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Impact of Orthologous Melan-A Peptide Immunizations on the Anti-Self Melan-A/HLA-A2 T Cell Cross-Reactivity

Sara Colombetti,* Theres Fagerberg,*† Petra Baumgartner,‡ Laurence Chapatte,* Daniel E. Speiser,*¶ Nathalie Rufer,§¶ Olivier Michielin,¶ and Frédéric Lévy*¶

In HLA-A2 individuals, the CD8 T cell response against the differentiation Ag Melan-A is mainly directed toward the peptide Melan-A26–35. The murine Melan-A24–33 sequence encodes a peptide that is identical with the human Melan-A26–35 decamer, except for a Thr-to-Ile substitution at the penultimate position. Here, we show that the murine Melan-A24–33 is naturally processed and presented by HLA-A2 molecules. Based on these findings, we compared the CD8 T cell response to human and murine Melan-A peptide by immunizing HLA-A2 transgenic mice. Even though the magnitude of the CTL response elicited by the murine Melan-A peptide was lower than the one elicited by the human Melan-A peptide, both populations of CTL recognized the corresponding immunizing peptide with the same functional avidity. Interestingly, CTL specific for the murine Melan-A peptide were completely cross-reactive against the orthologous peptide, whereas anti-human Melan-A CTL recognized the murine Melan-A peptide with lower avidity. Structurally, this discrepancy could be explained by the fact that Ile32 of murine Melan-A24–33 created a larger TCR contact area than Thr31 of human Melan-A26–35. These data indicate that, even if immunizations with orthologous peptides can induce strong specific T cell responses, the quality of this response against syngeneic targets might be suboptimal due to the structure of the peptide-TCR contact surface. The Journal of Immunology, 2006, 176: 6560–6567.

Effective anticancer immunotherapy critically depends on the induction of high-avidity tumor-specific T cells. The T cell specificity is restricted by the expression of appropriate HLA class I ligands at the surface of tumor cells. These ligands are peptides produced by the intracellular degradation of tumor-associated Ags. Tumor-associated Ags can be classified into four categories: tumor-specific Ags, also known as cancer/testis Ags, differentiation or lineage-specific Ags, Ags overexpressed in tumors, and Ags resulting from tumor-specific mutations (www.cancerimmunity.org/peptidedatabase/Tcelleetopes.htm#references). Differentiation Ags have attracted great attention because of their frequent and specific expression pattern. However, differentiation Ags are self-proteins expressed both in normal and transformed cells. Some of them being also expressed in the thymus, the specific T cell repertoire is likely to be partially tolerized (1, 2). In line with this, immunization of mice with xenogeneic, but not syngeneic, melanoma-associated Ags was shown to elicit detectable CD8+ T cell responses (3–7). Because of the high degree of homology between murine and human differentiation Ags, some of the CTL elicited by xenogeneic Ags were cross-reactive against the syngeneic Ags, and, in some cases, mediated antitumor immunity and autoimmunity.

The differentiation Ag Melan-A/MART1 (denoted Melan-A hereafter) is a membrane-embedded protein of 118 aa of unknown function that is expressed both in melanocytes and melanomas (8). In HLA-A2 individuals, processing of Melan-A gives rise to an immunodominant peptide. This peptide, with sequence EAAGILTV, encompasses aa 26–35 of the protein and is produced directly by the proteasome (9). Murine Melan-A is a slightly shorter protein (113 aa) sharing 68.6% amino acid identity with the human protein (7). The sequence of a peptide encompassing aa 24–33 of murine Melan-A is identical with the human Melan-A26–35 except for a Thr-to-Ile substitution at the penultimate position (aa 32 for murine Melan-A and 34 for the human Melan-A).

The crystal structure of human Melan-A26–35/HLA-A2 complexes has been recently solved (10). It indicates that the side chain of residue Thr32 is exposed to the solvent and is able to contact the TCR. The potential HLA-A2-restricted peptide derived from murine Melan-A, Melan-A24–33, contains an Ile at position 9 of the decamer, which differs from Thr by the exchange of the hydroxyl group with a methyl group and the presence of an additional methyl group. It is therefore likely that the different structure of these two amino acids will influence peptide-TCR contacts and CTL cross-reactivity upon immunization with human vs murine Melan-A peptide. To test this hypothesis, we have analyzed human and murine CTL against human and murine Melan-A peptides restricted by HLA-A2. As previously shown, immunization of HLA-A2/H-2Kb (A2Kb) transgenic mice with the human Melan-A peptide elicited a strong CD8+ T cell response readily detectable ex vivo (11). Here, we show that murine Melan-A24–33 is naturally processed and presented by HLA-A2 and is immunogenic in A2Kb mice, even if to lower extent than the human peptide. Interestingly, murine CTL generated with the murine epitope cross-reacted completely with the human epitope. In contrast, CTL specific for the human epitope showed a reduced level of cross-reactivity against the murine epitope. The nonreciprocal degree of cross-reactivity between anti-murine and anti-human Melan-A CTL could be explained by structural analyses and predictions of the peptide-HLA...
surfaces contacting TCR. Together, this study shows how immunizations with an orthogonal peptide, such as human Melan-A\(_{25-34}\) in mice, may increase the magnitude of the T cell responses but may simultaneously reduce the functional avidity of the elicited CTL against syngeneic targets.

**Materials and Methods**

**Mice and cell lines**

HLA-A2/H-2\(^k\) transgenic mice and EL-4 cells transfected with the HLA-A2/H-2\(^k\) gene (EL-4/A2K\(^k\)) were described previously (11, 12). Mice were bred in a pathogen-free animal facility and experiments were performed in compliance with the rules of the local veterinary office. COS-7 cells were purchased from American Type Culture Collection. The cell lines ME-290 (HLA-A2\(^\ast\) Melan-A\(^{-}\)) and ME-260 (HLA-A2\(^\ast\) Melan-A\(^{-}\)) were provided by Dr. D. Rimoldi (Ludwig Institute for Cancer Research (LICR), Lausanne Branch, Lausanne, Switzerland).

EL-4/A2K\(^k\) cells and COS-7 cells were maintained in DMEM supplemented with 1% HEPES, 1% strepto-penicillin, and 1% heat-inactivated FCS. ME-290 and ME-260 cells were maintained in RPMI 1640 medium supplemented with 1% HEPES, 1% strepto-penicillin, and 1% heat-inactivated FCS.

The HLA-A\(^2\) human mutant cell line T2 used as APCs in chromium release assays was cultured in RPMI 1640 medium containing 10% FCS.

**Plasmids, transfections, and IFN-\(\gamma\) release assay**

The cDNA of HLA-A2/H-2\(^k\) (A2\(^k\)) was obtained by RT-PCR from EL-4/A2K\(^k\) cells and was cloned in the PEGFP-C1 vector (BD Clontech). The plasmid pcDNA3 (BD Clontech) served as backbone for all the Melan-A constructs used in this study. pcDNA3 containing the human Melan-A sequences were a gift from Dr. D. Rimoldi. Murine Melan-A was obtained by RT-PCR of RNA extracted from the murine melanoma cell line B16F10. The PCR product was digested with EcoRI and BamHI and cloned into the EcoRI-BamHI sites of pcDNA3. Substitution of Ala\(^25\) in the murine Melan-A by Leu was performed using the PCR-based QuikChange site-directed mutagenesis kit (Stratagene) and appropriate oligonucleotides, following the manufacturer’s protocol. The sequence of all constructs was confirmed by DNA sequencing.

COS-7 cells were transfected with the A2\(^k\) gene by using FuGene (Roche Diagnostics). Transfected cells were subjected to Geneticin (G418) selection, and the pools of resistant cells were used in subsequent experiments.

To test T cell reactivity, COS-7/A2K\(^k\) cells were transiently transfected by FuGene with pcDNA3 plasmids encoding either the human Melan-A or the murine Melan-A proteins or with the empty pcDNA3 plasmid as control. After 24 h, the cells were washed before the addition of E-V10/9/9-specific CTL at an E:T ratio of 3:1. In parallel, E-V10/9/9-specific CTL were cocultured with COS-7/A2K\(^k\) cells pulsed with 1 \(\mu\)M E-V10/9/9 or E-V10/9/9 peptides for 1 h at 37°C. After 24 h, culture supernatants were harvested, and the concentration of IFN-\(\gamma\) was measured by ELISA, as previously described (9).

ME-260 cells were transiently transfected with the A2\(^k\) gene by FuGene. To test T cell recognition of the endogenously expressed Melan-A protein, ME-290 and ME-260/A2K\(^k\) cells were cocultured with E-V10/9/9-specific CTL at an E:T ratio of 3:1 for 24 h, and the concentration of IFN-\(\gamma\) in culture supernatants was then measured by ELISA. In parallel, E-V10/9/9-specific CTL were cocultured with ME-260/A2K\(^k\) cells pulsed with 1 \(\mu\)M E-V10/9/9 peptide for 1 h at 37°C.

**Synthetic peptides and CpG oligodeoxynucleotides (ODN)**

Peptides were synthesized by solid-phase chemistry and reached over 90% homogeneity, as indicated by analytical HPLC. Lyophilized peptides were diluted in DMSO and stored at \(-20^\circ\)C. Synthetic CpG containing ODN (TCTTGACGTTCTGACGTT) optimized for murine vaccination (ODN 1826; Coley Pharmaceutical Group) were dissolved in sterile PBS.

**Immunization of mice**

Peptide vaccinations were conducted by s.c. injection, at the base of the tail, of 50 \(\mu\)g of the relevant peptide admixed with 50 \(\mu\)g of CpG ODN emulsified in 50 \(\mu\)l of IFA (Sigma-Aldrich) and 50 \(\mu\)l of PBS. Ten days after injection, mice were sacrificed and lymphocyte suspensions from draining lymph nodes were prepared for in vitro stimulations with the different peptides.

**Enumeration of peptide-specific CD8\(^+\) T cells by tetramer staining**

PBMC obtained by tail bleedings of immunized mice were incubated with PE-conjugated A2K\(^k\)-E-V10/9/9 or A2K\(^k\)-E-V10/9/9 tetramers (prepared in our laboratory) for 45 min at 20°C, washed, and then incubated with FITC-conjugated anti-CD8a mAb (53-6.7) for 25 min at 4°C. Flow cytometry was performed on FACSCalibur using CellQuest software (BD Biosciences).

**Generation of peptide-specific murine CTL lines by in vitro restimulations**

Lymph node cells (1–2 \(\times\)10\(^6\)) were cultured with 2 \(\times\)10\(^4\) irradiated (10 krads) EL-4/A2K\(^k\) cells pulsed with 2.5 \(\mu\)M stimulating peptides for 1 h at 37°C, in 24-well cell culture plates in 2 ml of DMEM supplemented with 1% HEPES, 1% strepto-penicillin, 10% heat-inactivated FCS, 50 \(\mu\)M 2-ME, and 30 IU/ml rIL-2. After at least two rounds of weekly stimulation, cells were tested for cytolytic activity.

**Assessment of in vitro cytolytic activity**

Cytolytic activities of the CTL were determined by standard \(^{31}\)Cr release assays. Target cells were labeled with \(^{31}\)Cr for 1 h at 37°C, washed, and coincubated with CTL at the indicated E:T ratio in the presence of the indicated concentrations of the relevant peptide, in V-bottom 96-well plates in a volume of 200 \(\mu\)l of DMEM. Chromium release was measured after 4-h incubation at 37°C. The percentage of specific lysis was calculated as follows: percentage specific lysis = ([experimental release – spontaneous release]/total release – spontaneous release) \(\times\) 100.

**Competition assay**

Various concentrations of the competitor peptides (50 \(\mu\)l) were incubated with \(^{31}\)Cr-pulsed T2 cells (50 \(\mu\)l) (1000 cells/well) for 15 min at room temperature. The antigenic Influenza matrix peptide, FluMP5 8–6 6, was added at a concentration (100 pM) (50 \(\mu\)l) inducing 50% lysis of target cells (data not shown), together with a FluMP5 8–6 6-specific human CTL clone (50 \(\mu\)l) (5000 cells/well). Chromium release was measured after 4-h incubation at 37°C.

**Structural predictions of the E-V10/9/9-HLA-A2 and E-V10/9/9-HLA-A2 epitopes**

An ab initio method has been designed to theoretically predict the structure of peptides bound to the MHC class I molecule from the amino acid sequence alone. This method is composed of two steps. First, the peptide conformational space in the MHC environment is sampled. Second, the conformers are evaluated by using a combination of clustering and energy/entropy calculations. The conformational sampling was performed using a simulated annealing protocol in which 1000 heating-cooling cycles were completed. The CHARMM (13) molecular modeling program and the all-atom CHARMM 22 protein parameter set (14) were used to perform the simulated annealing protocol. At the end of each cycle, a conformation of the peptide in the MHC molecule was kept after energy minimization.

The simulations were performed in vacuum, using a distance-dependent dielectric constant that accounts in part for the solvent screening of the electrostatic interactions. For long-range nonbonded interactions, an atom-based force switching was applied from 14 to 15 Å. During the entire simulation, the MHC molecule was kept fixed, but no constraints were applied to the peptide.

For the evaluation of the conformers, clustering according to geometric similarities was performed. Using additional energy and entropy calculations, involving solvation free energies computed with the Poisson-Boltzmann continuum models, the mean effective energy and the conformational free energy of clusters were computed. The final conformation, i.e., the prediction, was chosen as the center of the lowest conformational free energy cluster.

**Human T cell cloning, culture, and cytolytic activity**

Melan-A-specific CD8\(^+\) T cell clones were derived from PBMC of patient LAU 444. Characteristics of this patient have been reported elsewhere (15). Monoclonal T cells were obtained by limiting-dilution cloning (data not well) following staining with Melan-A multimers and cell sorting using a FACSVantage SE (BD Biosciences). Cells were expanded in RPMI 1640 medium supplemented with 8% human serum, 150 U/ml rIL-2, 1 \(\mu\)g/ml
PHASE (Sodiag), and 1 × 10^6/ml irradiated (3000 rad) allogeneic PBMC as feeder cells. Positive Melan-A-specific T cell clones were restimulated bivweekly in 24-well plates with PHA (1 μg/ml), irradiated feeder cells (×10^6/ml), and rIL-2 (150 U/ml).

For each human Melan-A-specific T cell clone, the functional avidity of Ag recognition was assessed in standard 4-h ^31Cr release assays using T2 target cells.

**Results**

**Human and murine Melan-A peptides are immunogenic in HLA-A2/H-2K^b transgenic mice**

Human and murine Melan-A proteins share 68.6% amino acid identity (Fig. 1A). Murine Melan-A is a protein of 113 aa, slightly shorter than human Melan-A (118 aa). From sequence alignment, we observed that the murine Melan-A_{26-35} sequence (EAAGIGILTV) was almost identical with the human Melan-A_{26-35} sequence (EAAGIGILTV).

Immunization of HLA-A2/H-2K^b (A2K^b) transgenic mice with the natural Melan-A_{26-35} peptide in adjuvant was previously shown to elicit rather weak T cell responses (11). This peptide has low intrinsic affinity to HLA-A2, due to the absence of a strong anchor residue at position 2 of the decamer. Substitution of Ala_27 for Leu dramatically increased the affinity of the Melan-A_{26-35} peptide to HLA-A2 in vitro and led to an increased immunogenicity in vivo (16, 17). Importantly, both murine and human CTL elicited by A27L-mutated Melan-A_{26-35} were completely cross-reactive to the natural peptide and were tumor-reactive (11, 17, 18). In the current study, all Melan-A-derived peptides contained that particular substitution at position 2. These peptides will be denoted throughout the text as E-V10/T9 for the human A27L Melan-A_{26-35} analog peptide and E-V10/I9 for the corresponding murine Melan-A_{26-35} peptide.

We first compared the immunogenicity of E-V10/I9 and E-V10/T9 peptides in A2K^b transgenic mice. Both synthetic peptides were mixed with IFA and Cpg containing ODN and injected s.c. This vaccine formulation was shown to be highly efficient in the induction of strong CD8^+ T cell responses in vivo (18). After immunization of mice with E-V10/T9 or E-V10/I9 peptides, the specific CD8^+ T cell response was monitored by ex vivo staining of peripheral blood cells with A2K^b-E-V10/T9 or A2K^b-E-V10/I9 tetramers, respectively. As shown in Fig. 1B, the specific CD8^+ T cell response induced by E-V10/T9 peptide peaked at day 6, reaching an average of 5.2% tetramer^+ cells among total circulating CD8^+ T cells. Thereafter, the frequency of tet^+ cells decreased and was no longer detectable by day 21. The CD8^+ T cell response induced by E-V10/I9 peptide immunization also peaked at day 6, but was significantly lower (p = 0.005) in magnitude (average of 1.5% tet^+ among total CD8^+ T cells). With both peptide immunizations, ~80% tet^+ CD8^+ T cells displayed an activated phenotype (CD62Llow, typical of CD8^+ effector T cells (data not shown). It is noteworthy that, despite the high magnitude of the T cell response after E-V10/T9 or E-V10/I9 immunization, no sign of depigmentation was detectable.

These results demonstrate that, similar to the human Melan-A peptide, the murine peptide containing an Ala-to-Leu mutation is immunogenic in A2K^b transgenic mice and induces a specific CD8^+ T cell response that is readily detectable ex vivo.

**The E-V10/I9 peptide is processed and presented by HLA-A2^+ cells**

Having shown that E-V10/I9 is immunogenic, we then asked whether this peptide was naturally processed and presented by cells expressing A2K^b molecules. To this end, A2K^b expressing COS-7 cells (COS-7/A2K^b) were transfected with plasmids encoding either the wild-type or the A25L substituted murine Melan-A protein. In parallel, COS-7/A2K^b cells were also transfected with plasmids coding for the human wild-type or A27L Melan-A protein. As control, COS-7/A2K^b cells were pulsed with saturating amounts of exogenous peptides and recognition was assessed by measuring the amount of IFN-γ released by the CTL after 24 h of coculture (Fig. 2A). CTL incubated with COS-7/A2K^b cells expressing human or murine Melan-A released 7–30 times more IFN-γ than CTL incubated with mock-transfected cells, indicating that human and murine Melan-A peptides were processed and presented. As expected, cells pulsed with saturating amounts of exogenous peptides were efficiently recognized. Interestingly, no significant differences were observed between the recognition of cells expressing the natural or mutated form of mouse or human Melan-A protein.

To confirm that the murine CTL raised against E-V10/I9 could recognize melanoma cells expressing endogenous levels of Melan-A, HLA-A2^+ melanoma cells expressing wild-type Melan-A (ME-290; Fig. 2A), or HLA-A2-negative, Melan-A^- melanoma cells (ME-260) transfected with A2K^b (Fig. 2B) were incubated with E-V10/I9-specific CTL. In both cases, the CTL were able to recognize the HLA-A2^+ melanoma cells expressing Melan-A.

Together, these data indicate that the murine E-V10/I9 peptide is efficiently produced and presented by HLA-A2 molecules.
Moreover, these results also show that CTL specific for the E-V10/I9 epitope cross-react with the E-V10/T9 epitope.

**Ex vivo cross-recognition of E-V10/T9 and E-V10/I9 peptides by specific CD8\(^+\) T cells**

Based on the previous observation, we analyzed the extent of cross-recognition of the two epitopes by immunizing A2Kb mice with E-V10/T9 or E-V10/I9 peptides in adjuvant, and by staining the CD8\(^+\) T cells of each group of immunized mice directly ex vivo with A2Kb-E-V10/T9 and A2Kb-E-V10/I9 tetramers (Fig. 3). Both E-V10/T9- and E-V10/I9-specific peptide immunizations induced CD8\(^+\) T cells that were stained by both A2Kb-E-V10/T9 and A2Kb-E-V10/I9 tetramers. No detectable tet\(^+\)CD8\(^+\) T cells were induced by immunizing mice with adjuvant only. As before, the frequency of T cells elicited by E-V10/I9 immunization was significantly lower than that elicited by E-V10/T9. Interestingly, the proportion of CD8\(^+\) T cells elicited by E-V10/T9 or E-V10/I9 that were stained by tetramers containing either the immunizing peptide or the nonimmunizing, related Melan-A peptide was comparable (Fig. 3B).

Taken together, these data suggested a high degree of cross-recognition of the E-V10/T9 and E-V10/I9 peptides by specific CD8\(^+\) T cells.

**Analysis of CD8\(^+\) T cell cross-reactivity to E-V10/T9 and E-V10/I9 peptides**

To measure more accurately the extent of cross-reactivity between the anti-E-V10/T9 and anti-E-V10/I9 T cell populations, we raised CTL lines specific for the two peptides. After immunization of A2Kb mice with E-V10/T9 or E-V10/I9 peptides, draining lymph nodes were isolated and cells were stimulated at least twice in vitro with the immunizing peptides. The lytic activity of each CTL line was tested in a standard \(^{51}\)Cr assay against EL-4/A2Kb cells in the presence of titrating amount of either E-V10/T9 or E-V10/I9 peptides. As expected, CTL lines derived from mice immunized with E-V10/T9 efficiently lysed target cells presenting E-V10/T9, with a half-maximal lysis at peptide concentration of 1 nM (Fig. 4A, left panel, and B). A similar concentration of E-V10/I9 peptide was required for half-maximal lysis of target cells by E-V10/I9-specific CTL (Fig. 4A, right panel, and B). The E-V10/T9-specific CTL lines showed some degree of cross-reactivity against E-V10/I9 but 10\(^x\) higher peptide concentrations were required to reach a similar degree of lysis (Fig. 4B). In contrast, CTL lines derived from mice immunized with E-V10/I9 peptide not only recognized the immunizing peptide but also showed a complete cross-reactivity toward the E-V10/T9 epitope.

To exclude that the observed difference in the recognition of E-V10/T9 and E-V10/I9 by E-V10/T9-specific CTL was caused by a different affinity of the two peptides to HLA-A2, we performed...
A competition assay using either peptide as competitor. $^{51}$Cr-labeled T2 cells (HLA-A$^{2+}$) were pulsed with 0.1 nM FluMP58–66 peptide, together with increasing concentrations of the competitor peptide E-V10/T9 or E-V10/I9. The cells were then cocultured with a FluMP58–66-specific human CTL clone for 4 h. Mean percentages of specific lysis $\pm$ SD of four independent experiments are shown.

FIGURE 4. Analysis of the functional cross-reactivity of Melan-A-specific CTL toward E-V10/T9 and E-V10/I9. A, CTL lines specific for E-V10/T9 (left panel) and E-V10/I9 (right panel) were generated and propagated in vitro. The reactivity of each CTL line was independently tested in a standard cytotoxic assay against target cells pulsed with titrating amounts of E-V10/T9 (□) or E-V10/I9 peptide (○) at an E:T ratio of 10. The lysis of nonpulsed cells. Results represent the mean $\pm$ SEM of three independent CTL lines per group. The dashed lines indicate the peptide concentration required to reach half-maximal lysis. B, Bar graph representation of the concentration (in nanomolar) of E-V10/T9 and E-V10/I9 peptides required for half-maximal lysis of target cells by specific CTL lines. The values were calculated from the data shown in A. Mean concentrations $\pm$ SEM of peptides are shown. Note that the concentration of E-V10/T9 and E-V10/I9 peptide required for half-maximal lysis by E-V10/T9-specific CTL is significantly different ($p = 0.02$; Student’s t test). C, $^{51}$Cr-labeled T2 cells (HLA-A$^{2+}$) were pulsed with 0.1 nM FluMP58–66 peptide, together with increasing concentrations of the competitor peptide E-V10/T9 or E-V10/I9. The cells were then cocultured with a FluMP58–66-specific human CTL clone for 4 h. Mean percentages of specific lysis $\pm$ SD of four independent experiments are shown.

Structural features of the E-V10/T9-HLA-A2 and E-V10/I9-HLA-A2 epitopes

Next, we assessed whether the nonreciprocal cross-reactivity described above could be explained by the different structures of E-V10/T9- and E-V10/I9-HLA-A2 complexes. Relying on the existing crystallographic resolution of the E-V10/T9-HLA-A2 complex, we modeled the E-V10/I9-HLA-A2 complex, using an ab initio approach based on simulated annealing cycles, clustering of the conformers, and conformational free energy computation as described in Materials and Methods. This method was recently validated for peptide-HLA-A2 complexes by demonstrating that >90% of the 14 predicted peptide-HLA-A2 complexes matched the crystallized structure with root-mean-square deviation (RMSD) values of $<1.5 \text{ Å}$ (19).

The final structure of E-V10/I9-HLA-A2 was selected as the center of the cluster associated with the lowest conformational free energy computed using the Poisson-Boltzmann continuum solvent model. As shown in Fig. 5, the conformations of E-V10/T9 and E-V10/I9 complexed to HLA-A2 were very similar, with main chain atom RMSD values of 0.47 Å. The main chain atom average global displacement was 0.09 Å at the substitution site, T9/I9.

Formally, the substitution of T9 for I9 corresponded to the replacement of a polar hydroxyl group by a nonpolar, bulky ethyl group. Resolution of the crystal structure of E-V10/T9-HLA-A2 demonstrated that the hydroxyl group of T9 was pointing down into the HLA-A2 binding groove (hydrogen bonding to Asp$^{77}$ of HLA-A2) while its methyl group pointed up, toward the solvent. In the predicted structure of E-V10/I9-HLA-A2, the ethyl group was pointing up, toward the solvent (Fig. 5). These results indicate that the major structural difference between E-V10/T9 and E-V10/I9 is the presence, in the latter, of an additional methyl group. Consequently, the different degree of cross-reactivity revealed by the anti-E-V10/T9 and anti-E-V10/I9 CTL (Fig. 4A) can be explained by the different sensitivities of their TCR to the presence or absence of the extra methyl group.

Murine CTL specific for the E-V10/T9 peptide efficiently recognize the E-V10/I9 peptide

The functional and structural analyses described above suggested that the avidity of Melan-A-specific CTL might be less affected by the loss of a methyl group at position 9 than by the addition of such a group. To further substantiate this postulate, we tested to what extent the shortening of the side chain T9 of human E-V10/T9 to Ala affected T cell cross-reactivity. First, we compared the existing structure of E-V10/T9-HLA-A2 to the predicted structure of E-V10/A9-HLA-A2 (Fig. 6A). The average main chain atom RMSD value was 0.68 Å. The average global displacement values at the substitution site were small, 0.31 Å for main chain. In this case, the T9/A9 substitution resulted in the loss of one hydroxyl group pointing into the HLA-A2 binding groove and one methyl group from the solvent-exposed region. As before, both structures
were largely superimposable, except for the solvent-accessible surface surrounding residue 9.

Next, we compared the cross-reactivity of E-V10/T9-specific T cells against E-V10/A9 and E-V10/I9 presented by HLA-A2 (Fig. 6B). As earlier, the degree of functional cross-reactivity was determined by measuring the concentration of peptide required to reach half-maximal lysis of target cells. In accordance with our hypothesis, we observed that E-V10/T9-specific T cells were highly cross-reactive toward E-V10/A9 peptide. Again, the functional avidity of the E-V10/T9-specific T cells for the E-V10/I9 peptide was significantly lower.

These results supported the hypothesis that the shortening of amino acid side chains at the TCR-peptide interface does not affect the overall peptide recognition, whereas the opposite, i.e., lengthening of the side chain, has a much more negative effect.

Analysis of the cross-reactivity of human CTL clones specific for the E-V10/T9 peptide toward E-V10/A9 and E-V10/I9

To confirm the impact of the side chain of residue 9 on T cell recognition at the clonal level and to extend our findings to human settings, we tested the level of cross-reactivity between the different Melan-A peptides, using human CTL clones derived from an HLA-A2+ melanoma patient vaccinated with E-V10/T9 peptide in adjuvant. This patient mounted a very high, specific T cell response following peptide vaccination, with frequency reaching close to 4% tet+ cells among circulating CD8+ T cells. Two representative high-avidity clones (50% maximal lysis at peptide concentrations of $10^{-11}$ M) were incubated with 53Cr-labeled target cells pulsed with decreasing concentrations of Melan-A peptides. As shown in Fig. 7, we observed that the recognition of E-V10/T9 and E-V10/A9 by the human CTL clones was indistinguishable. In contrast, the reactivity against E-V10/I9 peptide was reproducibly lower. Indeed, 50% target cell lysis was reached at E-V10/I9 peptide concentrations of $10^{-8}$–$10^{-9}$ M, whereas 10–100× lower concentrations of E-V10/T9 or E-V10/A9 were sufficient to reach 50% cell lysis. In conclusion, our data show that human CTL clones have the same bias in their cross-reactivity toward epitopes incorporating amino acid substitutions at position 9 as their murine counterparts.

FIGURE 6. Structure of the E-V10/A9-HLA-A2 complex and CTL recognition. A, Comparison between the crystal structure of the HLA-A2-E-V10/T9 epitope (left panel) (10) and the predicted structure of the HLA-A2-E-V10/A9 epitope (see Materials and Methods). The HLA-A2 binding groove is visualized from the C-terminal end of the peptide. Both HLA-A2 and peptide are shown in blue with the substitution site residue colored in green (E-V10/T9) or red (E-V10/A9). B, E-V10/T9-specific CTL were reacted, at an E:T ratio of 10, with target cells pulsed with titrating amounts of E-V10/T9 (◻), E-V10/I9 (❍), or E-V10/A9 (■) peptides. ●, The lysis of nonpulsed cells. The dashed lines indicate the peptide concentration required to reach half-maximal lysis. Results are representative of two independent experiments.

FIGURE 7. Analysis of the functional cross-reactivity of human anti-Melan-A CTL clones toward E-V10/I9 and E-V10/A9. Two independent E-V10/T9-specific CTL clones were derived from the LAU 444 melanoma patient, as described in Materials and Methods. Each clone was reacted with 53Cr-labeled target cells in the presence of titrating amounts of E-V10/T9 (◻), E-V10/I9 (❍), or E-V10/A9 (■) peptides, or in the absence of peptides (●). Means of triplicate cultures are shown for each CTL clone.
Discussion

In this work, we identified a naturally processed HLA-A2-restricted antigenic peptide derived from murine Melan-A. This decameric peptide differs from the orthologous human peptide by a single amino acid at the penultimate position. Immunization of A2K* transgenic mice with the murine Melan-A peptide E-V10/I9 elicited CTL that were completely cross-reactive to the human Melan-A peptide E-V10/T9. In contrast, CTL induced by immunization of A2K* transgenic mice with the human Melan-A peptide only partially cross-reacted with the murine Melan-A peptide. This discrepancy could be explained by the different impact of the amino acids in position 9 of the two peptides on the TCR contact surface. Similar results were obtained with human CTL clones derived from an immunized melanoma patient.

Differentiation Ags constitute promising targets for the induction of antitumor T cell responses, because they are very frequently expressed in a selective type of cells. In the context of melanoma, differentiation Ags such as tyrosinase, TRP1 and -2, gp100/pmel17, and Melan-A were shown to encode several MHC class I-restricted peptides that could elicit antitumor responses in vivo. However, due to partial T cell tolerance against most self-Ags, induction of specific T cell responses in mice could only be achieved by immunization with vaccine modalities incorporating peptide of xenogeneic origin (3–7, 20). A recent report described the vaccination of dogs with DNA coding for the xenogeneic human tyrosinase and showed potential therapeutic effects (21). Contrary to most of these in vivo studies, where T cell responses were evaluated qualitatively, based on tumor rejection, we performed qualitative and quantitative measurements of the anti-Melan-A T cell response. First, we demonstrated that the magnitude of the T cell response was significantly lower following E-V10/I9 immunization compared with E-V10/T9. Whereas an average of 5% tet^C D8^ T cells were detectable at the peak of the anti-E-V10/T9 response, only 1.5% were detectable after E-V10/I9 immunization. Second, by measuring the concentration of peptides required to reach half-maximal target cell lysis, we showed that, despite a difference in specific CTL frequencies, the functional avidity of these CTL against the immunizing peptides was similar. Based on these findings, we conclude that the consequence of the expression of self-Ags, such as Melan-A, on the specific T cell population is a reduction in the frequency of potentially reactive T cell precursors rather than in the selection of low-avidity T cells. However, one cannot formally exclude that the expansion of T cells specific for self-Ags might be reduced as a result of partial T cell anergy or unresponsiveness. Among the specific T cell repertoire elicited by E-V10/T9 or E-V10/I9 peptides, a high degree of cross-recognition was revealed by staining T cells with A2Kb-E-V10/T9 and -E-V10/I9 tetramers. This result indicated that the two T cell repertoires were largely similar, and it predicted a high degree of functional cross-reactivity. However, the latter hypothesis was not completely accurate. Indeed, we showed that CTL elicited by E-V10/I9 were completely cross-reactive against E-V10/T9, but not the opposite. In contrast, EV-10/T9-specific CTL cross-reacted to the E-V10/A9 peptide.

Analyses of the existing crystal structure of the E-V10/T9-HLA-A2 complex and structural predictions showed that the overall conformation of the different Melan-A peptide-HLA complexes, including the C beta atoms of the three side chains Thr, Ile, and Ala at position 9, were all superimposable. The sole difference between E-V10/A9, E-V10/T9, and E-V10/I9 was the presence of 0, 1, and 2 methyl groups, respectively, pointing toward the TCR. Based on previously published structures of peptide-HLA-TCR complexes, the removal of a methyl group from I9 to T9 or from T9 to A9 should result in the loss of only few van der Waals contacts between the peptide and the TCR. Whereas such a loss may be of little consequence for the CTL avidity, the elimination of additional van der Waals contacts will eventually affect peptide-TCR interactions and results in decreased avidity. This interpretation is also compatible with findings by others that the elimination of protruding features emerging from the peptide affects T cell recognition (22). Preliminary observations indicate that the recognition of E-V10/A9 by CTL raised against E-V10/I9 is not as efficient as the recognition of E-V10/T9 (our unpublished results).

In contrast to the small effect caused by the removal of single methyl groups on CTL recognition, CTL raised against E-V10/T9 cross-reacted poorly against E-V10/I9. Based on our structural predictions, addition of extra methyl groups is likely to produce a sterically hindrance at the TCR contact site, which will result in a much larger drop in the binding free energy and will limit TCR engagement.

Alanine scans of the E-V10/T9 peptides showed that many clones derived from tumor-infiltrating lymphocytes, tumor-infiltrated lymph node, or PBL of normal donors cross-reacted only poorly with the analog E-V10/A9 (23). In contrast, our results, obtained with two representative CTL clones derived from a melanoma patient immunized with E-V10/T9 in adjuvant, showed that these clones completely cross-reacted with E-V10/A9. Natural E-V10/T9-HLA-A2 complexes were shown to be unstable (23). Recognition of these complexes by specific CTL will require extensive interactions between the TCR and the peptide-HLA complex. The interactions between the heteroclitic A27L analog peptide and HLA-A2 being significantly more stable (17), the necessity for a tight interaction between all possible residues of the peptide-HLA complex and TCR might not be as strong. Consequently, CTL elicited by the natural E-V10/T9 peptide might be much more sensitive to small energetic losses caused by the absence of a methyl group (as in E-V10/A9) than CTL elicited by the A27L analog. Whether this will impact on the overall avidity of the T cell repertoire elicited by natural vs analog peptide immunizations of a melanoma patient remains open.

Cross-reactivity of anti-Melan-A CTL against epitopes derived from proteins of microorganisms was previously demonstrated (24, 25). In one study, it was demonstrated that a human CTL clone specific for E-V10/T9 was cross-reactive against many bacterial and viral peptides (24). Importantly, it was also found that microbial peptides sharing significant homologies to E-V10/T9 within their central part could stimulate CTL that were at least partially cross-reactive with E-V10/T9-HLA-A2. Using as query the core sequence XAGIGILUX, where X is any amino acid and U any of the three amino acids A, T, or I, we identified several proteins of microorganisms containing that particular sequence (F. Lévy, unpublished results). Most interestingly, the peptide sequence LAGIGILIV was identified in the sequence of a sulfate transporter of Pseudomonas aeruginosa (Swissprot: Q9I729). This particular peptide sequence was not identified in previous reports, but because of its recent addition to the public databases and in part because of the methods used to identify such peptides. Most interestingly, preliminary results indicate that human anti-Melan-A CTL clones cross-react with this peptide (L. Derré and D. E. Speiser, unpublished results). Together, our results exemplify how orthologous peptide immunizations could impact on the specific anti-Melan-A T cell repertoires and activities.

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Disclosures
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References


