Self-Tolerance to the Murine Homologue of a Tyrosinase-derived Melanoma Antigen: Implications for Tumor Immunotherapy

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Abstract

The human tyrosinase-derived peptide **Y**MDGTMSQV is presented on the surface of human histocompatibility leukocyte antigen (HLA)-A*0201$^+$ melanomas and has been suggested to be a tumor antigen despite the fact that tyrosinase is also expressed in melanocytes. To gain information about immunoreactivity and self-tolerance to this antigen, we established a model using the murine tyrosinase-derived homologue of this peptide **F**MDGTMSQV, together with transgenic mice expressing the HLA-A*0201 recombinant molecule AAD. The murine peptide was processed and presented by AAD similarly to its human counterpart. After immunization with recombinant vaccinia virus encoding murine tyrosinase, we detected a robust AAD-restricted cytotoxic T lymphocyte (CTL) response to **F**MDGTMSQV in AAD transgenic mice in which the entire tyrosinase gene had been deleted by a radiation-induced mutation. A residual response was observed in the AAD$^+$tyrosinase$^+$ mice after activation under certain conditions. At least some of these residual CTLs in AAD$^+$tyrosinase$^+$ mice were of high avidity and induced vitiligo upon adoptive transfer into AAD$^+$tyrosinase$^+$ hosts. Collectively, these data suggest that **F**MDGTMSQV is naturally processed and presented in vivo, and that this presentation leads to substantial but incomplete self-tolerance. The relevance of this model to an understanding of the human immune response to tyrosinase is discussed.

Key words: tyrosinase • self-tolerance • MHC class I • cytotoxic T lymphocytes • immunotherapy

Introduction

CTLs derived from patients with melanoma have been shown to recognize Ags expressed on allogeneic melanoma in addition to autologous tumor (1–8). Characterization of these "shared" melanoma Ags has established that they are peptides restricted by class I MHC molecules and are derived from a variety of source proteins. These include developmentally regulated proteins that are silent in most normal tissues, with the exception of their expression in spermatogonia and primary spermatocytes of the testis, but become activated in several types of tumor cells (MAGE [9, 10], BAGE [11], and GAGE [12]). Shared melanoma antigens also include melanocyte differentiation proteins (MDPs)[1] that are normally expressed only in cells of the melanocytic lineage (tyrosinase [13–15], pMEL17/gp100 [2, 3, 16], gp75/tyrosinase-related protein [TRP]-1 [17], MART-1/Melan-A [4], and TRP-2 [18]). CTLs specific for MDP-derived Ags have been found in the metastatic LNs of melanoma patients (19, 20), but the presence of these CTLs sometimes correlates with the loss of MDP expression (21). Although MDPs are commonly expressed in

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[1]*Abbreviations used in this paper:* IC_{50}, half-maximal inhibitory concentration; M1, matrix protein 1; MDP, melanocyte differentiation protein; rvv-hu tyr, full-length human tyrosinase recombinant vaccinia virus; rvv-M1, full-length matrix protein 1 from influenza A/PR/8; rvv-mu tyr, full-length murine tyrosinase recombinant vaccinia virus; TRP, tyrosinase-related protein.

primary tumors, they are absent in ~30% of metastatic melanomas (22, 23). Nevertheless, immunotherapies that target MDP^+ tumors and utilize MDP-derived Ags induce positive clinical responses (24, 25). Taken together, these results suggest that an immune response against MDP-derived Ags may be an important aspect of the patient's ability to control tumor outgrowth.

Several ongoing clinical trials for melanoma immunotherapy are focused on targeting the immune response to MDP-derived Ags (24, 25). Since spontaneous regression of melanomas as well as clinical responses after IL-2 therapy correlate with the destruction of normal melanocytes (vitiligo) (26, 27), it is important to understand which of the MDP-derived Ags are associated with the development of vitiligo (28). Insight is provided by studies demonstrating that CTLs specific for some MDP-derived peptide Ags recognize human melanocyte cell lines cultured in the presence of growth factors (29–32; and our unpublished results), suggesting that these Ags may be targets of the immune response that mediates vitiligo. However, the influence of such growth factors on the profile of Ags presented is not clear. In addition, MHC tetramers have been used to show an accumulation of MDP-specific CTLs in lesions of vitiligo patients (33). Although these studies have provided some information about the Ags that may lead to the development of vitiligo, they have not examined the influence of normal melanocyte expression and/or presentation of MDP-derived Ags on an antitumor immune response.

To address these issues, we wished to evaluate whether MDP-derived Ags are presented by normal melanocytes in vivo and to determine how self-tolerance and autoreactivity to these Ags influences the immune response. We have taken advantage of a recently described albino strain in which the entire coding sequence for tyrosinase has been deleted (34). This deletion precludes processing and presentation of Ags derived from tyrosinase. Because no tyrosinase-derived epitopes have been described that are presented by murine class I MHC molecules, we have also used transgenic mice that express a chimeric human MHC class I transgene encoding the α1 and α2 domains from HLA-A*0201 and the α3, transmembrane, and cytoplasmic domains from D^d (AAD) (35). We have previously identified a peptide (**Y**MDGTMSQV) that is derived from the MDP tyrosinase, presented by HLA-A*0201^+ human melanomas, and recognized by patient CTLs (15). In this study, we have examined whether **F**MDGTMSQV, the murine homologue of **Y**MDGTMSQV, is presented in vivo by murine melanocytes and have evaluated the impact of tyrosinase expression on the development of a **F**MDGTMSQV-specific CTL immune response.

Materials and Methods

Cell Lines and Transfectants. EL4-A2/K^b (gift of Dr. Linda Sherman, The Scripps Research Institute, La Jolla, CA) is a transfectant of the murine thymoma EL4 (H-2^b haplotype) that expresses A2/K^b. The transfectant of the B lymphoblastoid cell line C1R expressing AAD has been described previously (36). AAD is a hybrid MHC class I molecule that contains the α1 and α2 domains from HLA-A*0201 and the α3 domain of the H-$2D^d$ molecule, and has been described previously (37).

Peptides. Synthetic peptides were made by standard Fmoc chemistry using a peptide synthesizer (model AMS422; Gilson Company, Inc.). All peptides were purified to >98% purity by reverse-phase HPLC on a C-8 column (Vydac). Purity and identity were established using an LCQ Finnigan Mat mass spectrometer with electrospray ionization.

Recombinant Vaccinia Viruses. Viruses encoding full-length human tyrosinase (rvv-hu tyr) have been described previously (38). The full-length murine tyrosinase recombinant vaccinia virus (rvv-mu tyr) has also been described (39). Virus encoding the full-length matrix protein 1 (M1) from influenza A/PR/8 (rvv-M1) was a gift from Jack Bennink (National Institutes of Health). Purified vaccinia virus stocks were titered and tested for proper expression using specific murine HLA-A*0201–restricted CTLs.

Class I Peptide Binding Affinity Assays. The relative affinities of peptides for HLA-A*0201 molecules were measured as described (40). In brief, affinity-purified HLA-A*0201 molecules were incubated at room temperature with an iodinated indicator peptide (FLPSDYFPSV) and graded doses of test peptides in PBS, pH 7.0, containing 0.05% NP-40, 1 μM human β2-microglobulin (Calbiochem), 1 mM PMSF, 1.3 mM 1,10-phenanthroline, 73 μM pepstatin A, 8 mM EDTA, and 200 μM N^{α}-*p*-tosyl-l-lysine chloromethyl ketone (TLCK). After 48 h, class I peptide complexes were separated from free peptides by gel filtration, and the dose of individual test peptides that reduced the binding of indicator peptide by 50% (IC_{50}) was calculated.

Mice. C57BL/6 mice transgenic for the AAD gene have been described previously (41). Mice carrying the 38R145L neutron radiation–induced deletion at the tyrosinase (*c*) locus on mouse chromosome 7 ($c^{38R145L}/c^{38R145L}$) have been described previously (34). Transmission of this deletion in heterozygous form was determined by PCR analysis using MapPair primers that define *D7Mit62* and *D7Mit301* (Research Genetics). These markers lie <2 cM from either side of the tyrosinase gene (42).

Generation of CTL Lines. 8-wk-old mice were immunized intraperitoneally with 10^7 PFU of recombinant vaccinia virus expressing murine tyrosinase, human tyrosinase, or influenza M1. After 3 wk, 7.5×10^6 splenocytes from primed mice were cocultured in 24-well plates with 3.5×10^6 irradiated (2,000 rads) autologous splenocytes that had been pulsed with various concentrations of the indicated synthetic peptide antigens. After 6 d of coculture, activity was measured by standard ^{51}Cr-release assay. CTL lines were established from these primary cultures in 12-well plates (Corning Costar) by weekly culture of 5×10^5 CTLs/well with 5×10^6 irradiated (2,000 rads) C57BL/6 AAD^+ peptide-pulsed and washed spleen cells. CTL lines specific for **F**MDGTMSQV are referred to as FMD 10, FMD 1, FMD 0.1, or FMD 0.01 to indicate the concentration of peptide (in μg/ml) used to pulse the stimulators. Murine CTL lines specific for the HLA-A2–restricted peptides **Y**MDGTMSQV (human tyrosinase$_{369–377}$) or GILGFVFTL (influenza $M1_{58–66}$) (43) are referred to as CJL and M1, respectively. All murine CTL lines were grown in RPMI 1640 supplemented with 2 mM L-glutamine, sodium pyruvate, essential and nonessential amino acids, penicillin/streptomycin, 50 μM β-mercaptoethanol, 10% FBS, 15 mM Hepes, and 10 U/ml of IL-2 in a humidified 8% CO_2 atmosphere at 37°C.

In Vitro Cytotoxicity Assay. Standard ^{51}Cr-release assays were performed to determine CTL recognition of murine and human

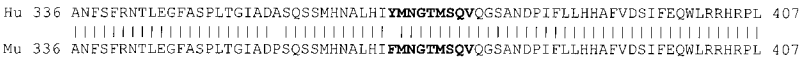

Figure 1. Sequence homology of murine and human tyrosinase in the region surrounding residues 369–377.

tyrosinase$_{369–377}$ peptides. For peptide dose–response assays, ^{51}Cr-labeled EL4-A2/K^b cells were incubated with the indicated concentrations of synthetic peptides for 3 h at room temperature. Con A blasts were generated by incubating 5×10^6 spleen cells/ml in 10 ml of RPMI medium containing 2 μg/ml of Con A for 72 h in a 25-cm^2 upright tissue culture flask in a humidified 5% CO_2 atmosphere at 37°C. For vaccinia-infected target cells, 10 PFU/cell were added to 10^6 target cells in 1 ml HBSS supplemented with 0.1% BSA, 1.6 mM $MgSO_4$, and 1.8 mM $CaCl_2$ for 1 h and then 4 ml of RPMI 1640 supplemented with 10% FBS was added for 8 h.

Enrichment of CD8$^+$ T Cells. CD8$^+$ T cells from spleens of immunized mice were isolated from a StemSep column after incubation with a cocktail of antibodies to enrich for CD8$^+$ cells (StemCell). Preparations were consistently 85–95% CD8$^+$ as assessed by flow cytometry conducted on a FACScan™ using CELLQuest™ software (Becton Dickinson).

Analysis of Intracellular IFN-γ Production. Peptide-pulsed stimulator cells were incubated with CD8$^+$ T cells for 5 h at a ratio of 1:1 in media supplemented with 50 U/ml IL-2 and 10 μg/ml brefeldin A (Sigma Chemical Co.). Stimulated cells were stained with PE-conjugated anti-CD8 (PharMingen), washed, fixed and permeabilized in Cytofix/Cytoperm (PharMingen), further stained with FITC-conjugated anti–IFN-γ (PharMingen) or isotype-matched controls, and analyzed by flow cytometry. Results are presented as percent positive cells after subtraction of isotype control values.

Adoptive Cell Transfer. 2 d after the last in vitro stimulation, 10^7 CJL, FMD 1, or M1 CTLs were subcutaneously transferred into irradiated (700 rads) AAD$^+$ or AAD$^-$ C57BL/6 mice with or without 5,000 Cetus U of Proleukin (IL-2; Chiron). Mice that received the initial dose of IL-2 received additional IL-2 (5,000 Cetus U) intraperitoneally each day for 4 d after CTL transfer. Mice were examined each week for 4 wk for evidence of coat color changes.

Results

*Murine Tyrosinase$_{369–377}$ Is Homologous to Human Tyrosinase$_{369–377}$ and Binds to HLA-A*0201.* The amino acid sequences of murine and human tyrosinase proteins are 82% homologous, and almost identical in the region surrounding residues 369–377,[2] from which the HLA-A*0201–restricted melanoma Ag **Y**MDGTMSQV is derived. With the exception of position 369 of this sequence, in which Y is substituted by an F residue, the 11 amino acids upstream and 30 amino acids downstream of the human epitope are identical to those in the murine protein (Fig. 1). As the human epitope undergoes a posttranslational modification in which N_{371} is converted to a D (15, 38), it seemed likely that the homologous murine peptide would also undergo this modification and would be presented by HLA-A*0201 similarly to the human tyrosinase Ag. When the relative affinity of the murine tyrosinase$_{369–377}$ peptide containing a D at position 371 (**F**MDGTMSQV) for purified HLA-A*0201 was measured in a competitive binding assay (15), the concentration that inhibited the binding of the iodinated standard peptide to purified HLA-A*0201 by 50% (IC_{50}) was nearly identical to the IC_{50} value of the human tyrosinase$_{369–377}$ peptide (**Y**MDGTMSQV) (Table I). Thus, this murine homologue of the human tyrosinase-derived melanoma Ag could potentially be presented by HLA-A*0201$^+$ cells and recognized by CTLs.

*HLA-A*0201–restricted CTLs Specific for Human Tyrosinase$_{369–377}$ Cross-react on the Homologous Murine Peptide.* To provide direct evidence for the immunological recognition of **F**MDGTMSQV, we used CTLs that had been elicited against **Y**MDGTMSQV (38). We found that several such CTL lines recognized EL4-A2/K^b target cells that had been pulsed with similar concentrations of either the human or murine peptides (Fig. 2 A, and data not shown). Similarly, we elicited CTLs from AAD$^+$ mice against **F**MDGTMSQV (FMD 1) and found that they had a similar affinity for both peptides, as determined from the concentration required to give half-maximal lysis (Fig. 2 B). Collectively, these results confirm that the murine and human tyrosinase-derived peptides bind to HLA-A*0201 and are sufficiently homologous to be recognized by the same CTLs.

Murine Tyrosinase$_{369–377}$ Is Naturally Processed and Presented. The preceding results demonstrated that a synthetic peptide corresponding to the sequence derived from murine tyrosinase$_{369–377}$ could be presented by HLA-A*0201 when added to cells. To determine whether this peptide could be naturally processed and presented, we infected cells with rvv-mu tyr. For this purpose we used C1R-AAD, a tyrosinase$^-$ B lymphoblastoid cell line that had been transfected with AAD. When infected with either the rvv-mu tyr or rvv-hu tyr, these cells were lysed by FMD 1 CTLs, which recognize murine tyrosinase$_{369–377}$ (Fig. 2 C). These results confirmed that murine

Table I. *Binding Affinities of Human and Murine Tyrosine-derived Peptides for HLA-A*0201*

Origin	Sequence	IC_{50}
		nM
Human	YMDGTMSQV	74
Murine	FMDGTMSQV	65

IC_{50} values were determined by measuring the binding of the indicated synthetic peptides to purified HLA-A*0201, as described in Materials and Methods.

[2]The sequence **Y**MNGTMSQV from the tyrosinase gene was initially identified as residues 368–376 (13), and this laboratory had previously used that numbering system (38). The correct numbering for the **Y**MNGTMSQV peptide is 369–377.

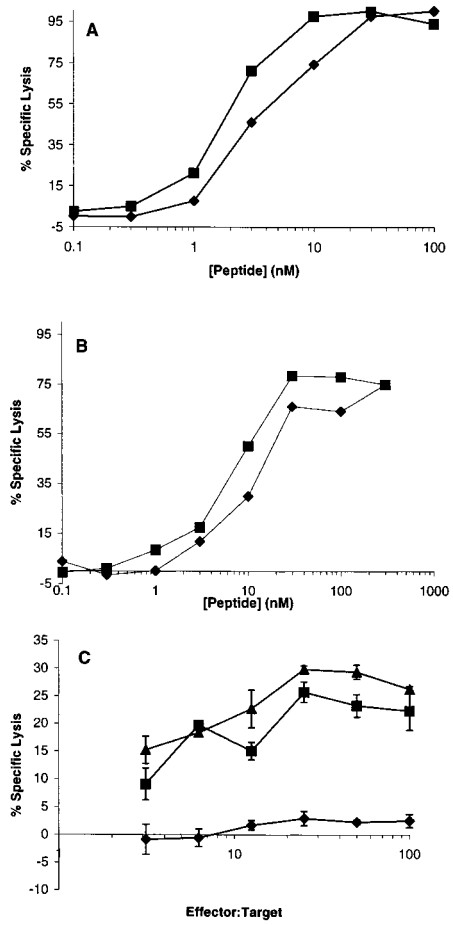

Figure 2. Murine tyrosinase$_{369–377}$ is naturally processed and presented in vivo. (A) ^{51}Cr-labeled EL4-AAK cells that had been pulsed with the indicated concentration of either murine (◆) or human (■) tyrosinase peptides were incubated with CJL CTLs stimulated by and reactive with human tyrosinase$_{369–377}$ at an E/T ratio of 20:1. (B) ^{51}Cr-labeled EL4-AAK cells that had been pulsed with the indicated concentration of either murine (◆) or human (■) tyrosinase peptides were incubated with FMD 1 CTLs stimulated by and reactive with murine tyrosinase$_{369–377}$ at an E/T ratio of 20:1. (C) C1R-AAD target cells were infected for 8 h with 10 PFU/cell of either rvv-mu tyr (■), rvv-hu tyr (▲), or rvv-M1 (◆). Cells were then labeled with ^{51}Cr and incubated with a murine tyrosinase$_{369–377}$–reactive CTL line.

tyrosinase$_{369–377}$ can be naturally processed and presented in association with HLA-A*0201 molecules.

T Cell Responses to Murine Tyrosinase$_{369–377}$ in Tyrosinase$^+$ and Tyrosinase$^-$ Mice. To test the impact of tyrosinase expression on CTL response to murine tyrosinase$_{369–377}$, we compared AAD$^+$ mice (which are of C57BL/6 origin and tyrosinase$^+$) with 38R145L mice, from which the tyrosinase coding sequence at the *c* locus has been deleted (34). The latter were crossed to AAD$^+$ mice, and progeny positive for both markers were intercrossed to produce AAD$^+$ mice that were c^+/c^+, $c^+/c^{38R145L}$, or $c^{38R145L}/c^{38R145L}$. While the first two genotypes were normally pigmented, animals with the third genotype were albino. After immunization of each type of animal with rvv-mu tyr, spleen cells were cultured with autologous splenocytes pulsed with **F**MDGTMSQV, and the CTL response was measured 6 d later. CTLs from immunized AAD$^+c^{38R145L}/c^{38R145L}$ mice (hereafter referred to as AAD$^+$ albino) recognized **F**MDGTMSQV peptide–pulsed EL4-A2/K^b targets (Fig. 3 A). In contrast, T cells from immunized AAD$^+$ mice that were either $c^+/c^{38R145L}$ or c^+/c^+ (hereafter referred to as AAD$^+$tyrosinase$^+$) exhibited no significant response to targets presenting **F**MDGTMSQV (Fig. 3, B and C). These results indicate that expression of tyrosinase is associated with a dramatic reduction in the response to this tyrosinase-derived peptide.

Several additional experiments were done to confirm that the CTL response observed in AAD$^+$ albino mice was dependent on the AAD molecule. First, albino mice that did not express AAD also failed to mount a specific CTL response after immunization with rvv-mu tyr and in vitro restimulation with **F**MDGTMSQV peptide (Fig. 3 D). Second, although **F**MDGTMSQV-specific CTLs from AAD$^+$ albino mice recognized peptide-pulsed EL4-A2/K^b transfectants, they failed to recognize peptide-pulsed but untransfected EL4 cells (Fig. 3 A). Similarly, these CTLs recognized peptide-pulsed Con A blasts from AAD$^+$, but not AAD$^-$ littermates (Fig. 4). These results establish that the CTL response in rvv-mu tyr–immunized AAD$^+$ albino mice is AAD restricted and confirms that **F**MDGTMSQV cannot be presented by murine restriction elements expressed in the outbred albino strain.

We also examined the impact of tyrosinase expression on the T cell response to **F**MDGTMSQV by the quantitation of IFN-γ production by CD8$^+$ cells from spleens of rvv-mu tyr–immunized mice. As observed for cytotoxic activity, a significant number of CD8$^+$ cells from AAD$^+$ albino mice produced IFN-γ at the peak of the response to rvv-mu tyr, whereas no IFN-γ production was discernible in CD8$^+$ cells from AAD$^+$tyrosinase$^+$ mice (Fig. 5). Based on the observation that the AAD-restricted murine and human tyrosinase epitopes were recognized in a cross-reactive manner by CTLs, we also examined responses in AAD$^+$ albino mice and AAD$^+$tyrosinase$^+$ mice after immunization with rvv-hu tyr. A strong response was observed in AAD$^+$ albino mice that was largely cross-reactive on the murine tyrosinase-derived peptide. In contrast, a much weaker response after immunization with rvv-hu tyr was observed in AAD$^+$tyrosinase$^+$ mice, and no significant cross-reactivity on the murine tyrosinase epitope was detectable. Taken together, we conclude that in vivo presentation of **F**MDGTMSQV by the AAD molecule in AAD$^+$tyrosinase$^+$ mice results in a substantially diminished CTL response to both the murine tyrosinase-derived peptide and its human homologue.

A Residual Tyrosinase-specific Response Exists in Tyrosinase$^+$ Mice. The preceding results demonstrated that there was no detectable **F**MDGTMSQV-specific CTL response from immunized AAD$^+$tyrosinase$^+$ mice either at the peak of the primary response or after secondary in vitro stimulation. These results appeared at odds with our previous demonstration that CTL lines elicited against the human tyrosinase epitope recognize the murine tyrosinase-derived pep-

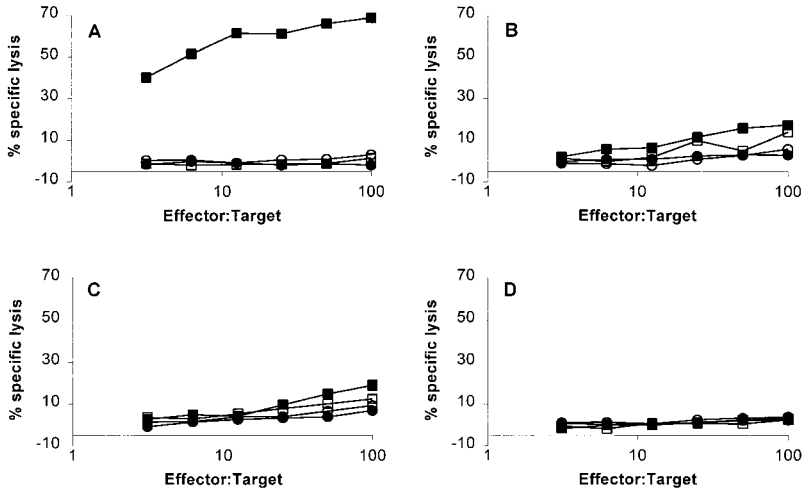

Figure 3. Cytotoxic T cell responses to murine tyrosinase$_{369–377}$ in tyrosinase$^+$ and tyrosinase$^-$ AAD$^+$ mice. Splenocytes from (A) AAD$^+c^{38R145L}/c^{38R145L}$ mice, (B) AAD$^+c^+/c^{38R145L}$ mice, (C) AAD$^+c^+$ mice, or (D) AAD$^-c^{38R145L}/c^{38R145L}$ mice that had been immunized with rvv-mu tyr were cultured for 6 d in the presence of 1 μM **F**MDGTMSQV. CTL activity was measured on ^{51}Cr-labeled EL4-AAK (■, □) or EL4 (●, ○) cells that had been pulsed with 1 μM **F**MDGTMSQV (filled symbols) or left unpulsed (open symbols). Data are representative of six independent experiments.

tide in a cross-reactive manner (Fig. 2 A). To further investigate the immunogenicity of murine tyrosinase in AAD$^+$tyrosinase$^+$ mice, spleen cells from AAD$^+$tyrosinase$^+$ and AAD$^+$ albino mice immunized with either rvv-mu tyr or rvv-hu tyr were cultured with stimulators pulsed with either the murine or human peptides and analyzed for their ability to make IFN-γ in response to either peptide (Fig. 6). As expected, AAD$^+$ albino mice responded well to both rvv-mu tyr and rvv-hu tyr. The T cells elicited were largely cross-reactive, based on their activation and differentiation in response to either peptide during 7 d of in vitro culture, as well as their subsequent ability to be induced to produce IFN-γ in a 5-h incubation. In contrast, AAD$^+$tyrosinase$^+$ mice failed to respond to rvv-mu tyr based on the lack of any detectable response in spleen cells cultured for 7 d with either the murine or human peptides. Consistent with our previous observations (Fig. 5, and reference 44), spleen cells from AAD$^+$tyrosinase$^+$ mice immunized with rvv-hu tyr and cultured in vitro for 7 d with the human peptide led to their activation and differentiation as measured by their ability to produce IFN-γ when induced with the human peptide for 5 h. Significantly, many of these T cells also recognized the murine tyrosinase-derived peptide as judged by their ability to make IFN-γ when induced with the murine peptide for 5 h. However, in parallel cultures of T cells from these same rvv-hu tyr–immunized AAD$^+$tyrosinase$^+$ mice, this murine tyrosinase-derived peptide did not cause activation and differentiation during a 7-d in vitro culture. These data establish that there are residual murine tyrosinase-specific T cells in AAD$^+$ tyrosinase$^+$ mice but that their ability to respond to the murine peptide is impaired.

Induction of Vitiligo following the Adoptive Transfer of FMDGTMSQV-reactive T Cells into Normal Mice. The preceding results indicated that the expression of tyrosinase in AAD$^+$ animals led to the development of partial tolerance towards the **F**MDGTMSQV peptide presented by AAD. However, they did not clearly establish whether this Ag was directly presented by melanocytes. Therefore, we conducted adoptive transfer experiments to determine whether **F**MDGTMSQV-specific CTLs could mediate melanocyte destruction in vivo (vitiligo). An **F**MDGTMSQV-specific CTL line that was derived from AAD$^+$ albino mice (FMD 1) was transferred subcutaneously into ir-

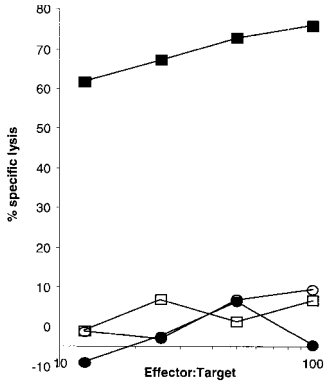

Figure 4. The T cell response to murine tyrosinase$_{369–377}$ in tyrosinase$^-$ mice is AAD restricted. CTLs from AAD$^+$ albino mice that had been generated as in the legend to Fig. 3 A were assayed for activity using Con A blast targets from either AAD$^+$ albino mice (■, □) or AAD$^-$ albino mice (●, ○) that had been either pulsed with **F**MDGTMSQV (filled symbols) or left unpulsed (open symbols).

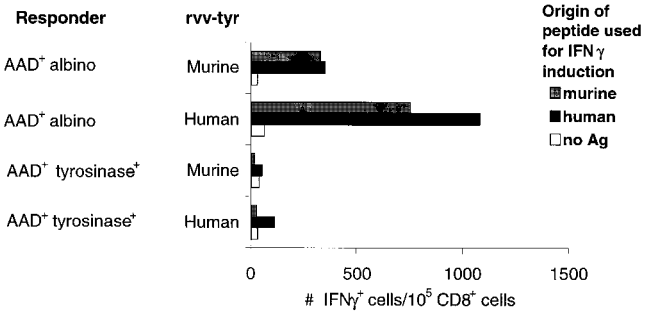

Figure 5. Ex vivo T cell response to murine tyrosinase$_{369–377}$ in AAD$^+$tyrosinase$^+$ and AAD$^+$ albino mice as measured by IFN-γ production. 7 d after intravenous immunization with 10^7 rvv-mu tyr or rvv-hu tyr, CD8$^+$ cells were enriched from splenocytes, stimulated for 5 h with synthetic **F**MDGTMSQV (murine peptide) or **Y**MDGTMSQV (human peptide), and assayed for the intracellular accumulation of IFN-γ as described in Materials and Methods.

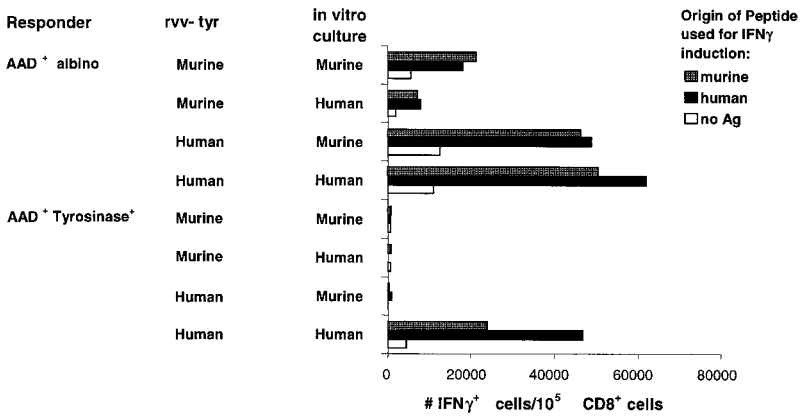

Figure 6. Characteristics of residual tyrosinase-specific response in AAD$^+$tyrosinase$^+$ mice. 3 wk after immunization with either rvv-mu tyr or rvv-hu tyr, splenocytes from AAD$^+$tyrosinase$^+$ mice or AAD$^+$ albino mice were cultured in vitro with an irradiated feeder layer that had been pulsed with 1 μg/ml of either synthetic **F**MDGTMSQV (murine) or **Y**MDGTMSQV (human) peptides. 7 d later, cultures were harvested, stimulated for 5 h with C1R-AAD cells that had been pulsed with synthetic **F**MDGTMSQV or **Y**MDGTMSQV, and assayed for the intracellular accumulation of IFN-γ.

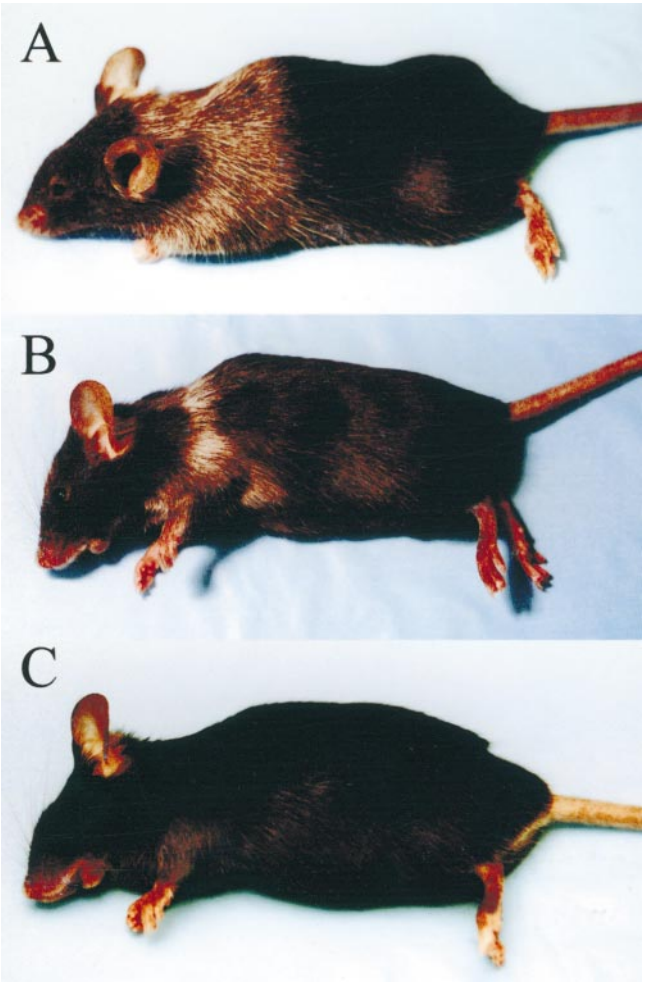

Figure 7. Induction of vitiligo after the adoptive transfer of tyrosinase-specific T cells into AAD$^+$tyrosinase$^+$ mice. FMD 1, a CTL line derived from AAD$^+$ albino mice, was transferred subcutaneously into AAD$^+$ tyrosinase$^+$ mice (A). CJL, a CTL line derived from AAD$^+$tyrosinase$^+$ mice, was transferred subcutaneously into either AAD$^+$tyrosinase$^+$ (B) or AAD$^-$tyrosinase$^+$ (C) mice. Photographs were taken 5 wk after transfer; those shown are representative of multiple mice in each group (for *n* values, see Table II).

radiated AAD$^+$tyrosinase$^+$ recipients together with IL-2. 4 wk after subcutaneous transfer, mice exhibited significant vitiligo as evidenced by depigmentation of the hair in the vicinity of the injection site (Fig. 7 A, and Table II). At 4 wk after treatment, vitiligo was not observed in mice that received IL-2 alone or were irradiated but did not receive either CTLs or IL-2 (Table II). As expected, transfer of the same number of CTLs specific for the HLA-A2–restricted influenza $M1_{58–66}$ epitope in combination with IL-2 failed to cause vitiligo. These results indicate that normal melanocytes in AAD$^+$ tyrosinase$^+$ mice express the tyrosinase Ag in the context of AAD and are targets for CTL-mediated destruction.

We also tested the ability of a tyrosinase-specific CTL line derived from AAD$^+$tyrosinase$^+$ mice to mediate vitiligo. CJL CTL was induced by immunization with rvv hu tyr, but showed equivalent recognition of both human and murine peptides (Fig. 2 A). In addition, the doses of the murine peptide required for half-maximal recognition are comparable to those of intermediate avidity CTLs obtained from albino AAD mice (compare Fig. 2, A and B; see also Table III). Using the same number of transferred CTLs,

Table II. *Induction of Vitiligo in AAD$^+$Tyrosinase$^+$ Mice by Adoptive Transfer of Tyrosinase-specific CTLs*

CTLs	Origin	Specificity	Host	IL-2	Vitiligo	*n*
FMD 1	Albino-AAD	Antityrosinase	AAD	+	+++	6
None	NA	NA	AAD	+	−	15
None	NA	NA	AAD	−	−	6
M1	B6-AAD	Anti-influenza M1	AAD	+	−	12
CJL	B6-AAD	Antityrosinase	AAD	+	+++	15
CJL	B6-AAD	Antityrosinase	B6	+	−	2
CJL	B6-AAD	Antityrosinase	AAD	−	+	6

All hosts were irradiated (700 rads) and received one of the regimens described above (see Materials and Methods). Vitiligo intensity was scored as the relative number of depigmented follicles within the 1-cm^2 area of transfer. Mice were scored 4 wk after transfer as: −, none; +, <30%; +++, >70%. B6, C57BL/6.

Table III. *Avidity of CTL Lines for Murine Tyrosinase Peptide*

CTL line	SD_{50}
	μg/ml
CJL	7.25
FMD 10	30
FMD 1	10
FMD 0.1	8.75
FMD 0.01	2

CTL lines were generated as described (see Materials and Methods). SD_{50} values are the average of three (FMD 10) or four (CJL, FMD 1, FMD 0.1, and FMD 0.01) independent determinations.

vitiligo occurred in these mice with a comparable time course and intensity (Fig. 7 B, and Table II). In contrast, these CTLs did not mediate vitiligo in AAD^{-}tyrosinase^{+} mice, and their induction of vitiligo in the absence of exogenous IL-2 was significantly diminished (Fig. 7 C, and Table II). Interestingly, we have not observed vitiligo in any animals immunized with recombinant vaccinia virus expressing either human or murine tyrosinase ($n > 20$), nor in mice immunized with either **F**MDGTMSQV or **Y**MDGTMSQV peptides pulsed onto dendritic cells ($n = 8$) (data not shown). Collectively, these results demonstrate that at least some of the residual tyrosinase-specific CTLs in AAD^{+}tyrosinase^{+} mice are capable of mediating vitiligo, but only when appropriately activated.

Discussion

In this report, we have established and characterized a murine model system to further our understanding of the human CTL response to the HLA-A*0201–restricted Ag derived from tyrosinase. Subsequent to the discovery that Ags derived from MDPs, such as tyrosinase, constitute a major target for melanoma-reactive CTLs, it was also established that murine MDPs encode peptide Ags that are presented in the context of murine class I molecules (45). While these models offer the possibility to determine the impact of these Ags on both antitumor immune responses and self-tolerance, the advantage of the system described in this report is that it permits the evaluation of an Ag that is highly homologous in structure, behavior, and expression to a human Ag that is currently in clinical trials for the treatment of melanoma.

The homologous murine peptide derived from tyrosinase differs from the human Ag by a single conservative amino acid change at P1. As a consequence, the binding affinities of the two peptides for HLA-A*0201 are nearly identical, and CTLs raised against one Ag frequently cross-react on the other. Most important, murine tyrosinase is endogenously processed, leading to the presentation of the murine peptide in association with HLA-A*0201. These similarities enabled us to use AAD^{+} albino mice to evaluate whether the tyrosinase-derived Ag is expressed on melanocytes in vivo and how it affects the development of an HLA-A*0201–restricted Ag-specific CTL response. This is the first model system established for studying self-tolerance to an Ag expressed on melanocytes. Using this unique model, we determined that the murine homologue of tyrosinase is naturally processed and presented in the context of AAD in tyrosinase^{+} mice in vivo and that this presentation has a tolerizing effect on the immune response.

The expression of tyrosinase protein and/or RNA is confined to neural crest–derived melanocytes (14, 46, 47), substantia nigra and forebrain (48), and chorioid and retinal pigment epithelium (46, 49). Because tyrosinase expression appears limited to a small number of tissues, self-tolerance to the tyrosinase-derived Ag is likely to be mediated by a peripheral rather than a central mechanism. However, one study has demonstrated that tyrosinase transcripts can be detected in a wide range of tissues after 60 cycles of PCR amplification (50), suggesting that very low-level tyrosinase expression is widespread. It was hypothesized that this might be due to the presence of melanocytes in these tissues, resulting from the arrested migration of neural crest cells during development. This observation leaves open the possibility that self-tolerance to tyrosinase might arise through a central mechanism. Studies are currently under way to evaluate the role of the thymus in the mechanism of self-tolerance to tyrosinase and to determine whether tolerance to the tyrosinase-derived Ag can be mediated directly by melanocytes or indirectly through cross-presentation by bone marrow–derived cells (51–53).

Regardless of the exact mechanisms responsible, our data show convincingly that self-tolerance is incomplete. Low levels of cytotoxic and IFN-$\gamma^{+}CD8^{+}$ T cells specific for **F**MDGTMSQV are demonstrable in AAD^{+}tyrosinase^{+} mice after priming and in vitro restimulations, and these T cells induce vitiligo upon adoptive transfer into AAD^{+} tyrosinase^{+} mice. In addition, peptide dose–response curves show that the avidity of these CTLs is comparable to that of high avidity CTLs generated in AAD^{+} albino mice under similar priming conditions. Collectively, these results demonstrate that at least some of the murine tyrosinase-specific T cells in AAD^{+}tyrosinase^{+} mice are of high avidity. This appears to be at odds with previous observations that peripheral expression of influenza HA under the control of the insulin promoter leads to a selective loss of high avidity CTLs (54). However, it is clear that the site of protein expression, level of protein expression, and the accessibility to the immune system contribute to the mechanism of tolerance to self-Ags expressed in the periphery (55, 56). In addition, it is also clear that the detection of murine tyrosinase-specific T cells in AAD^{+}tyrosinase^{+} mice is not straightforward. These cells do not appear to activate and/or differentiate in response to murine tyrosinase in vivo or in vitro. However, this failure can be circumvented by the use of human tyrosinase, and subsequently, the ability of these cells to recognize murine tyrosinase can be demonstrated either by cytolysis or IFN-γ secretion. These observations are reminiscent of the responses of T cells to altered

peptide ligands that function as partial agonists (57–59). It is frequently observed that partial agonists fail to induce proliferation, although they do induce cytolysis and the secretion of at least some cytokines (60, 61). Further work will be required to fully understand the recognition of the murine tyrosinase peptide by these cells.

The results of this study are also particularly interesting in light of recent work on tolerance to a keratinocyte-specific Ag (62). It was demonstrated that Ag-specific CTL precursors that arise during the first 4–6 wk of life are rendered tolerant due to circulation through the epidermis. Although access to the epidermis ceases after this time, subsequently arising T cells are tolerized by an undefined alternate mechanism. The major sites of expression of tyrosinase, as well as other MDP-derived Ags, are in the melanocytes of the skin, or tissues sequestered behind the blood–brain barrier. It will be of interest to determine if access to the epidermis is necessary for tolerance to these Ags, and whether the activation requirements of residual high avidity **F**MDGTMSQV-specific CTLs in tyrosinase$^+$ mice are a reflection of this alternate tolerizing mechanism.

In this study, the development of vitiligo in tyrosinase$^+$ mice after the transfer of **F**MDGTMSQV-reactive CTLs indicates that this murine tyrosinase-derived peptide is expressed in the context of AAD on normal melanocytes. This direct in vivo analysis complements and extends earlier work in which the expression of the homologous human tyrosinase-derived peptide was demonstrated on melanocytes cultured in the presence of growth factors and phorbol esters (32; and our unpublished results). It has previously been demonstrated that vitiligo in mice can be induced as a consequence of antibody responses to TRP-1 (63–65) or CD8$^+$ CTL responses against an unknown antigen expressed on B16 melanoma (66). Interestingly, it has been demonstrated that vitiligo in both models is associated with tumor regression and protective antitumor immune responses (57–59). Because of the similarities between human and mouse tyrosinase, the expression of the tyrosinase-derived peptide in the context of the AAD molecule on murine melanocytes in the present study strongly suggests that the human tyrosinase peptide is also expressed in the context of HLA-A*0201 on normal human melanocytes. This leads us to suggest that immune responses to tyrosinase may also lead to the hypopigmentation that is often observed in association with clinical responses in melanoma patients. In addition, it suggests that the use of this Ag in immunotherapeutic treatment of melanoma may result in vitiligo. Our results offer the possibility of developing a model system in which vitiligo-inducing CTL responses against a known human antigen can be studied for their impact on tumor regression.

The observations made in the murine model system described here allow us to make inferences about the importance of human tyrosinase as a tumor Ag. Our results suggest that the expression of tyrosinase in human melanocytes will lead to a diminished number of activatable CTLs that recognize the peptide **Y**MDGTMSQV in the context of HLA-A*0201. Nonetheless, it remains important to define the conditions for optimal activation and expansion of these cells in vivo to engender an active therapeutic response. The model system described here offers an important and useful approach to this issue.

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