the CHARMM22 all-atoms force field (22).

Results

A subset of Melan-A-specific CD8 T cell clones fail to bind to Melan-A tetramers

Previous studies have shown that the vast majority of Melan-A-specific CD8 T cell clones recognize the decapeptide EAAGIGILTV more efficiently than the nonamer AAGIGILTV (Table I), as determined by cytotoxicity assays using target cells labeled with titrated peptide doses (5, 6). In the following we will refer to these clones as "majority" clones. However, there are a few exceptional clones with superior recognition of nonapeptide, but they also seem to recognize synthetic decapeptide. In the following we will refer to the latter as "minority" clones, because they appear less frequent, despite that they have been actually described earlier (10, 23) than the majority clones (24). Based on current knowledge, it is believed that the two peptides represent two variants of the same HLA-A2-restricted epitope. To challenge this view, we first tested the ability of representative clones to bind to A2/Melan-A tetramers constructed with the parental decapeptide Melan-A_{26-35} (tEAA), the parental nonapeptide Melan-A_{27-35} (tAA), or the Melan-A_{26-35}(A27L) decapeptide analog (tELA; Table I). We used several T cell clones displaying different recognition pattern of Melan-A peptides, i.e., the majority clones BC25-R3 and LAU 337–6B7 preferentially reacting to the natural decapeptide (EAA), and the minority clones A42 and M77–80 preferentially recognizing the natural nonapeptide (AA) (5, 9). As shown in Fig. 1*A*, the tetramer tAA successfully labeled A42 and M77–80 clones, while tetramers tEAA and tELA surprisingly did not stain these minority clones. As expected, (Fig. 1*A*), majority clones were efficiently stained by Melan-A tetramers tELA and tEAA. The clone BC25-R3 displayed a low tAA staining. This is

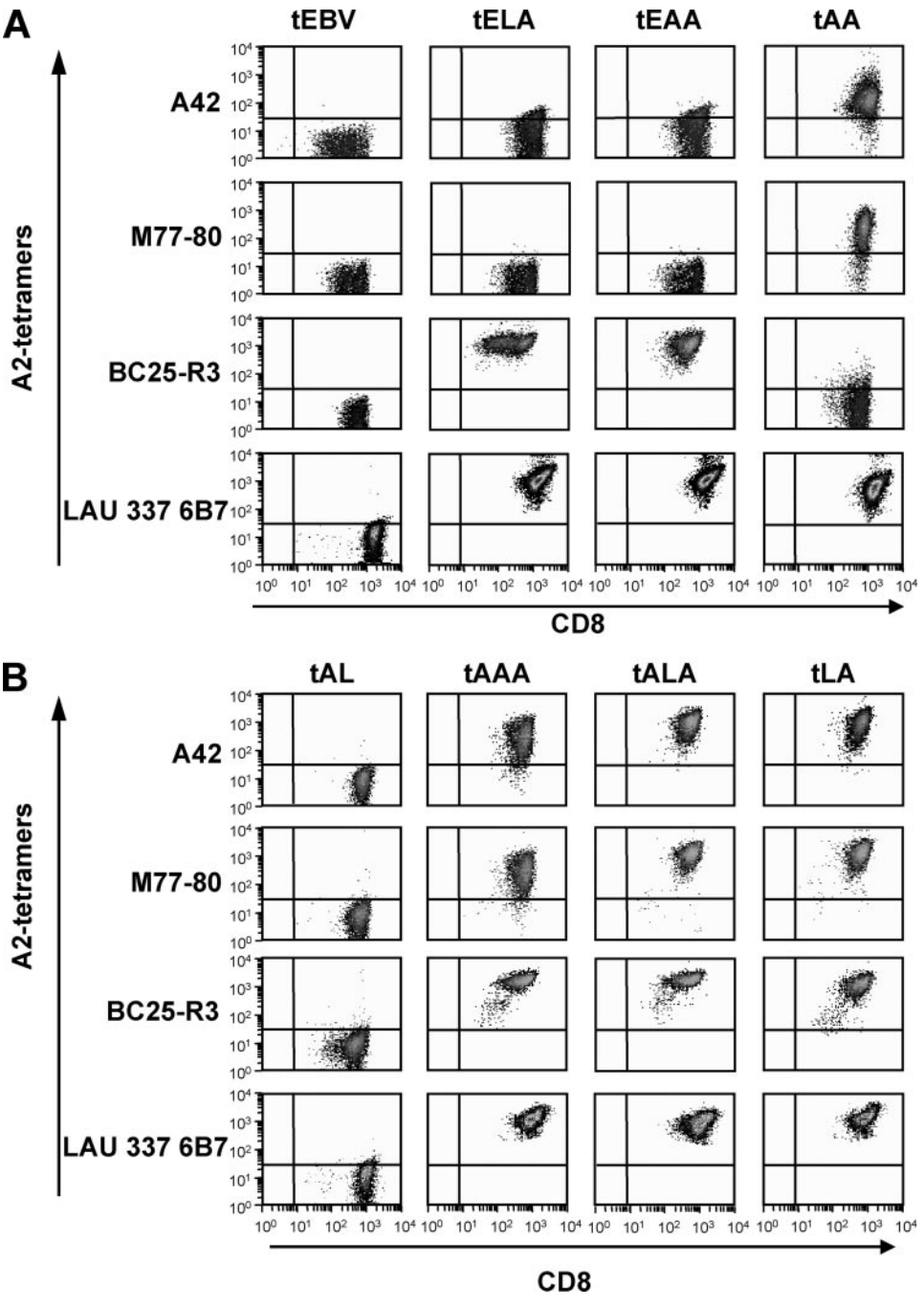

FIGURE 1. Tetramer labeling of minority and majority Melan-A-specific T cell clones. T cell clones were stained with anti-CD8-FITC and PE-labeled A2/Melan-A tetramers (t) prepared with various peptides (Table I), i.e., either ELA, EAA, AA (*A*, *upper panels*) or AL, AAA, ALA, LA (*B*, *lower panels*).

likely due to its weak avidity, supported by the relatively high peptide concentrations required for target cell recognition (Fig. 2*A* and Table II), and the fact that this clone was derived from a healthy donor of whom it is known that they frequently give rise to low avidity clones (25). Differential binding to tetramers tEAA vs tAA suggested that the glutamic acid (E) at the first position of the peptide prevented binding of the tEAA tetramer to minority clones. To explore this possibility, we generated tetramers loaded with a series of modified peptides (Table I). Although the tetramer prepared with the modified nonamer tAL (ALGIGILTV) was recognized neither by majority nor by minority clones, all the three other tetramers tAAA (AAAGIGILTV), tALA (ALAGIGILTV) and tLA (LAGIGILTV) labeled the Melan-A-specific clones efficiently (Fig. 1*B*), showing that the minority clones are able to recognize various Melan-A modified decapeptides without glutamic acid in position one. The data thus suggest that glutamic acid at the N terminus may hinder recognition by the minority clones.

Assessment of peptide and melanoma cell recognition by minority clones

To determine structure-function relationships, the relative antigenic activity of various peptide variants (Table I) was quantified by cytotoxicity assays with titrated amounts of peptides (Fig. 2*A*). As shown in previous studies (5–7, 10), majority Melan-A-specific clones displayed the usual pattern of recognition hierarchy because parental and modified immunodominant decamers EAA and ELA were better recognized than the natural nonapeptide AA (Fig. 2*A* and Table II). Together with data shown in Fig. 1, these findings confirmed that the two majority clones bore the usual fine specificity that is frequently observed in HLA-A2/Melan-A^{MART-1}-specific human T cell clones (5, 7). The highest functional avidity (recognition at lowest peptide concentrations) was observed to ELA and EAA peptides by the majority clones LAU 337 6B7 and BC25-R3, even if the latter clone showed

Table II. *Recognition of Melan-A natural and modified peptides*

	EC_{50} of T Cell Clones[a]			
Peptide	A42	M77–80	LAU 337 6B7	BC25-R3
ELA (ELAGIGILTV)	3.1	3.2	0.021	0.044
EAA (EAAGIGILTV)	262.5	331.8	0.06	2.5
AA (AAGIGILTV)	10.4	99.6	6.2	61.1

[a] EC_{50} values correspond to peptide concentrations (nanomoles) required to obtain 50% of maximal lysis of peptide-loaded target cells.

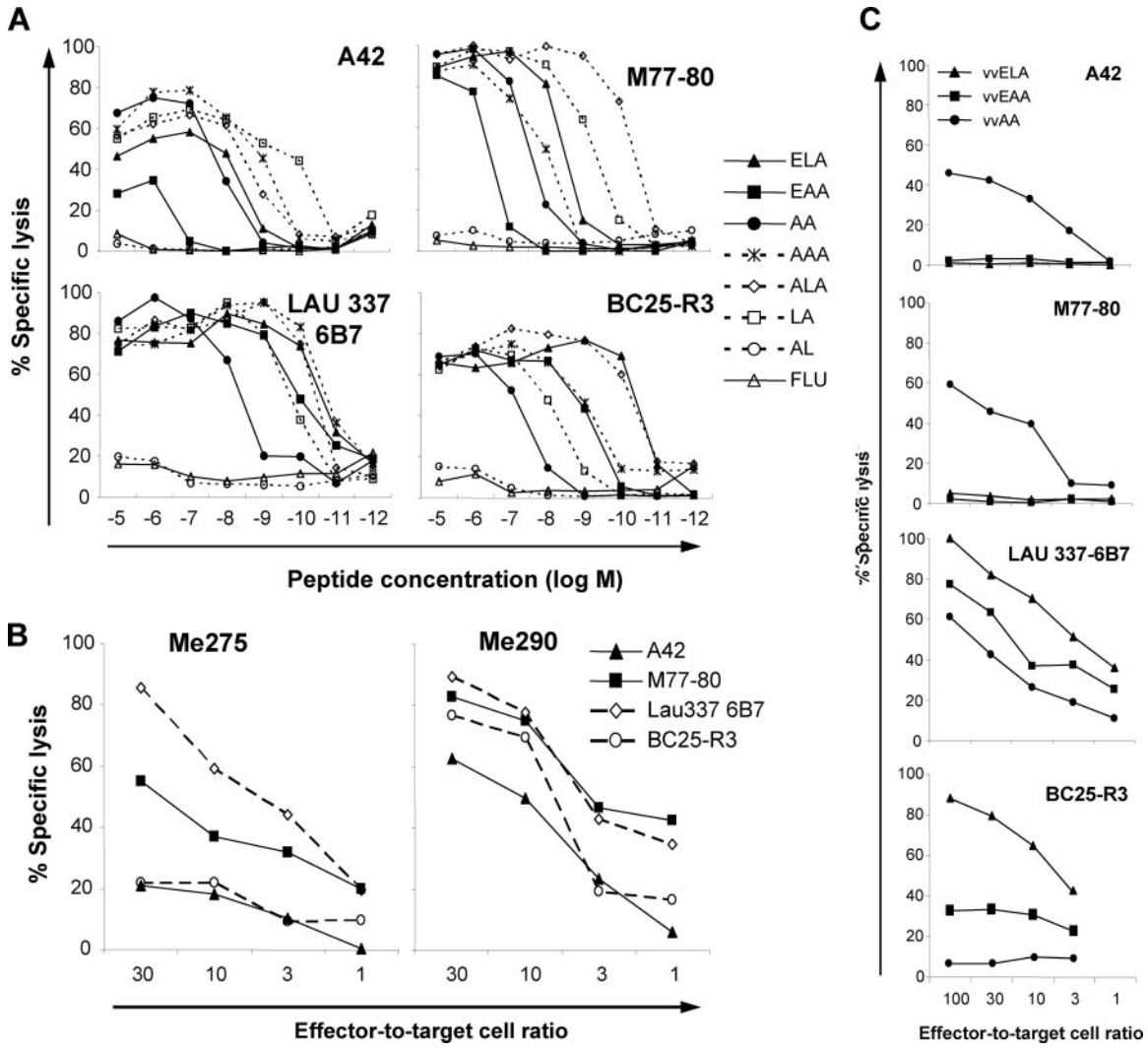

FIGURE 2. Ag recognition by T cell clones assessed in cytotoxicity assays. *A*, The relative antigenic activities of natural and modified Melan-A peptides were determined in cytotoxicity assays with T2 target cells (E:T ratio 10:1) in presence of increasing peptide concentrations. *B*, Tumor cell lysis was assessed using Me 275 and Me 290 melanoma cell lines (HLA-A*0201^{pos}/Melan-A^{pos}). *C*, Lysis of NA8-MEL cells infected with recombinant vaccinia viruses coding for ELA, EAA, or AA. Similar results were obtained in two independent experiments.

lower avidity to most peptides (Fig. 2*A* and Table II). Interestingly, despite that the two minority clones A42 and M77–80 were not stained by the corresponding tetramers, they nevertheless recognized target cells loaded with EAA and ELA decapeptides, albeit at much lower avidities than the majority clones (Fig. 2*A* and Table II). Furthermore, none of the clones recognized the modified nonamer AL (Fig. 2*A*). In contrast, AAA, ALA, and LA peptides were recognized by both types of clones, confirming tetramer binding results (Fig. 1*B*). The latter two peptides were particularly well recognized by minority clones.

Next, we tested the ability of the clones for specific recognition and killing of melanoma cells. The two Melan-A^{pos} cell lines Me 275 and Me 290 were efficiently lysed by both minority clones and majority clones (Fig. 2*B*), while Melan-A^{neg} melanoma cells were not lysed (data not shown). The data confirm that all four clones expressed TCRs capable to recognize and kill melanoma cells naturally processing and presenting A2/Melan-A epitopes.

Reactivity of minority Melan-A-specific T cell clones correlates with absence of glutamic acid (E) at the first position of the peptide

The above data revealed that majority and minority clones showed preference for deca- and nonapeptides, respectively. However, we were left with the paradox that minority clones also killed target cells loaded with EAA and ELA decamers whereas they were not labeled by the corresponding tetramers. It has been shown that Melan-A peptides are rapidly degraded in human serum by amino- and dipeptidyl-peptidases (26, 27). Because we used FCS in our assays, peptides may have been cleaved during our experiments, leading to the generation of nonamers in culture wells assessing decamer reactivity. This would explain the recognition of the natural and modified decamers by minority clones that we observed in killer assays (Fig. 2*A*). First, we performed cytotoxicity assays in serum free medium, but still obtained some recognition of decamers (data not shown). Alternative explanations are that peptides may have been cleaved by membrane proteases, or that the decapeptides synthesis contained trace amounts of nonapeptides that coeluted during HPLC separation (data not shown). Therefore, we designed an approach whereby Melan-A Ags are synthesized, processed and presented physiologically by target cells, and therefore should not be exposed to exopeptidases other than those involved in natural Ag processing. We prepared recombinant vaccinia viruses with minigenes coding for AA, EAA and ELA peptides (28, 29), and used them to infect NA8-MEL target cells. As expected, target cells infected with vaccinia virus-expressing antigenic nonapeptide were efficiently lysed by the clones, except for the

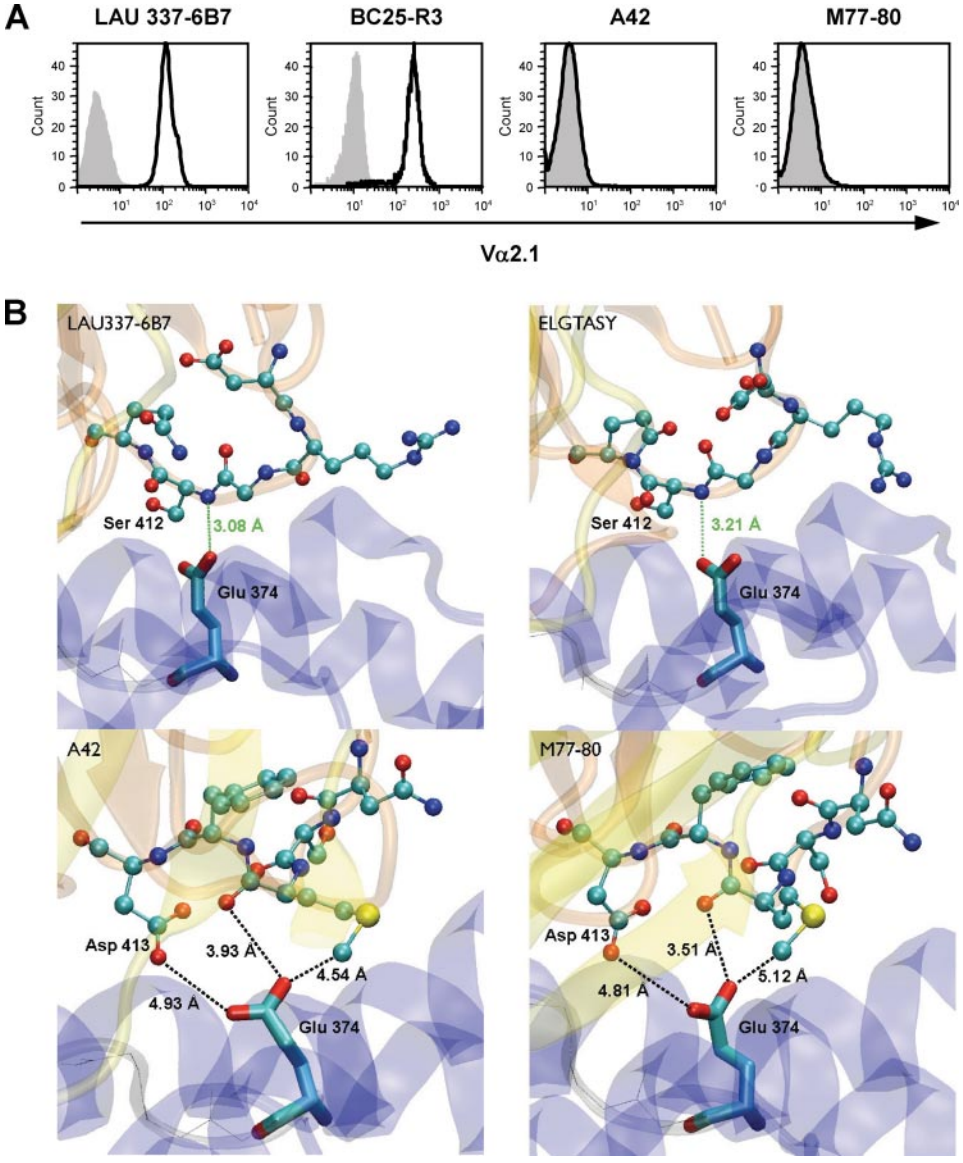

FIGURE 3. Structural differences between nona- vs decamer-specific clones. *A*, Vα2.1 expression by minority and majority clones. Melan-A specific clones were stained with anti-Vα2.1 mAb followed by FITC-labeled goat anti-rat IgG Ab (bold line) or with FITC-labeled conjugate alone (filled histogram). *B*, Structural modeling of nonamer vs decamer-specific TCRs bound to ELA/HLA-A*0201. Comparison of the predicted structure of ELA/HLA-A2 complex and TCRs Vα CDR1 loop from decamer-specific clones (*upper panels*) and from nonamer-specific clones (*lower panels*). HLA-A2 structures are shown in blue ribbon, the side chains of TCRs Vα CDR1 loop in ball and stick representation, and the side chains of the glutamic acid (Glu^{374}) from ELA peptide in blue stick. Hydrogen bonds are represented by green dotted lines and the distance between TCR Vα2.1 Ser^{412} and peptide Glu^{374} is given in Å. Shortest distances between the CDR1α of nonamer specific clones and peptide Glu^{374} are represented by black dotted lines.

Table III. *MM-GBSA contributions of CDR1α side chains (sc)*[a]

	E^{sc}_{vdw}	E^{sc}_{elec}	$\Delta G^{sc}_{elec,desolv}$	$\Delta G^{sc}_{np,desolv}$	ΔG^{sc}_{bind}		E^{sc}_{vdw}	E^{sc}_{elec}	$\Delta G^{sc}_{elec,desolv}$	$\Delta G^{sc}_{np,desolv}$	ΔG^{sc}_{bind}
ELGTASY						LAU 337–6B7					
Tyr^{407}	−0.06	0.01	0.02	0.00	−0.03	Tyr^{407}	−0.05	−0.06	−0.02	0.00	−0.13
Ser^{408}	−0.07	−2.10	2.05	−0.03	−0.15	Ser^{408}	−0.06	1.72	−1.52	0.00	0.14
Asp^{409}	−0.46	60.21	−59.10	−0.01	0.64	Asp^{409}	−0.15	51.91	−49.16	0.00	2.60
Arg^{410}	−1.13	−108.06	108.92	−1.03	−1.29	Arg^{410}	−1.61	−75.96	73.40	−0.81	−4.98
Gly^{411}	0.00	0.00	0.00	0.00	0.00	Gly^{411}	0.00	0.00	0.00	0.00	0.00
Ser^{412}	−0.27	−2.20	−0.91	−0.46	−3.84	Ser^{412}	0.35	−2.09	−1.77	−0.43	−3.94
Gln^{413}	−0.35	0.71	−0.89	−0.02	−0.55	Gln^{413}	−0.35	−4.44	4.46	0.00	−0.33
Ser^{414}	−0.23	−0.27	−0.11	−0.02	−0.64	Ser^{414}	−0.24	−0.64	1.00	−0.06	0.07
Phe^{415}	−0.15	−0.47	0.51	0.00	−0.11	Phe^{415}	−0.13	−0.38	0.50	0.00	−0.01
M77–80						A42					
Tyr^{407}	−0.11	−1.22	1.06	0.00	−0.27	Tyr^{407}	−0.12	−1.38	1.18	0.00	−0.31
Thr^{408}	−0.05	−0.42	0.50	0.00	0.03	Thr^{408}	−0.04	1.07	−1.31	0.00	−0.28
Asn^{409}	−0.41	0.59	−0.15	−0.07	−0.04	Asn^{409}	−0.45	0.73	−0.77	−0.09	−0.58
Ser^{410}	−0.67	−2.93	4.99	−0.32	1.06	Ser^{410}	−0.13	−9.67	7.04	−0.42	−3.18
Met^{411}	−2.58	0.60	−0.39	−0.63	−3.00	Met^{411}	−4.13	0.43	−0.52	−0.64	−4.86
Phe^{412}	−0.23	0.32	−0.50	0.00	−0.41	Phe^{412}	−0.26	0.04	−0.19	0.00	−0.41
Asp^{413}	−1.01	47.00	−35.61	−0.49	9.88	Asp^{413}	−0.51	50.45	−37.23	−0.24	12.47
Tyr^{414}	−1.02	−0.83	1.22	−0.23	−0.86	Tyr^{414}	−0.93	−0.14	0.07	−0.14	−1.14
Phe^{415}	−0.15	−0.39	0.26	0.00	−0.28	Phe^{415}	−0.11	−0.22	−0.08	0.00	−0.41

[a] $\Delta G^{sc}_{bind}{}^{sc}{}_{bind}$, Contribution to the total binding free energy, ΔG_{bind}, for the four modeled clones. Energies are in kilocalories per mole. E^{sc}_{vdw}, E^{sc}_{elec}, $\Delta G^{sc}_{elec,desolv}$, and $\Delta G^{sc}_{np,desolv}$ are the van der Waals and electrostatic interaction energies, and the electrostatic and nonpolar desolvation energies calculated for the side chains, respectively.

BC25-R3 clone due to its weak avidity and to the low affinity of AA peptide to HLA-A*0201 molecules In contrast, target cells expressing decamers EAA or ELA were only killed by "majority" clones (Fig. 2*C*). The results indicate that majority Melan-A-specific clones indeed failed to recognize Melan-A natural and modified decamers due to the presence of glutamic acid at the N terminus of the peptide. Therefore, minority clones are exclusively specific for the nonapeptide, rather than cross-reactive to nona- and decapeptide. Thus, minority clones can be called nonamer specific, and majority clones decamer specific.

Structural differences between nonamer- and decamer-specific T cell clones

Structural properties of peptides and T cell receptors are likely responsible for this differential recognition. TCR Vβ usage appears to be diverse, because many different Vβ elements are found in Melan-A-specific clones (30, 31) such as the ones used above (data not shown). In contrast, Vα usage is more restricted in Melan-A-specific T cells. Indeed, a strong preference for Vα2.1 usage was found in the preimmune repertoire of circulating Melan-A-specific CD8 T cells as well as at early stages of T cell development (30, 32–34). We analyzed the expression of Vα2.1 by Melan-A-specific clones, and found that Vα2.1 was expressed by decamer-specific clones, but not nonamer-specific clones (Fig. 3*A*), suggesting a beneficial role for Vα2.1 for binding to the decamer but not the nonamer.

To better understand, the interactions between Vα portions and the glutamic acid in Melan-A decapeptides, we performed in silico modeling of the TCR-p-MHC structure and the decomposition of the binding free energy calculated with the MM-GBSA approach (17), for two $V\alpha2.1^{pos}$ decamer-specific clones (TCR from clones LAU 337–6B7 and ELGTASY (35)) and for two $V\alpha2.1^{neg}$

Table IV. *MM-GBSA contributions of CDR1α backbone (bb)*[a]

	E^{bb}_{vdw}	E^{bb}_{elec}	$\Delta G^{bb}_{elec,desolv}$	$\Delta G^{bb}_{np,desolv}$	ΔG^{bb}_{bind}		E^{bb}_{vdw}	E^{bb}_{elec}	$\Delta G^{bb}_{elec,desolv}$	$\Delta G^{bb}_{np,desolv}$	ΔG^{bb}_{bind}
ELGTASY						LAU 337–6B7					
Tyr^{407}	−0.03	−0.47	0.57	0.00	0.08	Tyr^{407}	−0.03	−0.53	0.36	0.00	−0.20
Ser^{408}	−0.14	1.45	−1.44	−0.01	−0.15	Ser^{408}	−0.29	4.67	−3.28	−0.02	1.09
Asp^{409}	−0.51	−5.31	3.92	−0.07	−1.97	Asp^{409}	−0.35	−1.49	0.98	0.00	−0.87
Arg^{410}	−0.90	−5.53	5.55	−0.12	−1.00	Arg^{410}	−1.02	−2.36	4.44	−0.17	0.88
Gly^{411}	−1.14	−5.03	3.65	−0.24	−2.76	Gly^{411}	−0.94	−3.77	2.07	−0.09	−2.74
Ser^{412}	−1.51	−6.36	5.89	−0.13	−2.10	Ser^{412}	−1.43	−5.66	4.38	−0.07	−2.78
Gln^{413}	−0.38	2.61	−2.33	−0.02	−0.12	Gln^{413}	−0.37	1.96	−1.89	0.00	−0.31
Ser^{414}	−0.19	0.31	−0.52	0.00	−0.41	Ser^{414}	−0.16	0.06	0.24	0.00	0.14
Phe^{415}	−0.05	0.15	−0.05	0.00	0.04	Phe^{415}	−0.05	0.07	−0.04	0.00	−0.02
M77–80						A42					
Tyr^{407}	−0.02	−0.40	0.57	0.00	0.15	Tyr^{407}	−0.02	−0.60	0.99	0.00	0.37
Thr^{408}	−0.05	−0.26	0.32	0.00	0.01	Thr^{408}	−0.06	0.23	−0.34	0.00	−0.16
Asn^{409}	−0.16	−0.66	0.93	0.00	0.11	Asn^{409}	−0.19	−0.98	0.88	0.00	−0.29
Ser^{410}	−0.71	1.75	−0.68	−0.06	0.30	Ser^{410}	−1.01	0.37	1.13	−0.09	0.40
Met^{411}	−1.26	−1.35	3.19	−0.12	0.45	Met^{411}	−1.05	−1.63	1.65	−0.05	−1.09
Phe^{412}	−0.36	2.64	−3.40	0.00	−1.12	Phe^{412}	−0.27	1.71	−1.23	0.00	0.20
Asp^{413}	−0.54	1.45	−2.99	0.00	−2.08	Asp^{413}	−0.32	0.49	−1.79	0.00	−1.62
Tyr^{414}	−0.27	−0.31	−0.89	0.00	−1.47	Tyr^{414}	−0.18	−0.10	0.19	0.00	−0.09
Phe^{415}	−0.06	0.05	0.13	0.00	0.12	Phe^{415}	−0.05	0.12	−0.05	0.00	0.02

[a] ΔG^{bb}_{bind} Contribution to the total binding free energy, ΔG_{bind}, for the four modeled clones. Energies are in kilocalories per mole. E^{bb}_{vdw}, E^{bb}_{elec}, $\Delta G^{bb}_{elec,desolv}$, and $\Delta G^{bb}_{np,desolv}$ are the van der Waals and electrostatic interaction energies, and the electrostatic and nonpolar desolvation energies calculated for the backbone, respectively

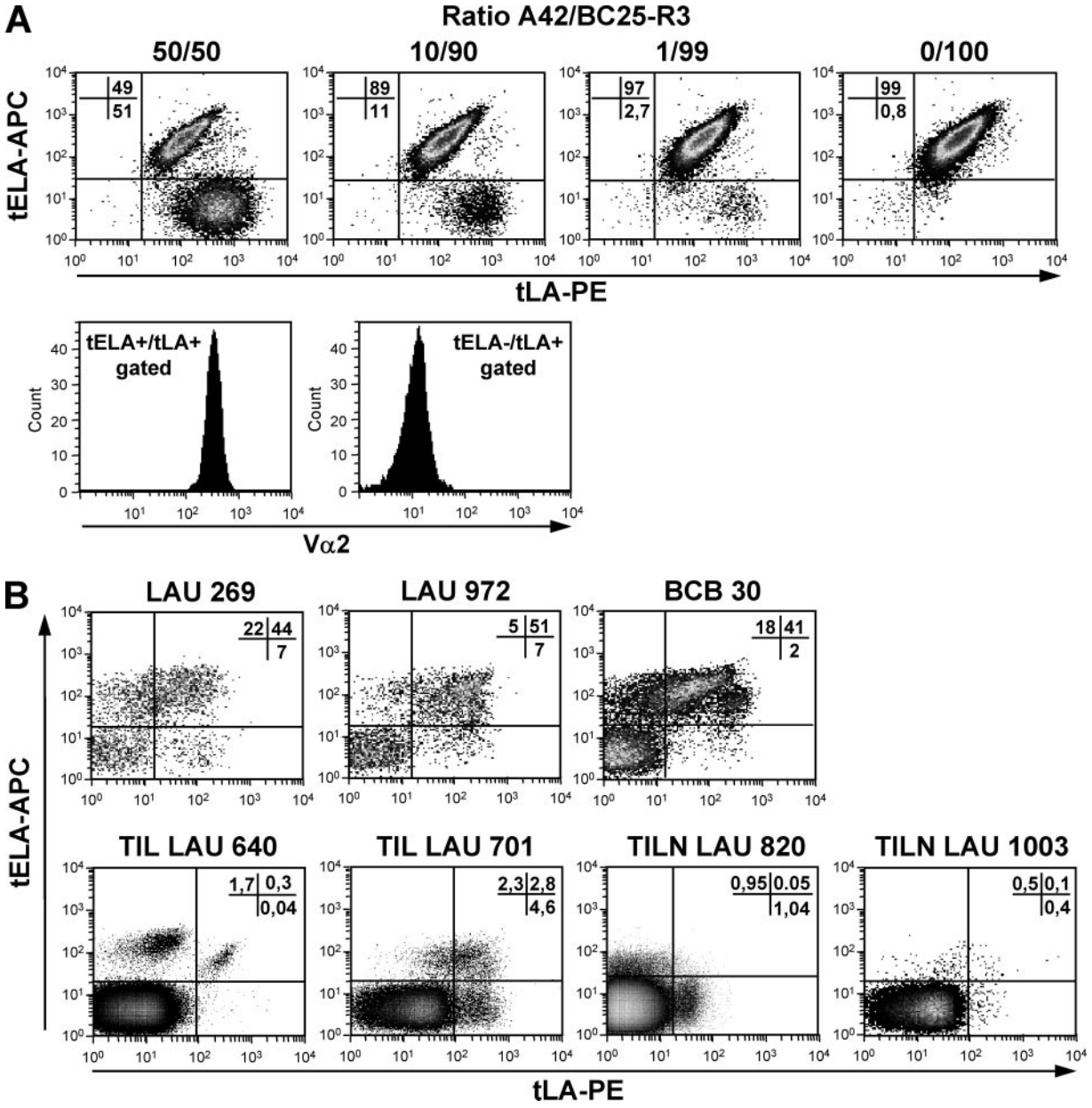

FIGURE 4. Strategy to distinguish nonamer from decamer specific T cells by staining with two tetramers and Vα2.1 specific Ab. *A*, Mixtures of A42 and BC25-R3 clones at different ratios were stained with anti-Vα2.1 mAb and tetramers prepared with peptides LAGIGILTV (tLA-PE) and ELAGIGILTV (tELA-allophycocyanin). *B*, Similar double tetramer stainings were performed on peptide stimulated PBMCs from healthy donors and patients, and on short-term cultured TIL or TILN cells analyzed directly ex vivo from metastases of melanoma patients. Graphs are $DAPI^{neg}CD8^{pos}$ gated.

nonamer-specific clones (TCR from clones A42 and M77–80). Fig. 3 shows the interactions between the glutamic acid at the first position of the ELA peptide (Glu^{374}) and the TCR CDR1α forming the CDR loop closest to Glu^{374}. This analysis revealed that similar CDR1α sequences gave similar conformations (Fig. 3*B*). For each model, we calculated side chain contributions (Table III) and backbone contributions (Table IV) to the binding free energy made by the CDR1α residues, respectively.

For the nonamer-specific clones, the shortest distances between the heavy atoms of the CDR1α and Glu^{374} are 3.51 and 3.93 Å, respectively. No hydrogen bond is formed between CDR1α and the peptide. The TCR Asp^{413} is one of the closest residues from Glu^{374}. The distance between the two corresponding side chain carboxylates is lower than 5Å. This interaction between two negatively charged residues is strongly unfavorable for the stability of the complex. We calculated a large unfavorable contribution for Asp^{413} in both cases, i.e., +9.88 kcal/M and + 12.47 kcal/M for M77–80 and A42, respectively (Tables III and IV). The electrostatic part, $E^{sc}_{elec} + \Delta G^{sc}_{elec,desolv}$, of the binding free energy contribution is highly positive for both models. Such high values of side chain contributions are rarely observed experimentally. This indicates that this unfavorable interaction probably prevents TCR-p-MHC binding, or requires a significant and energetically costly conformational rearrangement, in agreement with the low activity found in cellular assays. On the contrary, for both decamer-specific clones, a strong favorable hydrogen bond is formed between the TCR Ser^{412} backbone and the peptide Glu^{374} side chain. The distances between the heavy atoms are 3.21 and 3.08 Å, respectively (Fig. 3*B*), in good agreement with the favorable contributions that were calculated for the Ser^{412} backbone, and with the results found in cellular assays. In both cases, the unfavorable electrostatic desolvation, $\Delta G^{bb}_{elec,desolv}$ is more than compensated by the favorable

Table V. *Detection of nona- and decamer-specific T cells*[a]

TIL(N) from Patient	% tELA$^+$/LA$^-$	% tELA$^+$/LA$^+$	% tELA$^-$/LA$^+$
LAU 640	83.3	14.7	2
LAU 701	23.7	28.9	47.4
LAU 820	46.6	2.4	51
LAU 1003	50	10	40

[a]Analysis of TIL/TILN from four patients. Percentages of Melan-A-specific populations positively stained with one or both tetramers (tELA and tLA), by calculating tLA^{pos} plus tELA^{pos} cells as 100%.

electrostatic interactions, E^{bb}_{elec}. The backbone contributions of Ser^{412} are −2.10 kcal/mol and −2.78 kcal/mol for the clones ELGTASY and LAU 337–6B7, respectively (Tables III and IV).

Detection of Melan-A nonamer-specific CD8 T cells following in vitro peptide stimulation of PBMC

The question arose whether the two nonamer-specific clones represented a rare species of melanoma-specific T cells, or whether this specificity existed more frequently. To generate a larger number of nonamer-specific CD8 T cells, we developed a double tetramer staining strategy by using allophycocyanin-labeled tetramers bearing the decapeptide ELAGIGILTV (tELA-allophycocyanin) and PE-labeled tetramers bearing the nonapeptide LAGIGILTV (tLA-PE), allowing a quantitative dissection of the TCR repertoire of polyclonal Melan-A-specific CD8 T cell populations. For validation of this approach, we stained mixtures of A42 and BC25-R3 clones. Indeed, this allowed to discriminate nonamer from decamer-specific clones. The two clones were mixed at different ratios, demonstrating that the double tetramer approach was capable of distinguishing clone A42 (single tLA^{pos}) from clone BC25-R3 (tLA^{pos} and tELA^{pos}) in a quantitative manner (Fig. 4*A*, *upper panels*). The addition of Vα2.1 staining allowed to confirm that double tetramer^{pos} cells were Vα2.1^{pos} and single tetramer tLA^{pos} lymphocytes did not express Vα2.1 (Fig. 4*A*, *lower panels*). Subsequently, we used this labeling to analyze the frequency of Melan-A nona- vs decapeptide-specific cells in polyclonal populations. Total PBMC from two healthy donors were stimulated with Melan-A peptide and cultured for 15 days. As expected, the majority of Melan-A-specific cells were positive for the tELA tetramer and these cells were mostly Vα2.1^{pos} (Fig. 4*B*, *upper panels*, and data not shown), confirming the predominance of decamer-specific T cells in five donors analyzed. However, two patients and one healthy donor displayed, after two rounds of stimulation, a small but distinct tELA^{neg}/tLA^{pos} population, at frequencies of 2 and 7% (Fig. 4*B*, *upper panels*).

Ex vivo detection of Melan-A nonamer-specific CD8 T cells

In parallel, we also performed double tetramer stainings of TIL and TILN cells from metastases of four melanoma patients (two TIL cultured for 2–3 wk, and two TILN cells analyzed directly ex

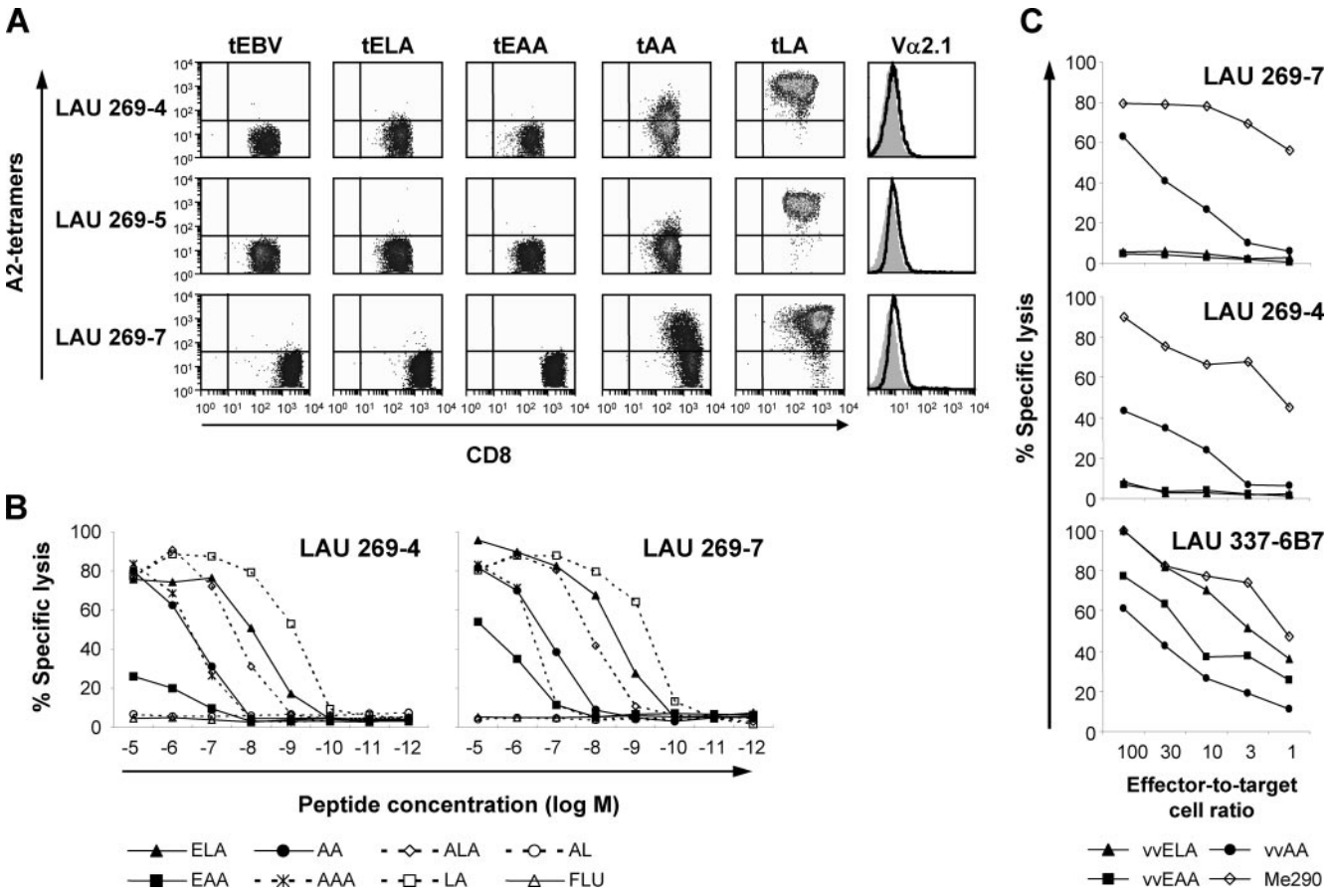

FIGURE 5. Functional assessment of newly generated nonamer-specific clones. *A*, Clones generated from PBMCs of patient LAU 269 were labeled with tetramers tELA, tEAA, tAA, tLA, and tEBV (as negative control) and anti-CD8 mAb, with anti-Vα2.1 mAb (bold line) or with FITC conjugate alone (filled histogram). *B*, Specificity of nonamer-specific clones was measured with T2 target cells (E:T ratio 10:1) in presence of increasing peptide concentrations. *C*, Tumor cell lysis was assessed using Me 290 melanoma cells (HLA-A*0201^{pos}/Melan-A^{pos}), and NA8-MEL cells infected with recombinant vaccinia viruses coding for ELA, EAA, or AA. Similar results were obtained in two independent experiments.

vivo), which was possible without in vitro Ag stimulation because TIL/TILN contain high frequencies of Melan-A-specific T cells due to spontaneous enrichment in vivo (2, 36, 37). Interestingly, $tELA^{neg}/tLA^{pos}$ populations were found in all four patients, ranging from 0.04 to 4.6% of $CD8^{pos}$ T cells. When we compared this nonamer-specific subset to the total percentages of Melan-A-specific CD8 T cells (by calculating tLA^{pos} + $tELA^{pos}$ cells as 100%.), we observed that $tELA^{neg}/tLA^{pos}$ cells could reach up to half of Melan-A-specific T lymphocytes (Fig. 4*B*, *lower panels*, and Table V). To generate nonamer-specific T cell clones and confirm their specificity, $tELA^{neg}/tLA^{pos}$ cells were sorted from TIL(N) or from stimulated PBMC and cloned by limiting dilution. After expansion, almost all clones obtained from PBMCs confirmed to be $tELA^{neg}/tLA^{pos}$ and $V\alpha2.1^{neg}$ (Fig. 5*A* and Table VI), and 8 of 13 clones from TIL(N) were also $tELA^{neg}/tLA^{pos}$ (Table VI). Thus, large numbers of nonamer-specific cells were not only detected ex vivo, but we were also able to isolate them and generate nonamer-specific T cell clones demonstrating wide existence of clones with specificity similar to A42 and M77–80. Functional analysis confirmed recognition of natural Melan-A nonapeptide as well as the nonapeptide LA and analog decapeptides (AAA, ALA), but the nonapeptide AL was still not recognized (Fig. 5*B*). As before, we also observed paradoxical recognition of EAA and ELA decapeptides (Fig. 5*B*). However, ^{51}Cr-release assays of NA8-MEL cells infected by vaccinia viruses confirmed that these clones were truly nonamer-specific, because they killed target cells expressing AA minigenes, but not EAA or ELA minigenes (Fig. 5*C*). Finally, these clones were able to efficiently lyse melanoma cells without adding exogenous peptides. All together we generated 17 additional nonamer-specific T cell clones with similar properties as clones A42 and M77–80. To obtain an overview, these 19 clones were compared with 69 decamer-specific clones with regard to tetramer binding and expression of Vα2.1 (30). The data show that the nonamer-specific clones (all $tELA^{neg}$) efficiently bound the tLA tetramer, and were mostly $V\alpha2^{neg}$ (Table VII). In contrast, decamer-specific clones ($tELA^{pos}$) were mostly $V\alpha2.1^{pos}$, while also staining with tLA tetramers.

Table VI. *Newly generated LAGIGILTV-specific T cell clones*[a]

tLA^+ Sorted Clones from	tELA	tLA	Vα 2.1
Stimulated PBMCs (n = 10)			
269-1	−	+	−
269-2	−	+	−
269-3	−	+	−
269-4	−	+	−
269-5	−	+	−
269-6	−	+	−
269-7	−	+	−
269-11	−	+	−
269-9	−	+	+
269-10	+	+	+
TIL(N) (n = 13)			
701-1	−	+	−
701-2	−	+	−
701-3	−	+	−
701-11	−	+	−
701-30	−	+	−
701-41	−	+	−
701-38	+	+	−
820-4	−	+	−
820-9	−	+	−
820-1	+	+	−
820-2	+	+	−
820-3	+	+	−
820-10	+	+	+

[a]The clones were derived from PBMCs and TIL(N) as indicated, and demonstrated systematic binding to tLA tetramers and infrequent expression of Vα2.

Discussion

Among the relatively large number of known tumor associated Ags which are recognized by human CD8 T cells, Melan-A is one of the most immunodominant in HLA-A2 melanoma patients (38). Although the Melan-$A_{27–35}$ nonapeptide was originally described as immunodominant antigenic peptide (10), it was subsequently shown that the Melan-$A_{26–35}$ decapeptide is the natural peptide that is recognized most frequently and most efficiently by HLA-A*0201 and -B*3501-restricted T cells from TIL(N) and PBMC (5–8). Several reports described that the dominant Melan-A-specific CD8 T cell response is diverse in terms of TCR usage, and that most appear to recognize both nona- and decapeptides, and cross-react with superior functionality with the defined peptide variant Melan-$A_{26–35}$(A27L) (6, 7). In contrast to these findings, we report here that some Melan-A-specific clones do not recognize the naturally processed decamer. Rather, they display a distinct specificity to the Melan-A nonamer. We show that a significant proportion of Melan-A-specific T cells is nonamer but not decamer specific. Therefore, lack of decamer recognition is due to presence of glutamic acid (E) in position one of the peptide.

We first compared tetramer staining and functional avidity to Melan-A natural and modified peptides between majority and minority clones. We found surprising discrepancies, because minority clones efficiently recognized decamer labeled target cells but did not bind to the corresponding tetramers. Using NA8-MEL cells infected by vaccinia viruses coding for Melan-A minigenes as target cells, we demonstrated that decamer peptide recognition in cytotoxicity assays was an experimental artifact, likely due to the uncontrolled presence of nonapeptides. Thus, our data demonstrate that synthetic peptide recognition data need to be interpreted with caution. All CD8 T cell clones successfully recognized the AAA, ALA, and LA analog peptides suggesting that it is the glutamic acid that prevents recognition by minority, i.e., nonamer-specific clones. A previous study described a similar phenomenon using HLA-B*4501-restricted clones specific for the 11-mer (AEEAAGIGILT) from Melan-A. The authors showed that the presence of threonine at the C terminus abrogated the recognition by a CTL clone, whereas another CTL clone recognized the peptide irrespective of the presence or absence of the threonine (39).

The structure of the wild-type Melan-A nonamer and decamer/HLA-A2 complexes are expected to diverge at the N terminus of the peptides (40). The clones distinguishing the nonamer from the decamer, as described in this report, appear to interact differentially with residues near the P1 pocket of the HLA-A2 molecules, which consists of glutamic acid in the decamer complex, but empty or more likely water filled moieties in the natural nonamer complex (40, 41), providing the structural basis for distinguishing the two complexes (40). Previous studies have emphasized the large diversity but the highly conserved Vα2.1 usage of the pre- and postimmune repertoire of A2/Melan-A-specific CD8 T cells. This was particularly striking in T cell clones generated with EAA or ELA decapeptides (30, 32, 33). An earlier study generated clones without peptide (i.e., with the help

Table VII. *Summary of tetramer binding and Vα 2.1 expression by decamer- and nonamer-specific clones*

	% $tELA^+$	% tLA^+	% $V\alpha2.1^+$
Nonamer-specific clones (n = 19)	0	100	5
Decamer-specific clones (n = 69) [a]	100	84[b]	85

[a]From Dietrich et al. (30) and our unpublished data.
[b]As determined in 19 clones.

of mixed lymphocyte tumor cell cultures) and found that six of nine clones expressed Vα2 (34), suggesting that naturally presented Melan-A peptides (likely nona- plus decapeptides) may already induce some Vα2 bias. Our structural modeling data show that Vα2.1 seems highly favorable for the canonical interaction between TCR and ELA/HLA-A2 complex (Fig. 3*B* and Tables III and IV), while CDR1α of nonamer-specific clones exhibit an unfavorable interaction between Glu^{374} of the peptide and Asp^{413} of TCR. This suggests that the glutamic acid at the N terminus of decapeptides may contribute to selection of TCRs bearing Vα2.1. One may speculate that already in the thymus, Vα2.1-expressing TCRs might preferentially interact with glutamic acid leading to more frequent selection of Vα2.1-positive T cells. The described extraordinary high frequency of naive Melan-A-specific cells which are frequently Vα2.1 and decamer specific (1, 30, 42–44) may however not be entirely explained by the existence of a single hydrogen bond between CDR1α and glutamic acid of the decapeptide, as several human TCR Vα might have similar favorable interactions. More extended structural analyses are necessary to test the hypothesis whether the overall sequences and structures of Vα2-negative TCRs may be inappropriate to interact with the ELA/HLA-A2 complex. Finally, absence of glutamic acid in the nonapeptide seems not to confer preferential recognition by Vα2.1-expressing TCRs, although Vα2.1 may occasionally be expressed by nonamer-specific T cells (Table VI and VII).

According to TCR function/structure relationship analysis, and to recent data from crystallized HLA-A*0201 peptide complexes (40), recognition of the various peptides studied here by Melan-A-specific T cell clones appears to be based on a the following peptide conformation: The second amino acid of the decamers is anchored in P2 pocket, and the C-terminal valine in P9 pocket of the peptide binding groove of HLA-A*0201. In this conformation the decamer forms a kink, i.e., it bends out of the groove toward the TCR (40). By contrast, the conformation of the natural nonamer is not known. A recent study with crystallized nonamer AL/HLA-A*0201 complexes revealed that the AL peptide lies in a flat conformation in the peptide binding groove (40). We used this peptide systematically in our peptide titration assay, and found that the majority of Melan-A-specific CD8 T cells do not recognized this AL nonamer (Figs. 2*A* and 5*B* and data not shown), suggesting that the AA peptide (i.e., the wild-type nonamer) is recognized when presented in a kink conformation, which is generated when the first alanine lies in pocket P2. The current available HLA-A2/Melan-A-specific clones, no matter whether they are nonamer or decamer-specific, may recognize the kink conformation. Thus, based on the available data, the kink but not the flat conformation of Melan-A peptides appears to be recognized by melanoma-specific T cells. However, future studies must focus on the question whether Melan-A-specific T cells with different fine specificities may exist that indeed can recognize melanoma cells based on the AA peptide presented in a flat conformation, i.e., with the second alanine in pocket P2.

We found high percentages of nonamer-specific CD8 T cells in TIL(N) from unvaccinated melanoma patients, demonstrating the frequent presence of such T cells in vivo (Fig. 4*B* and Table V). Specificity was confirmed by the analysis of 13 Melan-A-specific CD8 T cell clones derived from TIL(N) and generated in absence of synthetic peptide. These results indicate that nonamer-specific CD8 T cells are efficiently primed and expanded in vivo in melanoma patients. Thus, not only decamer but also nonamer-specific T cells are substantially enriched in melanoma lesions. This is remarkable, given the weak antigenicity of the nonamer, caused by the weak binding to HLA-A*0201. It is therefore possible that nonamer-specific TCRs are of higher affinity allowing to compensate for low p-MHC stability.

Several reasons may explain why most laboratories generate decamer but not nonamer-specific T cell clones. Most researchers use the Melan-A decamer peptides, because they are more strongly antigenic and thus lead to stronger proliferation than the nonapeptides. The enhanced p-MHC stability is also the reason why tetramers are mostly prepared with decamers. Consequently, T cell cloning is often done through sorting with decamer-tetramers, and they are subsequently also used to screen for positive and negative clones.

Immunotherapy of melanoma patients more often applies Melan-A decapeptides than nonapeptides (45), again because the former are more strongly antigenic. In deed, previous studies with the nonapeptide AAGIGILTV induced only weak immune responses (46). However, novel vaccine adjuvants substantially increase the likelihood of strong human T cell responses even with weakly antigenic peptides (Ref. 47 and D. E. Speiser et al., submitted for publication). Based on our present data, the use of Melan-A nonapeptides for vaccination should be encouraged (48), to elicit nonamer-specific T cell responses in vivo and evaluate their protective potential.

Together, our data reveal a new face of HLA-A*0201/Melan-A-specific CD8 T cells, revealing a population of T cells exclusively specific for the nonapeptide, representing a significant proportion of Melan-A-specific T cells. The specificity difference between nonamer and decamer-specific T cells is more pronounced than previously thought. Although some cells are cross-reactive, this has been largely overestimated in the past, based on functional experiments with peptides that do not allow precise distinction between recognition of nonamers and decamers. Tetramers are much better suited for this distinction, a remarkable finding given the usually high level of cross-specific binding by tetramers. We conclude that the two types of cells are specific for two different epitopes, rather than just cross-reactive.

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Disclosures

The authors have no financial conflict of interest.

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