The human tyrosinase gene codes for two distinct antigens that are recognized by HLA-A*0201-restricted CTLs. For one of them, tyrosinase peptide 368–376, the sequence identified by mass spectrometry in melanoma cell eluates differs from the gene-encoded sequence as a result of posttranslational modification of amino acid residue 370 (asparagine to aspartic acid). Here, we used fluorescent tetramer complexes (“tetramers”) of HLA-A*0201 and tyrosinase peptide 368–376 (YMDDGITMSQV) to characterize the CD8+ T-cell response to this antigen in lymphoid cell populations from HLA-A2 melanoma patients. Taking advantage of the presence of significant numbers of tetramer-positive CD8+ T cells in tumor-infiltrated lymph node cells from a melanoma patient, we derived polyclonal and monoclonal tyrosinase peptide 368–376-specific CTLs by tetramer-guided flow cytometric sorting. These CTLs efficiently and specifically lysed HLA-A*0201- and tyrosinase-positive melanoma cells. As assessed with tyrosinase peptide variants, the fine antigen specificity of the CTLs was quite diverse at the clonal level. Flow cytometric analysis of PBMCs stained with A2/tyrosinase tetramers showed that tyrosinase peptide 368–376-specific CD8+ T cells were hardly detectable in peripheral blood of melanoma patients. However, significant numbers of such cells were detected after short-term stimulation of CD8+ lymphocytes with tyrosinase peptide 368–376 in 6 of 10 HLA-A2 melanoma patients. Taken together, these findings emphasize the significant contribution of the naturally occurring tyrosinase peptide 368–376 to the antigenic specificities recognized by the tumor-reactive CTLs that may develop in HLA-A2 melanoma patients.

INTRODUCTION

Tyrosinase (monophenol, L-dopa: oxygen oxydoreductase; EC 1.14.18.1) is a single-chain glycoprotein enzyme, specifically expressed in melanocytes, which catalyzes the synthesis of the melanin precursor dihydroxypyphenylanine and is essential for pigment formation in mammals. In addition to melanocytes, tyrosinase is expressed in nearly all fresh melanoma tumor samples as well as in 70% of melanoma cell lines (1). The tyrosinase gene encodes antigens recognized on melanoma cells by autologous CTLs. Tyrosinase-derived peptides that are recognized by T cells in association either with HLA-A2 (2), HLA-A24 (3), HLA-B44 (4), HLA-A1 (5), and HLA-DR4 (6) or with HLA-DR15 (7) have been identified.

The naturally occurring HLA-A*0201-associated peptide corresponding to tyrosinase residues 368–376 was recently identified by mass spectrometry in eluates of melanoma cells (8). The sequence of the eluted peptide differed from the gene-deduced sequence at residue 370 (aspartic acid instead of asparagine) as the result of a posttranslational modification. Importantly, this difference was found to significantly affect T-cell recognition (8). However, functional analysis of recognition of tyrosinase peptide 368–376 by specific CTLs has, thus far, mostly focused on the gene-deduced sequence (9, 10). We recently produced fluorescent HLA-A*0201 peptide tetramers (11) using either the natural tyrosinase peptide 368–376 YMDDGITMSQV (A2/tyrosinase tetramers thereafter) or a modified Melan-A peptide 26–35 (12). Staining of short-term cultured TILNs3 from melanoma patients revealed the presence of relatively high numbers of A2/Melan-A tetramer-positive CD8+ T lymphocytes. A2/tyrosinase tetramer-positive CD8+ T lymphocytes were also detected, albeit in smaller numbers (11). To gain more insight on the recognition of the natural tyrosinase peptide 368–376 by HLA-A2-restricted specific CTLs, we used A2/tyrosinase tetramers to isolate and functionally characterize polyclonal and monoclonal specific CD8+ T-cell populations from TILNs of a melanoma patient. In addition, A2/tyrosinase tetramers were used to detect tyrosinase peptide 368–376-specific CD8+ T cells in PBMCs of 10 HLA-A*0201 melanoma patients. Although we found that the frequencies of such cells were close to or below the detection limit of the technique, the presence of a significant number of tetramer-positive CD8+ T cells was documented in 6 of these 10 patients after short-term culture with the natural tyrosinase peptide.

MATERIALS AND METHODS

Synthetic Peptides. Peptides were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer (Applied Biosystems, Foster City, CA), using a N-(9-fluorenyl)methoxycarbonyl group for transient NH2-terminal protection, and analyzed by mass spectrometry. All peptides were >90% pure, as indicated by analytical high-performance liquid chromatography. Lyophilized peptides were diluted in DMSO and stored at −20°C.

Cytokines. Human recombinant IL-2 (Glaxo, Geneva, Switzerland) was kindly provided by Dr. M. Naholz (Institut Suisse de Recherche Experimen- tale sur le Cancer, Epalinges, Switzerland), and human IL-7 was donated by Dr. N. Vita of Sanofi Recherche (Labège, France). One unit/ml of IL-2 was defined as the concentration that gives 50% maximal proliferation of CTLL-2 cells.

Cell Lines and Clones. N8-MEL tyr+, derived from transfection of the tyrosinase-negative cell line N8-MEL with the tyrosinase cDNA 123. B2 (1), was kindly provided by Dr. P. Coulie (Ludwig Institute for Cancer Research, Brussels Branch). Tumor cell lines and EBV-transformed B-cell lines were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS, 0.55 mM arginine, 0.24 mM asparagine, and 1.5 mM glutamine. The melanoma cell line Me 290 (HLA-A*0201- and Melan-A-positive) was established at the Ludwig Institute for Cancer Research, Lausanne Branch, from a surgically excised melanoma metastasis from patient LAU 203. HLA-A2 and Melan-A expression were assessed by flow cytometry analysis and Western blot analysis. Peptide-specific CTLs were generated as described previously (13), with minor modifications. Briefly, CD8+ lymphocytes were positively selected by magnetic cell sorting from PBMCs of HLA-A*0201-positive melanoma patients using a miniMACS device (Milten- yyi Biotec, Sunnyvale, CA). Purified CD8+ T cells were plated at 0.5 × 10^6.

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3 The abbreviations used are: TILN, tumor-infiltrated lymph node cell; IL, interleukin; PHA, phytohemagglutinin; mAB, monoclonal antibody; βm, β2-microglobulin.
to \(1 \times 10^6\) cells/well in 2 ml of Iscove’s medium supplemented with 10% human serum, asparagine, arginine, and glutamine (complete medium). Cells were initially stimulated by adding 100 \(\mu\)M peptide directly into the culture medium. Thereafter, cultures were stimulated weekly with autologous PMBCs (3 \(\times\) 10^6 cells/well) pulsed during 2 h at 37°C in serum-free medium (X-VIVO 10; BioWhittaker, Walkersville, MD) with the appropriate peptide (100 \(\mu\)M) and human \(\beta_2\)m (3 \(\mu\)g/ml; Sigma, Buchs, Switzerland). Peptide-pulsed PMBCs were then extensively washed, irradiated (3000 rad), and adjusted to the appropriate volume before addition to the responder cell population. IL-2 (100 units/ml) and IL-7 (10 ng/ml) were added during the first stimulation cycle, and thereafter, IL-2 alone (100 units/ml) was added. Melan-A-specific CTL clone 2.34 was derived from TILNs of melanoma patient LAU 203 from limiting dilution cultures in the presence of irradiated allogeneic PMBCs, EBV-transformed B lymphocytes, PHA, and recombinant IL-2 (14). Clones were derived from wells having a probability of clonality of >90%, according to single-hit Poisson distribution. A polyclonal tyrosinase monospecific CD8^+ T-cell population was obtained from TILNs of melanoma patient LAU 132 by flow cytometry sorting of CD8^+. HLA-A2/tyrosinase tetramer-positive lymphocytes was used to set up limiting dilution cultures, as described. Clones 1A12/1, 1B3/1, 1E12/0.3, 1F11/1, 1G4/1, and 1D5/1 were derived from these cultures. The tyrosinase-specific CTL clone LAU 132/2 was similarly derived, with minor modifications, from TILNs of patient LAU 132 after in vitro restimulation with the natural tyrosinase peptide 368–376.

**HLA-A*0201 Binding Assay.** The peptide binding capacity to HLA-A*0201 was assessed in a functional competition assay based on inhibition of recognition of the antigenic peptide Melan-A 27–35 (AAGIGILTV) by the HLA-A*0201-restricted CTL clone 2.34 (14). Briefly, T2 cells were 51Cr-labeled in presence of anti-MHC class I mAb W6/32. Various concentrations of competitor peptides (50 \(\mu\)M) were incubated with 51Cr-labeled T2 cells (50 \(\mu\)l; 1000 cells/well) for 15 min at room temperature. A suboptimal dose (125 \(nm\)) of the antigenic peptide (50 \(\mu\)M) was then added together with specific CTLs (5000 cells/well; 50 \(\mu\)l). Chromium release was measured after a 4-h incubation at 37°C. The concentration of each competitor peptide required to achieve 50% inhibition of target cell lysis was then determined and indicated as [nm] 50%. To facilitate comparison, we calculated the relative competitor activity of each peptide as the [nm] 50% of the competitor peptide.

**Assessment of the Stability of Peptide/HLA-A*0201 Complexes.** The stability of peptide-HLA-A*0201 complexes was assayed as described previously (12). Briefly, T2 cells were loaded with saturating concentrations (10 \(\mu\)M) of the different peptides by overnight incubation at room temperature in presence of \(\beta_2\)m (3 \(\mu\)g/ml) in serum-free medium (X-VIVO 10; BioWhittaker). After peptide removal and addition of emetine (10 \(\mu\)M; Sigma) to block protein synthesis, cells were incubated at 37°C for the indicated time periods. For each time point, an aliquot of cells was stained with mAb BB7.2 (HLA-A2 specific) to measure HLA-A2 antigen expression. Influenza matrix 58–66 peptide, which has been shown to bind to HLA-A2/tyrosinase tetramer-positive lymphocytes, was used as an internal standard. Results are expressed as relative complex stability = 100 \(\times\) [(mean fluorescence with test peptide – background mean fluorescence)/(mean fluorescence with influenza A matrix peptide – background mean fluorescence)], where background mean fluorescence represents the fluorescence value obtained on a sample of T2 cells treated in similar conditions except for the absence of exogenous peptide.

**Tetramers.** Complexes were synthesized as described (11, 16). Briefly, purified HLA heavy chain and \(\beta_2\)m were synthesized by means of a prokaryotic expression system (pET; R&D Systems, Inc., Minneapolis, MN). The heavy chain was modified by deletion of the transmembrane-cytosolic tail and COOH-terminal addition of a sequence containing the BirA enzymatic biotinylation site. Heavy chain, \(\beta_2\)m, and peptide were refolded by dialysis. The Mv, 45,000 refolded product was isolated by fast protein liquid chromatography and then biotinylated by BirA (AviDye, Denver, CO) in the presence of biotin, ATP, and Mg^2+ (all from Sigma Chemical Co., St. Louis, MO). Streptavidin-phycoerythrin conjugate (Sigma) was added in 1:4 molar ratio, and the tetramer-tetrameric product was concentrated to 1 mg/ml.

**mAbs and Flow Cytometry ImmunoFluorescence Assay.** Cells were stained with tetramers in 20 \(\mu\)l of PBS-2% BSA during 20 min at 4°C; 50 \(\mu\)l of anti-CD8^+ mAb (Becton Dickinson, San Jose, CA) were then added, and the mixture was incubated for an additional 20 min. Cells were washed once in the same buffer and analyzed by flow cytometry. Data analysis was performed using Cell Quest software. When indicated, CD8^+ lymphocytes were enriched from peripheral blood mononuclear cells by positive selection magnetic cell sorting using a miniMACS device (Miltenyi Biotec). The resulting cells, which were >98% CD3^+ CD8^+, were stained for flow cytometry analysis, as described above.

**Chromium Release Assay.** Antigen recognition was assessed using target cells (T2 or melanoma) labeled with 51Cr for 1 h at 37°C and washed twice. Labeled target cells (1000 cells in 50 \(\mu\)l) were then added to varying numbers of effector cells (50 \(\mu\)l) in V-bottomed microwells in presence or absence of 1 \(\mu\)g/ml of the antigenic peptide (50 \(\mu\)l). The effector cells were preincubated for at least 20 min at 37°C in the presence of unlabeled K562 cells (50,000 cells/well) to eliminate nonspecific lysis due to natural killer-like effectors present in stimulated T-cell populations. In the peptide titration experiments, target cells (1000 cells in 50 \(\mu\)l) were incubated in presence of various concentrations of peptide (50 \(\mu\)M) for 15 min at room temperature before the addition of effector cells. Chromium release was measured after incubation for 4 h at 37°C. The percentage specific lysis was calculated as: 100 \(\times\) [(experimental – spontaneous release)/(total – spontaneous release)]. The concentration of each peptide required to achieve 50% maximal lysis of target cells was then determined and indicated as [nm] 50%. To facilitate comparison, the relative activity of each peptide was calculated as the [nm] 50% of the parental nonapeptide Melan-A AAGIGILTV divided by the [nm] 50% of the tested peptide.

**RESULTS**

**Binding of Single Alanine-substituted Tyrosinase Peptide Variants and Assessment of the Natural Tyrosinase Peptide 368–376**

**Dissociation Rate from HLA-A*0201.** Previous reports on the binding properties of tyrosinase peptide 368–376 have shown that both peptide YMNGTMSQV and the natural sequence YMNGTMSQV bind to HLA-A*0201 with similar affinity (8). To determine the contribution of single-amino acid side chains to the interaction between tyrosinase peptide 368–376 YMNGTMSQV and the HLA-A*0201 molecule, single alanine-substituted variants of this peptide were synthesized and tested for binding to HLA-A*0201 in a functional peptide competition assay. We found that the natural tyrosinase peptide 368–376 displays an intermediate competitor affinity (Table 1; 50% inhibition of maximal lysis required a peptide concentration of ~100 nm, compared to the 6 nm required for the high-affinity binder influenza A matrix peptide 58–66). Surprisingly, substitution of wild-type residues with alanine (including putative anchor residues at positions 2 and 9) only marginally altered binding affinity of the variant peptides to HLA-A*0201 (Table 1). The stability of the

**Table 1** Binding of single alanine-substituted tyrosinase 368–376 peptide variants to HLA-A*0201

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence activity [nm] 50%</th>
<th>Relative competitor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosinase 368–376 analogues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YMNGTMSQV</td>
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<td>1</td>
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<table>
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<th>Influenza A matrix 58–66</th>
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<tbody>
<tr>
<td>GILGFVFTL</td>
<td>6</td>
<td>17</td>
</tr>
</tbody>
</table>

* Alanine substitutions are underlined.

* Competition activity was measured on the basis of the inhibition of recognition of the Melan-A 27–35 antigenic peptide by the control HLA-A*0201 by the peptide-specific CTL clone 2.34.

* The relative competitor activity was calculated as described in “Materials and Methods” using the tyrosinase 368–376 as the reference peptide with an arbitrary competitor activity of 1.

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complexes formed between HLA-A*0201 and tyrosinase peptide 368–376 was found on T2 cells, as described previously (12). Values were normalized relative to that observed for influenza A matrix 58–66 peptide-HLA-A*0201 complexes. As shown previously, HLA-A*0201 complexes formed with peptide Melan-A 26–35 (included as an internal control) were unstable, i.e., they completely dissociated in <1 h of incubation at 37°C. In contrast, complexes formed with the tyrosinase peptide 368–376 were relatively stable over a 6-h period (Fig. 1).

Direct Visualization and A2/Tyrosinase Tetramer-guided Cell Sorting of Tyrosinase 368–376-specific CD8+ T Lymphocytes from TILNs of a Melanoma Patient. We have recently shown that significant numbers of A2/tyrosinase tetramer-positive CD8+ T lymphocytes can be detected in short-term cultured TILNs from HLA-A*0201 melanoma patients (11). The highest proportion of HLA-A2/tyrosinase tetramer-positive lymphocytes was found in TILNs from patient LAU 132, in which up to 1% of the CD8+ T cells were tetramer positive (Fig. 2A). To assess the antigen and functional specificity of the tetramer-positive lymphocytes, we sorted the TILN CD8+ T lymphocytes according to tetramer staining into tetramer-positive and -negative fractions. Each fraction was expanded over 2 weeks by mitogen-driven stimulation and then tested for its ability to lyse target cells pulsed with the appropriate peptide antigen. As shown in Fig. 2B, the CD8+ tetramer-positive population but not the CD8+ tetramer-negative population specifically lysed target cells pulsed with the natural tyrosinase peptide 368–376.

Lytic Activity, Relative Avidity, and Fine Specificity of Polyclonal CTLs and CTL Clones Derived from A2/Tyrosinase Tetramer-sorted CD8+ T Cells. To obtain monoclonal tyrosinase peptide 368–376-specific T-cell populations, we cloned sorted HLA-A2/tyrosinase tetramer-positive lymphocytes by limiting dilution with PHA and allogeneic feeder cells, as described previously (14). We obtained six specific CTL clones from this cloning experiment. One additional clone was generated from unfractionated TILN of patient LAU 132 after in vitro restimulation with tyrosinase peptide 368–376 in an independent cloning experiment. Polyclonal monospecific and monoclonal T-cell populations were further analyzed for tumor antigen recognition, avidity, and fine specificity. All of the clones analyzed as well as the tetramer-positive polyclonal monospecific population specifically lysed the HLA-A*0201-positive tyrosinase-negative melanoma cell line NA8-MEL only in the presence of tyrosinase peptide 368–376 YMDGTMSQV. However, the melanoma cell line NA8-MEL tyr+, obtained from NA8-MEL after transfection of the tyrosinase cDNA, as well as the HLA-A*0201-positive tyrosinase+ melanoma cell line Me 290 were lysed equally well in the presence and in the absence of exogenously added peptide (Fig. 3). The fine antigenic specificity of polyclonal and monoclonal specific T-cell populations was analyzed by quantitating the relative antigenic activity of the single alanine-substituted tyrosinase 368–376 YM-DGTMSQV peptide variants in a standard CTL assay. Titration curves over a wide range of peptide concentrations (10^-5 to 10^-13 M) were generated for each peptide variant. For a quantitative comparison, the concentration required for 50% maximal activity was deter-

Fig. 1. Assessment of the stability of peptide tyrosinase YM-DGTMSQV-HLA*A0201 complexes. After overnight incubation with saturating amounts of peptide, T2 cells were treated with emetine (to inhibit protein synthesis) and incubated at 37°C. The indicated time points, cells were stained for HLA-A2 antigen expression. The stability of each peptide-HLA-A*0201 complex was calculated as detailed in "Materials and Methods" and normalized relative to that observed for the influenza A matrix 58–66 peptide-HLA-A*0201 complex. ○, tyrosinase 368–376; □, influenza A matrix 58–66 peptide-HLA-A*0201 complex; ■, Melan-A 26–35.

Fig. 2. Direct visualization of tyrosinase specific CTLs by A2/tyrosinase 368–376 tetramers and assessment of their functional specificity. A, TILNs from melanoma patient LAU 132 cultured for 3 weeks with cytokines only were stained with phycoerythrin-labeled A2/tyrosinase 368–376 tetramer together with anti-CD8FITC mAb. B, CD8+ TILNs were sterile-sorted into A2/tyrosinase 368–376 tetramer-positive and -negative populations. After expansion for 2 weeks in the presence of irradiated allogeneic PBMCs and PHA, each cell fraction was tested for their lytic activity against chromium labeled T2 cells in the absence (○) or presence (●) of natural tyrosinase peptide 368–376 (4 μM).
replacement of G371 with A resulted in a drastically reduced peptide.

...positions was highly variable among individual clones. For example, T-cell receptor (17). Apart from the importance of S374 for all CTL between the side chains of residues located at these positions and the cases, a result that is consistent with the lack of direct interaction V376 did not drastically affect antigen recognition in the majority of alanine replacement of the HLA-A*0201 anchor residues M369 and the analysis by flow cytometry. In three patients, no CD8+ A2/tyrosinase tetramer-positive lymphocytes were detected. However, in the other seven patients, frequencies of tetramer-positive cells ranged between ±0.01 and 0.03%. According to our current experience with tetramer staining, we consider that these frequencies are not significantly above our flow cytometer detection limit (0.04%)4. Thus, direct A2/tyrosinase tetramer staining of circulating CD8+ T lymphocytes did not allow us to demonstrate unambiguously the presence of tyrosinase peptide-specific cells in blood of melanoma patients. To further investigate this question, we stimulated CD8+ -enriched T lymphocytes from each patient with the natural tyrosinase peptide in presence of autologous PBMCs as antigen-presenting cells. On day 7 after stimulation, cultures were monitored for the presence of CD8+ A2/tyrosinase tetramer-positive lymphocytes. Fig. 4 illustrates the results obtained for patients LAU 132, LAU 203, and LAU 233 before (Fig. 4A) and after (Fig. 4B) in vitro stimulation. A compilation of the results obtained for the 10 melanoma patients tested is reported in Fig. 4C. Interestingly, A2/tyrosinase tetramer-positive CD8+ T lymphocytes were clearly detectable after short-term peptide stimulation in 6 of the 10 melanoma patients tested. To assess the capacity of tyrosinase peptide 368–376 YMGTMSQV-induced CTLs to specifically lyse tyrosinase-expressing tumor cells, we isolated CD8+ A2/tyrosinase tetramer-positive cells from patient LAU 233 (Fig. 4B) by cell sorting and further expanded the cells in vitro. The cell line obtained was able to specifically lyse not only peptide-pulsed target cells but also tumor cells endogenously expressing tyrosinase, including patient LAU 233 autologous tumor cell line (data not shown).

**DISCUSSION**

In this study, we used A2/tyrosinase tetramers to identify CD8+ T cells directed against the natural tyrosinase peptide 368–376 in lymphoid cell populations obtained from melanoma patients. As assessed by multiparametric flow cytometry, such cells were readily detected in cultured metastatic lymph node cell suspensions as well as in cultured circulating CD8+ T cells from a significant proportion of melanoma patients. Moreover, derivation of tyrosinase peptide 368–376-specific CD8+ CTLs clones by tetramer-guided sorting allowed direct demonstration of the specific tumor reactivity of these cells, thus emphasizing the potential role of the tyrosinase peptide in CTL-mediated control of melanoma growth in vivo.

It has been shown previously that the naturally presented tyrosinase peptide 368–376 differs from the tyrosinase gene-deduced sequence as a result of posttranslational modification (8). The difference in


**Fig. 3. Antigen specificity and tumor reactivity of polyclonal monospecific and monoclonal tyrosinase-specific CTLs.** Lysis of NA8-MEL, NA8-MEL tyr1*, and M290 by natural tyrosinase peptide 368–376 CTLs from melanoma patient LAU 132 was measured in a 4-h 51Cr release assay in the absence (○, □, and △) or presence (●, ■, and ▲) of exogenously added peptide tyrosinase 368–376 (1 μM). ○, NA8; ●, NA8 plus peptide; □, NA8-MEL tyr1*; ■, NA8-MEL tyr1* plus peptide; △, M290;▲, M290 plus peptide.

**Lymphocyte to target cell ratio**

The relative avidity of CTL clones for the antigen, defined as the concentration of tyrosinase peptide 368–376 required to obtain half maximal lysis at an E:T cell ratio of 10:1 ranged between 4 and 200 nM. Each clone displayed a unique pattern of fine specificity. The alanine replacement of the HLA-A*0201 anchor residues M369 and V376 did not drastically affect antigen recognition in the majority of the cases, a result that is consistent with the lack of direct interaction between the side chains of residues located at these positions and the T-cell receptor (17). Apart from the importance of S374 for all CTL clones, the effect of alanine replacement of residues at the remaining positions was highly variable among individual clones. For example, replacement of G371 with A resulted in a drastically reduced peptide recognition for four of seven CTL clones. In contrast, the remaining three CTL clones as well as the polyclonal monospecific line recognized this peptide variant more efficiently than the parental sequence. These results reveal a wide heterogeneity in epitope recognition by tyrosinase peptide 368–376-specific CTLs derived from a single tumor-infiltrated lymph node.

**A2/Tyrosinase Tetramer-positive CD8+ T Cells in PBMCs of HLA-A*0201 Melanoma Patients.** We recently used A2/Melan-A fluorescent tetramers to measure the frequency of CD8+ T lymphocytes specific for the immunodominant epitope Melan-A 26 (27–35) in PBMCs from melanoma patients. A2/Melan-A tetramer-positive lymphocytes were detected in all patients tested with frequencies ranging between 0.03 and 0.09% of CD8+ T cells (18). In a similar attempt to determine the frequencies of tyrosinase peptide 368–376-specific circulating T lymphocytes, highly enriched CD8+ T cells from PBMCs of 10 HLA-A*0201 melanoma patients were stained with A2/tyrosinase tetramers and anti-CD8 mAb and analyzed by flow cytometry. In three patients, no CD8+ A2/tyrosinase tetramer-positive lymphocytes were detected. However, in the other seven patients, frequencies of tetramer-positive cells ranged between ±0.01 and 0.03%.
GTMSQV failed to recognize target immunization with peptide YM
stimulated CD8 for 10 HLA-A*0201 melanoma patients for un-
eramer-positive relative to total CD8 bers.

Recognition of the natural tyrosinase peptide YMDGTMSQV by a specific CTL clone derived from an autologous mixed lymphocyte-
tumor cell culture was ~100-fold more efficient than that of peptide YMNGTMSQV (8). In contrast, the two peptides exhibited similar HLA-A*0201-binding activities (8). Although a single CTL clone was tested in this analysis, the data suggested that the substitution of residue 370 could selectively and significantly affect CTL recognition of the tyrosinase peptide 368–376 expressed on the surface of HLA-
A*0201 melanoma cells. As shown here, all of the CTL clones tested, which were derived from precursor cells that were selected according to binding of tetramers containing the natural tyrosinase peptide 368–376, efficiently recognized HLA-A*0201 melanoma cells expressing the tyrosinase gene. In contrast, previous studies showed that most of the CTL clones specific for peptide YMNGTMSQV, which were derived from PBMCs of healthy donors, were unable to kill tyrosinase-expressing melanoma cells (9). Although the ability of tumoricidal and nontumoricidal CTL clones to recognize peptide YMDGTMSQV was not addressed in these studies, the results suggested that only a fraction of YMNGTMSQV-specific CTLs were able to cross-recognize the natural tyrosinase peptide 368–376. The poor cross-reactivity between YMNGTMSQV- and YMDGTMSQV-specific CTLs was recently confirmed in a HLA-A*0201/H2D\(^+\) transgenic mice model (19). Indeed, specific CTL populations derived after immunization with peptide YMNGTMSQV failed to recognize target cells pulsed with peptide YMDGTMSQV and *vice versa*. Furthermore, YMDGTMSQV- but not YMNGTMSQV-specific CTLs were able to lyse HLA-A*0201-positive melanomas expressing tyrosinase.

Each of the clones analyzed in our study displayed a unique pattern of fine specificity, thus suggesting functional heterogeneity in epitope recognition by specific CTLs. In addition, substitution of aspartic acid 370 with alanine resulted in significantly decreased peptide recognition for the majority of specific clones, thus emphasizing the importance of aspartic acid 370 for recognition by specific CTLs. Interestingly, two of the tyrosinase peptide variants resulted in heteroclitic T-cell recognition. However, these effects were limited to single-peptide variant/CTL clone combinations.

By performing a functional competition assay, we found that the natural tyrosinase peptide 368–376 binds to HLA-A2 with intermediate affinity, as compared to the high-affinity binder influenza matrix 58–66 and to the low-affinity binder Melan-A 26–35. More importantly, we observed that, in contrast to Melan-A 26–35 and similarly to influenza matrix 58–66, tyrosinase peptide 368–376 forms stable HLA-A2/peptide complexes. This provides support for this peptide as a candidate for a peptide-based vaccine because a strong correlation has been found between the ability of a peptide to form stable HLA-A2 complexes and its immunogenicity (20).

It is conceivable that self-reactive T lymphocytes that are specific for a peptide that is very efficiently processed and abundantly presented at the cell surface would be relatively rare due to tolerance mechanisms. Indeed, in a recent report, a specific CTL response against the natural tyrosinase peptide 368–376 was detected in only 1 of 26 melanoma patients and 10 normal donors tested (21). However, the analysis of peptide-specific CTL responses has, until recently, been limited by the difficulty of directly isolating T cells of defined antigen specificity, especially when they are present at a very low frequency.

The development of tetrameric complexes of antigenic peptide/ HLA class I molecules that allow direct identification of antigen-specific T cells has opened new possibilities (16). We have recently found that A2/Melan-A tetramer-positive lymphocytes are present at relatively high frequencies (up to 3%) in *ex vivo* tumor-invaded lymph

### Table 2 Recognition of single alanine-substituted tyrosinase 368–376 peptide variants by tyrosinase-specific CTLs\(^a\)

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Relative antigenic activity(^b) of CTL clone:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1A12/1 (70 ns)</td>
</tr>
<tr>
<td>YMDGTMSQV</td>
<td>1</td>
</tr>
<tr>
<td>YMDGTASQV</td>
<td>0.05</td>
</tr>
<tr>
<td>YMNGTMSQV</td>
<td>1</td>
</tr>
<tr>
<td>YMNGTASQV</td>
<td>0.006</td>
</tr>
<tr>
<td>YMDGANSQV</td>
<td>8</td>
</tr>
<tr>
<td>YMDGANSQV</td>
<td>0.08</td>
</tr>
<tr>
<td>YMDGANSQV</td>
<td>&lt;0.0004</td>
</tr>
<tr>
<td>YMDGATMQV</td>
<td>0.004</td>
</tr>
<tr>
<td>YMDGATMQV</td>
<td>0.05</td>
</tr>
<tr>
<td>YMDGMSQV</td>
<td>0.06</td>
</tr>
</tbody>
</table>

\(^a\) Alanine substitutions are underlined.

\(^b\) Numbers in parentheses represent the nanomolar concentration of the parental tyrosinase 368–376 peptide required for 50% maximal lysis by the corresponding CTL clone. These values were used as reference concentrations for calculating the relative potency of all the other peptides analyzed in this table. Relative antigenic activity of single A substituted tyrosinase 368–376 peptide variants as compared to tyrosinase 368–376. The relative antigenic activity for each peptide compared with that of the parental peptide tyrosinase 368–376 was calculated as described in “Materials and Methods.”

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![Fig. 4. Direct visualization of *in vitro* expansion of tyrosinase specific precursors from PBMCs of melanoma patients.](image-url)
nodes and that their percentage increases upon in vitro culture for 2–3 weeks with cytokines (11). In contrast, A2/tyrosinase tetramer-positive lymphocytes were detected at frequencies that were, in most cases, close to or below our estimated detection limit for tetramer staining of TILs (~0.2%; Ref. 11). Here, frequencies of tyrosinase peptide 368–376-specific cells detected by staining with A2/tyrosinase tetramers in circulating CD8+ T lymphocytes of melanoma patients were also close to or below our estimated detection limit in PBMCs (~0.04%). However, upon stimulation with peptide-pulsed autologous antigen-presenting cells, a significant expansion of CD8+1 circulating CD8+ association between the occurrence of vitiligo and the frequency of currently investigating in melanoma patients whether there is any remains to be seen whether such cells would be detectable in short-weeks with cytokines (11). In contrast, A2/tyrosinase tetramer-positive lymphocytes were detected at frequencies that were, in most cases, close to or below our estimated detection limit for tetramer staining of TILs (~0.2%; Ref. 11). Here, frequencies of tyrosinase peptide 368–376-specific cells detected by staining with A2/tyrosinase tetramers in circulating CD8+ T lymphocytes of melanoma patients were also close to or below our estimated detection limit in PBMCs (~0.04%).

However, upon stimulation with peptide-pulsed autologous antigen-presenting cells, a significant expansion of CD8+1 tetramer-positive lymphocytes was observed in 6 of 10 patients. These results suggest that a large fraction of HLA-A*0201 melanoma patients may respond to vaccination protocols aimed at stimulating tyrosinase peptide 368–376-specific CTLs. In addition, polyclonal T-cell populations specific for this peptide could be easily obtained from responder patients by tetramer-guided sorting of in vitro stimulated PBMCs and used for adoptive transfer therapy.

Although here we focused on CD8+ T-cell responses of HLA-A2+ melanoma patients to the natural tyrosinase peptide 368–376, there are other clinical conditions that may also lead to such responses. In particular, there is suggestive evidence that vitiligo, a common progressive depigmentary disease, may involve the destruction of epidermal melanocytes by melanocyte-specific CTLs. Indeed, the presence of relatively high frequencies of circulating A2/Melan-A tetramer-positive CD8+ T cells was recently demonstrated in 7 of 9 vitiligo patients (22). Although no detectable A2/tyrosinase peptide tetramer-positive CD8+ T cells were observed in the same populations, it remains to be seen whether such cells would be detectable in short-term cultures, as described here. Moreover, development of a vitiligo in untreated and treated melanoma patients has been reported to be associated with good prognosis and tumor responses, respectively (23). Using both A2/Melan-A and A2/tyrosinase tetramers, we are currently investigating in melanoma patients whether there is any association between the occurrence of vitiligo and the frequency of circulating CD8+ T cells directed against either of these antigenic peptides.

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Analysis of the Cytolytic T Lymphocyte Response of Melanoma Patients to the Naturally HLA-A*0201-associated Tyrosinase Peptide 368–376

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