Melanoma-specific CD4+ T Cells Recognize Nonmutated HLA-DR-restricted Tyrosinase Epitopes

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Summary

Tyrosinase was the first melanoma-associated antigen shown to be recognized by CD4+ T cells. In this study, we have identified two HLA-DRB1*0401–restricted peptides recognized by these T cells: Ty 56-70 and Ty 448-462. As with many of the MHC class I–restricted melanoma epitopes, both are nonmutated self peptides that have intermediate and weak MHC binding affinities, respectively. Mutated and truncated versions of these peptides were used to define their MHC binding anchor residues. Anchor residues were then modified to derive peptides with increased MHC binding affinities and T cell stimulatory properties. Ty 56-70 and Ty 448-462 enhance the list of immunogenic HLA-A2-, A24-, and B44-restricted tyrosinase peptides already described. Thus, tyrosinase provides a model for anti-melanoma vaccines in which a single molecule can generate multivalent immunization incorporating both CD4+ and CD8+ T cell responses.

In recent years, a number of human melanoma-associated antigens (Ag) recognized by CD8+ T lymphocytes have been identified, and their peptide epitopes characterized. In some cases, the epitopes were found to represent mutated gene products (1, 2), but most of the epitopes recognized by MHC class I–restricted T cells were nonmutated and derived from melanocyte lineage-specific proteins: MART-1/Melan-A, gp100, gp75, and tyrosinase (3–8). Although it seemed likely that these molecules might also contain epitopes recognized by CD4+ T cells, and CD4+ T cells with specific reactivity against human melanomas have been isolated repeatedly (9, 10), to date only tyrosinase has been identified as a specific target for CD4+ melanoma-reactive T cells (11). The importance of CD4+ T cells in antitumor immunity has been demonstrated in animal models in which these cells not only serve cooperative and effector functions but are also critical in maintaining immune memory (12). We have previously reported HLA-DR-restricted recognition of melanomas, normal melanocytes, and COS-7 transfectants expressing a full-length tyrosinase gene by CD4+ tumor-infiltrating lymphocytes (TIL)[1] from melanoma patient number 1088 (11). We now identify two tyrosinase peptides recognized by these CD4+ TIL and analyze their MHC binding properties and capacity for T cell stimulation.

Materials and Methods

Cell Cultures. CD4+ TIL 1088 were purified from a bulk TIL culture derived from a metastatic melanoma lesion and maintained in the presence of RPMI 1640 with 10% human AB serum and recombinant IL-2 (6,000 IU/ml; Chiron, Emeryville, CA) for up to 70 d as described (9). EBV-transformed B cell cultures and melanoma cultures were established in our laboratory and maintained in RPMI 1640 with 10% FCS (13).

HLA Typing. HLA serotypes and DNA genotypes of PBL and EBV-B cells were determined by the NIH HLA Laboratory, as described (9). The HLA-DR genotype of tyrosinase-specific CD4+ TIL 1088 was found to be HLA-DRB1*0301, DRB1*0401.

Peptide Synthesis. Peptides were synthesized using a solid phase method based on fluorenylmethoxycarbonyl (Fmoc) chemistry on a multiple peptide synthesizer (Model AMS 422; Gilson Co., Inc., Worthington, OH). The molecular masses of peptides were verified by laser desorption mass spectrometry (Bio-Synthesis, Inc., Lewisville, TX). Impure samples were further purified by reverse-phase HPLC. Synthesis of tyrosinase peptides was based on the sequence of a tyrosinase gene cloned from the melanoma cell line 501-mel, which was recognized by CD4+ TIL 1088 when transfected into COS-7 cells. This sequence was identical to a se-

[1] *Abbreviation used in this paper:* TIL, tumor-infiltrating lymphocytes.

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quence for tyrosinase published by Bouchard et al. (14) (GenBank accession number Y00819), with the exception of an I→M substitution at amino acid position 179.

Assessment of T Cell Responses to Peptides and Melanoma Cell Lysates. EBV-B cells were used as APC for peptide and protein Ag (9). EBV-B cells (2×10^5 cells/well in microtiter plates) were incubated overnight at 37°C in the presence of peptides or freeze/thaw lysates of melanoma cells, then T cells were added at $1–2.5 \times 10^5$ cells/well. Assay medium was RPMI 1640 with 10% AB serum and IL-2 120 IU/ml. After 18–24 h, cell-free culture supernatants were harvested and assessed for GM-CSF secretion by $CD4^+$ T cells using a commercially available ELISA kit (R & D Systems, Minneapolis, MN; detectable range 8–500 pg/ml). Cytokine secretion in response to Ag-pulsed B cells was considered significant if it was at least twofold above the background response of T cells to B cells alone.

Peptide Competition Assays. To assess the MHC restriction of the response of $CD4^+$ TIL 1088 to individual tyrosinase peptides, competition assays were performed using the influenza peptide HA 307-319 and the mycobacterial peptide MT_{65} 3-13. These peptides are known to bind with high affinities to HLA-DRB1*0401 and DRB1*0301, respectively (15). EBV-B cells were pulsed with a 10-fold excess of HA 307-319 or MT_{65} 3-13 for 3 h before adding tyrosinase peptides. After an overnight incubation, TIL were added and the cytokine secretion assay completed per routine.

Class II-Peptide Binding Assays. HLA-DRB1*0401 molecules were purified from Priess EBV-B cells using the mAb LB3.1 coupled to Sepharose 4B beads, as described (16). Purified class II molecules were incubated with 5 nM ^{125}I-radiolabeled HA 307-319 combinatorial peptide and various concentrations (1.2 ng/ml to 120 μg/ml) of tyrosinase (inhibitor) peptide for 48 h in the presence of protease inhibitors. Class II–peptide complexes were then separated from free radiolabeled peptide by gel filtration, and the concentration of tyrosinase peptide necessary to inhibit 50% of the binding of radiolabeled peptide to class II molecules was calculated as the IC_{50} (16). Tyrosinase peptides were tested in two to four completely independent experiments. Peptides were classified as high affinity binders (IC_{50} <50 nM), intermediate binders (50–500 nM), weak binders (500–5,000 nM), or non-binders (>5,000 nM).

Results and Discussion

Tyrosinase is a type I transmembrane protein which, including its signal sequence, contains 529 amino acids. We first attempted to characterize its class II–restricted epitopes by using exonuclease III to create nested deletions of the full-length gene from its 3′ terminus. The truncated genes were transiently transfected into COS-7 cells, which were subsequently lysed and pulsed onto EBV-B cells for presentation of Ag to $CD4^+$ T cells (11). Although T cell recognition of tyrosinase was abrogated by removal of the transmembrane region of the molecule, none of the synthesized 15-mer peptides spanning this region were recognized (data not shown). This might have resulted from instability of the truncated protein (17) or an artifact introduced by our COS assay system, in which the soluble form of tyrosinase might not be internalized and processed by EBV-B cells as efficiently as the membrane-bound form. We proceeded to screen the full-length tyrosinase molecule for class II restricted epitopes by synthesizing its component 15-mers overlapping by five amino acids. Peptides were tested at concentrations of 25–50 μM for recognition by $CD4^+$ TIL 1088. Two peptides were identified that repeatedly evoked specific cytokine secretion from $CD4^+$ TIL: Ty 56-70 (QNILLSNAPLGPQFP) and Ty 448-462 (DYSYLQDSDPDSFQD).

Recognition of tyrosinase by $CD4^+$ TIL 1088 is HLA-DR-restricted (11). Patient 1088 is heterozygous for HLA-DRB1, expressing the DRB1*0301 and DRB1*0401 alleles. To determine the restriction element(s) used for recognition of Ty 56-70 and Ty 448-462, the amino acid sequences of these two peptides were compared to published motifs for peptides binding to HLA-DRB1*0301 and DRB1*0401. MHC class II–restricted peptides are similar to class I–restricted peptides in having preferred anchor residues within an MHC binding core of 9–10 amino acids. However, unlike MHC class I–restricted peptides, class II–restricted peptides vary considerably in length and can tolerate extensions at both the amino and carboxy termini (18, 19), making their motifs more difficult to define than class I motifs. Most peptides binding to HLA-DRB1*0301 are characterized by an aspartic acid or glutamic acid at the P4 position in the binding core, and a hydrophobic amino acid at P1. This motif is distinct from that for DRB1*0401 which, in addition to an aromatic or hydrophobic P1 an-

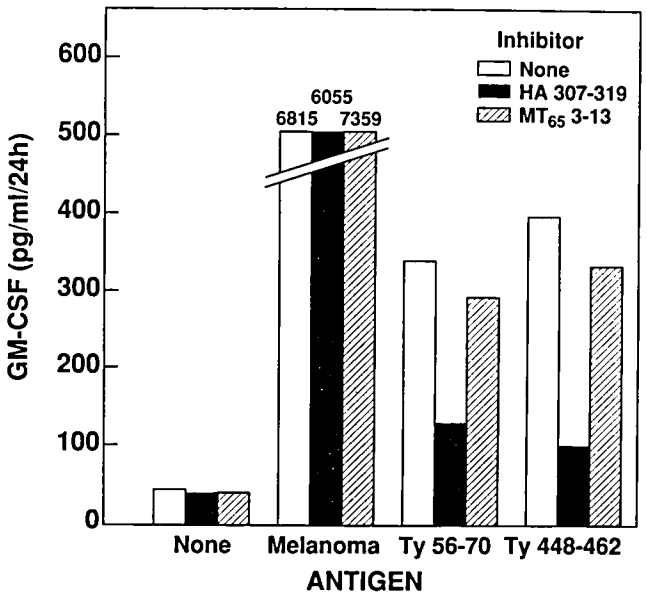

Figure 1. Recognition of Ty 56-70 and Ty 448-462 by $CD4^+$ TIL 1088 is restricted by HLA-DRB1*0401. 1088-EBV expressing both DRB1*0301 and DRB1*0401 (1×10^6 cells/ml) were cultured in the presence of 500 μM of inhibitor peptide, with 50 μM of the indicated tyrosinase peptide or with 1088-mel lysate (1×10^6 cell equivalents/ml). $CD4^+$ TIL reactivity was measured as GM-CSF secretion by 1.25×10^6 cells/ml. T cell recognition of both tyrosinase peptides was inhibited only by HA 307-319, which binds to HLA-DRB1*0401 with high affinity, and not by MT_{65} 3-13, which binds to DRB1*0301. Similar results were obtained using an allogenic HLA-matched EBV-B cell line as the APC (not shown). Neither inhibitor peptide affected TIL recognition of the melanoma cell lysate, which is internalized prior to Ag processing and presentation.

Table 1. *Determining the P1 and P6 Anchor Positions for Ty56-70*

		GM-CSF			
		Exp. 1	Exp. 2	Exp. 3	IC_{50}
			pg/ml/24 h		*nM*
Ty 56-70	Q N I L L S N A P L G P Q F P	295	524	903	225
I58	- - Q - - - - - - - - - - - - -	<8	<8	<8	6767
	- - F - - - - - - - - - - - - -	39	46	ND	55
	- - V - - - - - - - - - - - - -	ND	ND	154	957
L59	- - - Q - - - - - - - - - - - -	<8	<8	<8	750
	- - - F - - - - - - - - - - - -	<8	<8	ND	321
	- - - V - - - - - - - - - - - -	ND	ND	<8	95
L60	- - - - Q - - - - - - - - - - -	<8	62	<8	750
	- - - - F - - - - - - - - - - -	86	<8	ND	1098
	- - - - V - - - - - - - - - - -	ND	ND	10	612
A63	- - - - - - - Q - - - - - - - -	<8	<8	ND	7759
	- - - - - - - V - - - - - - - -	889	2080	ND	12
P64	- - - - - - - - Q - - - - - - -	<8	<8	ND	290
	- - - - - - - - V - - - - - - -	<8	48	ND	158
L65	- - - - - - - - - Q - - - - - -	24	50	ND	2000
	- - - - - - - - - V - - - - - -	2050	3816	ND	1500
Ty 57-70	- - - - - - - - - - - - - - - -	ND	ND	615	324
Ty 58-70	- - - - - - - - - - - - - - - -	ND	<8	<8	>10,000
Ty 59-71	- - - - - - - - - - - - - - ->	ND	<8	<8	>10,000
Ty 60-72	- - - - - - - - - - - - - - ->	ND	<8	<8	>10,000
Ty 61-75	- - - - - - - - - - - - - - ->	ND	<8	ND	>10,000
1088-mel Lysate		6762	>5500	ND	ND

Peptides pulsed onto EBV-B cells were tested at 100 μM for recognition by $CD4^+$T cells. Results of three separate experiments are shown. Background values of GM-CSF secretion by $CD4^+$ TIL 1088 cultured with 1088-EBV in the absence of peptide have been subtracted (53, 296, and 99 pg/ml, respectively, for experiments 1–3).
IC_{50}, concentration of tyrosinase peptide yielding 50% inhibition of binding of standard peptide to DRB1*0401. *ND*, not done.

chor, tolerates hydrophobic residues at P4 and contains a hydrophobic or hydroxyl amino acid at a critical P6 anchor position (15, 20–23). The Ty 56-70 peptide appeared to conform to the DRB1*0401 motif, with I58, L59, or L60 serving as the P1 anchor, whereas Ty 448-462 seemed to conform better to the DRB1*0301 motif, with Y451 as P1 and D454 as P4. However, when EBV-B cells of different HLA types were used as APC for T cell recognition, it seemed that both peptides were presented only in the context of DRB1*0401, and there was no indication of presentation by DRB3 or DRB4 alleles (data not shown). To confirm this, peptides with high binding affinities for either DRB1*0301 (MT_{65} 3-13) or DRB1*0401 (HA 307-319) were used to inhibit the binding of Ty 56-70 or Ty 448-462 to EBV-B cells expressing both HLA molecules. $CD4^+$ T cell responses to both tyrosinase peptides were inhibited when EBV-B cells were preincubated with HA 307-319 but not with MT_{65} 3-13, indicating that both Ty 56-70 and Ty 448-462 are restricted by HLA-DRB1*0401 (Fig. 1).

The amino acid sequences Ty 56-70 and Ty 448-462 are identical to those encoded by a nonmutated tyrosinase gene cloned from normal human melanocytes (24) and do not include any known sites of allelic variation. Titration of the $CD4^+$ T cell response to these peptides revealed that peptide concentrations ≥50 μM were needed to evoke significant reactivity, suggesting that peptide affinities for the MHC molecule and/or TCR might be low. This result is reminiscent of MHC class I–restricted nonmutated melanoma epitopes recognized by $CD8^+$ T cells (5) and may reflect selection of lower affinity epitopes by self tolerance mechanisms. To design peptides with enhanced T cell stimulatory properties, we first set out to identify the primary MHC binding anchors in each peptide. Experiments with Ty 56-70 are shown in Table 1. Based on published data describing the relative binding of HA 307-319 and its substituted analogues to HLA-DRB1*0401, it appeared that I58, L59 or L60, could serve as the P1 anchor residue in Ty 56-70 and thus A63, P64, or L65 could serve as P6 (22). Mutated peptides containing "favorable" (F or V) or "unfavorable" (Q) amino acid substitutions for MHC binding at

these positions were therefore synthesized and assessed for $CD4^+$ T cell recognition, which would simultaneously reflect peptide binding to both the MHC molecule and the TCR. Among the P1 candidates, only I58 could tolerate a F or V substitution but not a Q substitution as measured by T cell recognition. Direct peptide binding studies subsequently demonstrated that the native Ty 56-70 binds to HLA-DRB1*0401 with intermediate affinity (IC_{50} = 225 nM) and that MHC binding is reduced considerably by substituting Q at position I58. The observed high affinity of Ty 56-70 I58→F for DRB1*0401 would be expected if I58 were indeed the P1 anchor. The paradoxically diminished T recognition of this modified peptide might be due to the bulky F residue inducing a conformational change in Ty 56-70, affecting interaction of this peptide with the TCR. Assignment of I58 as the P1 anchor was further supported by data obtained with truncated peptides showing that Ty 58-70 was not recognized by T cells and also failed to bind HLA-DRB1*0401. This was in agreement with the observation that at least one amino acid NH_2-terminal to P1 is preferred for stable binding of peptide to MHC II (23). The combination of high affinity binding and enhanced T cell recognition of the A63→V analogue suggested that A63 was the P6 anchor. While Ty 56-70 L65→V was also a more potent T cell stimulant than the parent peptide, its weak affinity for class II molecules (IC_{50} = 1,500 nM) eliminated it as a potential P6 anchor, suggesting that it enhances TCR engagement through some other mechanism. The data presented in Table 1 also suggest that L59 and P64 are TCR contact residues, since substituted analogues which bound to MHC class II molecules with affinities comparable to the native Ty 56-70 peptide nevertheless failed to evoke cytokine secretion from $CD4^+$ TIL.

The primary binding anchor residues in Ty 448-462 were determined by assigning an aromatic or hydrophobic residue as the critical P1 anchor. Thus, Y449, Y451 and L452 were candidates for P1. Again using mutated and serially truncated peptides, Y451 was identified as the P1 anchor (Table 2). It was also observed that Ty 449-462 and Ty 450-462 seemed to evoke stronger T cell recognition than Ty 448-462, correlating with their relative binding affinities for HLA-DRB1*0401, and this was confirmed in peptide titration experiments (Fig. 2 and data not shown). Although Ty 451-462 was not recognized by T cells, its MHC binding affinity was somewhat higher than that of the native peptide (IC_{50} = 566 and 989 nM, respectively), suggesting that S450 might be a TCR contact residue. L452 is probably a TCR contact as well, since substituted analogues at this position bound MHC molecules at least as well as the parent peptide but failed to evoke a T cell response (Table 2).

Assignment of Y451 as the P1 anchor in Ty 448-462 revealed that the putative P6 anchor, D456, was not a preferred amino acid in this position. Fig. 2 shows that the mutated peptide Ty 448-462 D456→ V was ~1,000-fold more potent than the parent peptide in stimulating GM-CSF release from $CD4^+$ TIL 1088. This was reflected in the relative MHC binding affinities of the native and mutant peptides (IC_{50} = 989 and 155 nM, respectively). Combining the D456→V mutation with the Ty 450-462 truncation enhanced T cell recognition and MHC binding (IC_{50} = 63 nM) even further.

Table 2. *Determining the P1 Anchor Position for Ty 448-462*

		GM-CSF		
		Exp. 1	Exp. 2	IC_{50}
		pg/ml/24 h		*nM*
Ty 448-462	D Y S Y L Q D S D P D S F Q D	108	802	989
Y449	- Q - - - - - - - - - - - - -	74	520	918
	- F - - - - - - - - - - - - -	103	781	1475
Y451	- - - Q - - - - - - - - - - -	28	<8	>10,000
	- - - F - - - - - - - - - - -	324	1065	563
L452	- - - - Q - - - - - - - - - -	14	<8	989
	- - - - F - - - - - - - - - -	14	<8	506
Ty 449-462	- - - - - - - - - - - - - - -	485	1960	243
Ty 450-462	- - - - - - - - - - - - - - -	556	2749	328
Ty 451-462	- - - - - - - - - - - - - - -	21	<8	566
Ty 452-462	- - - - - - - - - - - - - - -	18	<8	10,000
1088-mel Lysate		>5500	5822	ND

In two separate experiments, peptides (100 μM) pulsed onto 1088-EBV were assessed for recognition by $CD4^+$ TIL1088. Background GM-CSF secretion by B cells cultured with T cells in the absence of peptide has been subtracted (52 and 196 pg/ml, respectively, for experiments 1 and 2). *IC_{50}*, concentration of tyrosinase peptide yielding 50% inhibition of binding of standard peptide to DRB1*0401. *ND*, not done.

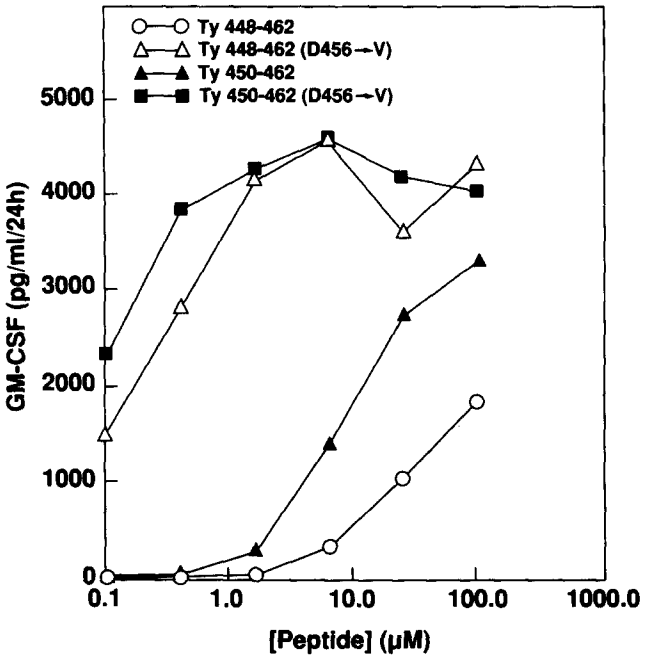

Figure 2. Truncated and mutated analogues of Ty 448-462 provide stronger stimulation for CD4+ TIL 1088 than the parent peptide. T cells and B cells were cultured at 1 × 10^6 cells/ml. Background GM-CSF secretion by TIL + EBV-B cells in the absence of peptide (112 pg/ml) has been substrated from the values shown. Similar results were obtained in repeat experiments.

Because the amino acid sequences of Ty 56-70 and Ty 448-462 were suboptimal for binding to HLA-DRB1*0401, we questioned whether patient 1088 might express a mutant allele of the tyrosinase gene encoding altered peptides. Total RNA isolated from the autologous melanoma cell line 1088-mel was used as a template for RT-PCR conducted with primers flanking the entire coding region of the tyrosinase gene. When the PCR products were sequenced, only one sequence encoding Ty 56-70 was identified, corresponding to the amino acid sequence presented in Table 1. However, for Ty 448-462, two distinct PCR products were identified, one encoding the amino acid sequence presented in Table 2 and the other encoding a mutant peptide with the substitution F460→S. This mutated peptide was synthesized and tested for T cell recognition, but it proved equivalent in potency to the original peptide (data not shown).

Melanoma-specific HLA-DR-restricted CD4+ T cells from two other melanoma patients failed to recognize the tyrosinase peptides, further demonstrating the specificity of the CD4+ TIL 1088 response.

In summary, we have identified two nonmutated tyrosinase peptides, Ty 56-70 and Ty 448-462, which are specifically recognized by tyrosinase-reactive CD4+ T cells from melanoma patient 1088. These bind with intermediate and low affinities to HLA-DRB1*0401, respectively, not unlike many of the immunogenic class I–restricted peptides derived from nonmutated melanocytic proteins (5). Mapping the MHC II binding anchor residues within Ty 56-70 and Ty 448-462 enabled us to modify these peptides to increase MHC binding affinity, such that their potency in stimulating CD4+ T cells was enhanced up to 1,000-fold. Future studies will address the question of whether these native or mutated peptides can be used to raise melanoma-reactive CD4+ T cells from the peripheral blood of melanoma patients. Studies with HLA-A2-restricted gp100 peptides have shown that mutated peptides with increased MHC binding affinities are more immunogenic than the native peptides in vitro (Parkhurst, M., M.L. Salgaller, S. Southwood, P.F. Robbins, A. Sette, S.A. Rosenberg, and Y. Kawakami, manuscript submitted for publication), and murine studies indicate that such peptides may be more immunogenic in vivo as well (25).

The recent cloning of a number of melanoma-associated genes whose protein products can be recognized by CD8+ T cells has revealed that the majority of melanoma patients respond to one or more of a family of nonmutated proteins specific for cells of the melanocyte lineage. The transmembrane proteins tyrosinase, gp75, and gp100 are located within melanosomes while the precise intracellular location of MART-1/Melan-A, also a transmembrane protein, has yet to be characterized. Melanosomes are believed to belong to the lysosomal lineage of organelles, by virtue of expressing LAMP-1 (26). As such, they may be part of the specialized intracellular compartment to which MHC class II molecules are transported for acquisition of peptide (27). One might speculate that the immunogenicity of melanosomal proteins stems from their ability to directly bind MHC class II molecules transported to the melanosomal/lysosomal compartment, generating a CD4+ T cell response which ultimately leads to CD8+ T cell immunity. As normal self transmembrane proteins, tyrosinase and the other members of the pigment-related protein family share common features with the majority of proteins whose peptides have been eluted from MHC class II molecules on the surface of murine or human B cells (18, 28–30). Human melanomas commonly express MHC class II molecules on their cell surface, and the relative abundance of the pigment-related proteins in melanoma cells might favor their presentation on class II molecules. Thus, perhaps it is not unexpected that a CD4+ T cell response against tyrosinase has been identified, and one might anticipate finding such reactivity against other melanosomal proteins in the future.

These experiments outline a general approach to melanoma vaccine design involving the identification of MHC class II–restricted peptides recognized by melanoma-specific CD4+ T cells, followed by targetted peptide modifications that increase the potency of T cell stimulation. The demonstration of HLA-DRB1*0401-restricted epitopes within tyrosinase adds to the list of HLA-A2-restricted (7), A24-restricted (8), and B44-restricted (31) epitopes already identified within this molecule. Thus, with its capacity to generate both CD4+ and CD8+ T cell responses, tyrosinase may provide optimal immunization against melanoma if used as a whole protein or multivalent peptide vaccine. A recent demonstration of the frequent and homogeneous expression of tyrosinase in melanoma specimens (32) supports its use as an anti-melanoma vaccine.

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