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Induction of Potent Antitumor CTL Responses by Recombinant Vaccinia Encoding a Melan-A Peptide Analogue

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There is considerable interest in the development of vaccination strategies that would elicit strong tumor-specific CTL responses in cancer patients. One strategy consists of using recombinant viruses encoding amino acid sequences corresponding to natural CTL-defined peptide from tumor Ags as immunogens. However, studies with synthetic tumor antigenic peptides have demonstrated that introduction of single amino acid substitutions may dramatically increase their immunogenicity. In this study we have used a well-defined human melanoma tumor Ag system to test the possibility of translating the immunological potency of synthetic tumor antigenic peptide analogues into recombinant vaccinia viruses carrying constructs with the appropriate nucleotide substitutions. Our results indicate that the use of a mutated minigene construct directing the expression of a modified melanoma tumor Ag leads to improved Ag recognition and, more importantly, to enhanced immunogenicity. Thus, recombinant vaccinia viruses containing mutated minigene sequences may lead to new strategies for the induction of strong tumor-specific CTL responses in cancer patients. *The Journal of Immunology,* **2000, 164: 1125–1131.**

CD8$^+$ CTL have the ability to specifically recognize and kill tumor cells. Induction of potent antitumor CD8$^+$ CTL responses by vaccination can, as demonstrated in experimental model systems, result in tumor regression and prevent the formation of tumor metastases. Thus, efforts toward the development of anti-cancer vaccines have recently focused on the generation of tumor-specific CD8$^+$ T lymphocyte responses. Short peptides corresponding to MHC class I-restricted CTL epitopes derived from melanoma-associated Ag have been identified (1, 2). These peptides are mostly derived from nonmutated self differentiation Ags such as gp100/pmel-17, MART-1/Melan-A, tyrosinase, and tyrosinase-related proteins. Self differentiation Ags, expressed by both normal and malignant melanocytes, are specifically recognized by HLA-A2-restricted tumor-infiltrating lymphocytes derived from melanoma patients and seem to be involved in tumor regression (3).

These findings have opened new possibilities for Ag-specific immunotherapy of melanoma. The more direct approach consists of the immunization of melanoma patients with the appropriate MHC class I allele with synthetic peptides corresponding to the defined melanoma-derived MHC class I-restricted Ags. However, the immunogenicity of short synthetic peptides corresponding to CTL epitopes in patients may be limited; on the one hand, even those peptides that bind MHC class I molecules with high affinity are generally weak immunogens, probably due to their susceptibility to rapid degradation by serum proteases (4–6), thus requiring repeated administration of relatively high doses (7, 8). On the other hand, although administration of CTL peptides in adjuvant generally results in expansion of specific CTL, experimental evidence for tolerance induction in this experimental setting has also been reported (9).

For these reasons there is a clear interest in designing alternative immunization protocols for the induction of CTL responses directed against melanoma-associated Ags. One attractive strategy consists of using recombinant viral vectors encoding the sequences of interest either as full genes or as minigenes as immunogens (10–16). Recombinant viral vectors encoding full-length differentiation Ags have to date shown limited immunogenicity in both mice and humans (10, 11, 17). Different factors may account for this result. These are 1) the existence of a high degree of tolerance to self Ags, 2) suboptimal processing and presentation of the relevant sequences following infection with recombinant vaccinia viruses (rVV),[3] and/or 3) the fact that immunodominant peptides from self Ags often bind to MHC with low affinity and are poorly recognized by specific T cells.

We and others have recently identified differentiation Ag-derived peptide analogues carrying single or multiple amino acid substitutions that have improved immunogenicity compared with the parental sequences (18–20). In particular, we have described Melan-A$_{26–35}$ immunodominant peptide analogues that exhibit improved binding to HLA-A2, form stable HLA-A2 peptide complexes, and are recognized with improved efficiency by Melan-A-specific CTL. We have also shown that Melan-A$_{26–35}$ analogues are more efficient than parental Melan-A peptides at inducing Melan-A-specific CTL responses both in vitro and in vivo (21, 22).

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[3] Abbreviations used in this paper: rVV, recombinant vaccinia viruses; Ub, ubiquitin; GFP, green fluorescence protein; UPR, ubiquitin/protein/reference; ER, endoplasmic reticulum.

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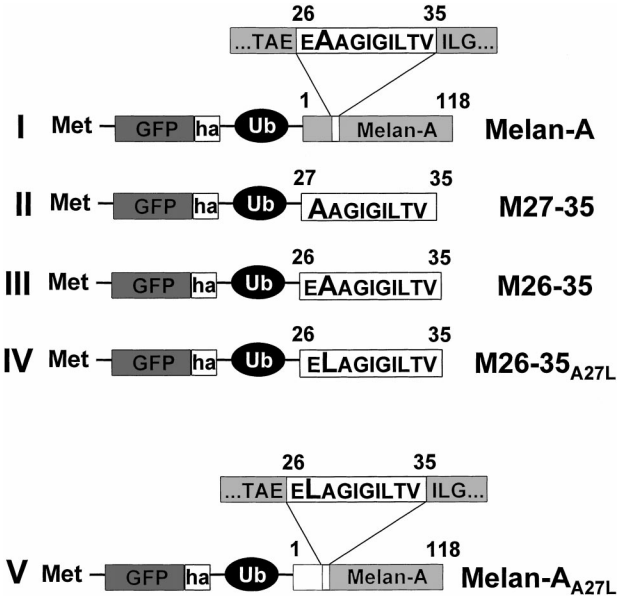

FIGURE 1. The UPR-based recombinant vectors. Five constructs were made. They contained sequences encoding Melan-A full gene (construct I), Melan-$A_{27–35}$ parental nonapeptide minigene (construct II), Melan-$A_{26–35}$ parental decapeptide minigene (construct III), Melan-$A_{26–35}$ A27L analogue decapeptide minigene (construct IV), or Melan-A full-length gene incorporating the A27L mutation (construct V). The term ha denotes a peptide sequence tag derived from the influenza hemagglutinin that is recognized by a specific Ab. The segment corresponding to the antigenic peptide in each construct is indicated as a white box. Recombinant VV containing UPR constructs were generated by recombination as detailed in *Materials and Methods*.

One of these sequences contains a single leucine to alanine substitution at position 2 of the Melan-$A_{26–35}$ decapeptide sequence (peptide A27L) (19).

In the present study we have analyzed the impact of introducing the A27L mutation into rVV encoding Melan-A-derived sequences. We observed that introduction of the A27L mutation into a rVV encoding the Melan-$A_{26–35}$ minigene resulted in a strongly increased immunogenicity in vitro and in vivo. However, when the mutation was introduced into the Melan-A full gene sequence, we observed specific lysis of infected cells but no induction of Melan-A-specific CTL. These findings suggest that both qualitative (the sequence of the peptide Ag generated), and quantitative (the efficiency of Ag processing) factors can be crucial for the generation of efficient recombinant virus vectors. In addition, they indicate that recombinant viruses encoding optimized minigene sequences from tumor Ags may be more effective than minigenes encoding parental sequences for the generation of anti-cancer immune responses.

Materials and Methods

Construction of plasmids and rVV

The generation of the plasmid pGFP/Ub (ubiquitin) that served as the vector for all constructs used in this study has been described previously (20), and details are available upon request. The open reading frame coding for the full-length human Melan-A protein was obtained by PCR amplification and was inserted between the *Sac*II and *Ava*I sites of pGFP/Ub (Fig. 1, construct I). Constructs II–IV were obtained by annealing complementary synthetic oligonucleotides. Ligation of these fragments into pGFP/Ub led to the generation of plasmids directing the expression of the reference protein GFP-Ub and the Melan-A fragments spanning aa 27–35 (construct II), aa 26–35 (construct III), or aa 26–35 with an A to L substitution at position 27 (construct IV). A fragment spanning the Melan-A open reading frame, in which the codon for amino acid A27 was substituted for one encoding L, was obtained by PCR amplification and was inserted between the *Bst*EII and *Ava*I of construct I, resulting in construct V. The sequence of the critical regions in all constructs was confirmed by DNA sequencing. Wild-type vaccinia virus (Copenhagen) strain was a gift from Dr. R. Drillien, (Strasbourg, France). The DNA fragments spanning GFP-Ub and the Melan-A sequences of constructs I–V were isolated and inserted into the plasmid pKT1030, placing them under the control of a mutant vaccinia virus promoter P 7.5 (23). For each construct, the cassette containing the promoter and insert was subcloned into plasmid pKT 1401. This plasmid contains flanking sequences homologous to the I4L locus of vaccinia virus. Recombination into the I4L locus of vaccinia virus was achieved following the transient dominant gpt selection protocol on CV-1 (American Type Culture Collection, Manassas, VA; CCL 70) and BSC-40 (derivative of BSC-1, American Type Culture Collection CCL26) cells (14). The rVV encoding the MAGE-$3_{271–279}$ peptide was generated by the same procedure. The MAGE-3 sequence was derived from pGFP/Ub-MAGE-3 described previously (24). After plaque purifications the recombinant viruses were PCR screened, amplified, and semipurified on sucrose cushions. Where indicated, the virus was psoralen-long-wave-UV-inactivated before infection of APC as previously described (23).

Cells

Tumor cell lines were maintained in culture as previously described (19). The human melanoma cell line Na-8 MEL was provided by Dr. F. Jotereau (Unit 211, Institut National de la Santé et de la Recherche Médicale, Nantes, France). Melanoma cell line Me 290 (HLA-A*0201 and Melan-A^+) has been previously described (19). In Ag recognition assays we used the CTL line LAU 203 EAA as effector. This CTL line was derived after in vitro stimulation of $CD8^+$ highly enriched lymphocytes from melanoma patient LAU 203 with the parental decapeptide Melan-$A_{26–35}$, followed by isolation of A2/Melan-A tetramer-positive cells by tetramer-guided cell sorting and further mitogen-driven expansion (21). In CTL induction experiments $CD8^+$ lymphocytes were positively selected by magnetic cell sorting from PBMC of HLA-A*0201 melanoma patients using a mini-MACS device (Miltenyi Biotec, Sunnyvale, CA). From the $CD8^-$ fraction $CD14^+$ cells were then similarly selected and used as APCs. After infection with recombinant or wild-type viruses at 10 multiplicity of infection during 2 h, $CD14^+$ cells were extensively washed, irradiated (3000 rad), and mixed with $CD8^+$ responder T cells at a 1:1 ratio in 2 ml of CTL medium containing human rIL-2 (100 U/ml; Glaxo, Geneva, Switzerland; provided by Dr. M. Nabholz, ISREC, Epalinges, Switzerland), and human rIL-7 (10 ng/ml; donated by Sanofi Recherche, Labege, France). Where indicated, synthetic peptides were added at a final concentration of 1 μM. For mouse experiments, the murine EL-4 cell line transfected with the HLA-A*0201/K^b gene (EL-4 A2/K^b transfectants) was provided by Dr. Linda Sherman (Scripps Clinic and Research Foundation, La Jolla, CA). HLA-A*0201/K^b transgenic mice (line 6) were provided by Harlan Sprague Dawley (Indianapolis, IN). Two- to three-month-old mice were immunized i.p. with 10^7 PFU rVV encoding the Melan-A minigene 26–35, Melan-A minigene 26–35 A27L, or an irrelevant Ag. Three weeks later, T cells were purified from spleens by passage on nylon wool columns. Purified T cells (2×10^6) were then cultured with 2×10^5 irradiated (10,000 rad) EL-4 A2/K^b cells previously pulsed with peptide Melan-$A_{26–35}$ A27L (1 μM) for 1 h at 37°C in 12-well cell culture plates in 2 ml of DMEM/10% FCS containing 30 U/ml of IL-2 as previously described (22). Cultures were tested for peptide-specific cytolytic activity after two rounds of weekly stimulation.

Flow cytometric immunofluorescence analysis

Tetramers were synthesized as previously described (25). As the antigenic peptide, the Melan-$A_{26–35}$ A27L analogue (ELAGIGILTV), which has a higher binding stability to HLA-A*0201, was used. This results in a much more efficient generation of stable tetramer preparations compared with parental peptides. The Melan-$A_{26–35}$ A27L analogue also has a higher T cell antigenicity and immunogenicity than the natural Melan-A decapeptide EAAGIGILTV or the nonapeptide AAGIGILTV (19). We have recently extensively validated the use of Melan-A A27L-containing tetramers for the visualization and isolation of Melan-A-specific, tumor-reactive CTL from either tumor-infiltrated lymph nodes or peptide-stimulated PBMC of melanoma patients (21, 25). Cells were stained with tetramers (200 ng/sample) in 20 μl of PBS/2% FCS for 20 min at room temperature, then 20 μl of anti-$CD8^{FITC}$ mAb (Becton Dickinson, Basel, Switzerland) was added, and cells were incubated for an additional 30 min at 4°C. Cells were washed once in the same buffer and analyzed by flow cytometry. Data analysis was performed using CellQuest software (Becton Dickinson).

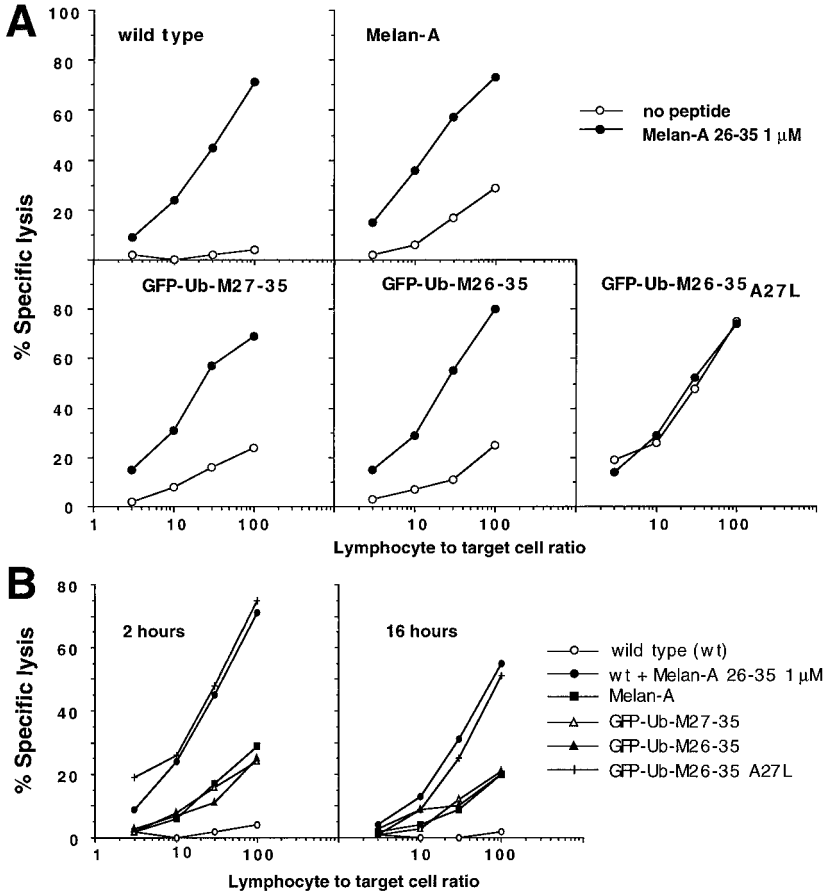

FIGURE 2. Recognition of Na-8 MEL cells infected with rVV by HLA-A*0201-restricted Melan-A-specific CTL. *A*, NA-8 MEL cells were ^{51}Cr labeled for 1 h, washed, and infected with wt or rVV at 10 multiplicity of infection for 2 h. Cells were then added to Melan-A-specific effectors (CTL line LAU 203 EAA) at different lymphocyte to target cell ratios in the absence or the presence of the peptide Melan-A_{26-35} (1 μM). Chromium release was measured after 4 h. Similar results were consistently obtained in several independent experiments. *B*, Wild-type or rVV were psoralen-long-wave-UV-inactivated before infection of APC as previously described (23). NA-8 MEL cells were infected with inactivated wt or rVV at 10 multiplicity of infection for 2 h. One-half of the cells were then ^{51}Cr labeled and tested as described above (not shown). The remaining cells were cultured overnight and then ^{51}Cr labeled before the test.

Cytotoxicity and TNF-α release assays

Cytotoxic activity was measured using standard 4-h chromium release assays as previously reported (19). NA8 melanoma cells (HLA-A*0201$^+$ Melan-A$^-$) were used as target cells. For mouse experiments chromium release assays were similarly performed using EL-4 A2/K^b transfectants as target cells. Transient transfection of NA8-MEL cells used in a TNF-α release assay was measured as previously described (24).

The IFN-γ release assay

CD8$^+$ lymphocytes were enriched, stimulated with rVV, and then cocultured (2×10^5) with either NA8-MEL or Me 290 melanoma cells at a lymphocyte to stimulator cell ratio of 2/1 in 96-well round-bottom microplates in 200 μl of RPMI/10% FCS containing 20 U/ml human IL-2. After overnight incubation at 37°C, IFN-γ production was measured in the culture supernatant using an ELISA as previously described (26).

Results

Construction of Ub/protein/reference (UPR)-based recombinant vectors

Vectors encoding either full-length or fragments of Melan-A were developed based on the previously described UPR constructs (24, 27). Ub is a 76-aa polypeptide that is naturally synthesized as a linear fusion product either to itself or to other cellular proteins (28). The UPR constructs exploit this finding and consist of linear fusion products in which Ub is located between a reference protein (here the GFP) and the protein of interest (Fig. 1). Cotranslational cleavage by Ub-specific proteases after the last residue of Ub yields two independent proteins in equimolar amounts, the protein of interest and the reference protein. It is noteworthy that the use of Ub fusion also bypasses the need for a Met residue at the N-terminus of minigene products, thus allowing direct generation of the relevant peptides. The rVVs containing UPR constructs were generated as described in *Materials and Methods*.

Infection of Melan-A$^-$ melanoma cells with rVV encoding the mutated, but not the parental, Melan-A_{26-35} minigene results in maximal lysis by Melan-A-specific CTL

To determine whether infection with rVV vectors coding for parental or mutated Melan-A sequences would render the Melan-A$^-$ HLA-A2$^+$ tumor line Na-8 MEL susceptible to killing by Melan-A-specific tumor-reactive CTL, we performed the experiment illustrated in Fig. 2*A*. We used the polyclonal monospecific CD8$^+$ T cell line LAU 203 EAA as effector cells (21). The fine specificity of Ag recognition of line LAU 293 EAA is similar to that of the majority of Melan-A-specific CTL previously described (19, 20). Infection of Na-8 MEL cells with rVV encoding the Melan-A gene protein, or the nona- or decapeptide minigene sequences resulted in low, but significant, levels of lysis by the Melan-A-specific CTL line. In contrast, infection with rVV carrying the Melan-A_{26-35} A27L analogue minigene sequence resulted in efficient lysis at levels comparable to those obtained in the presence of saturating amounts of exogenously added synthetic parental peptide Melan-A_{26-35} or Melan-A_{26-35} A27L analogue (data not shown). Similar results were obtained after transient transfection of NA8-MEL cells with UPR/Melan-A constructs using measurement of TNF-α secretion by specific CTL (data not shown).

The low level of specific lysis obtained after infection with rVV encoding parental Melan-A sequences could, in principle, be explained by the short time of the assay not allowing the synthesis of a sufficient amount of the Ag peptide. To address this question, we infected NA8-MEL cells for 2 h with psoralen-UV-inactivated rVVs (23) and then cultured them for 16 h at 37°C before use. This treatment prevents viral replication, thus allowing the transcription of early genes (including the I4L locus) to persist for a longer period than in cells infected by replicating virus. As illustrated in

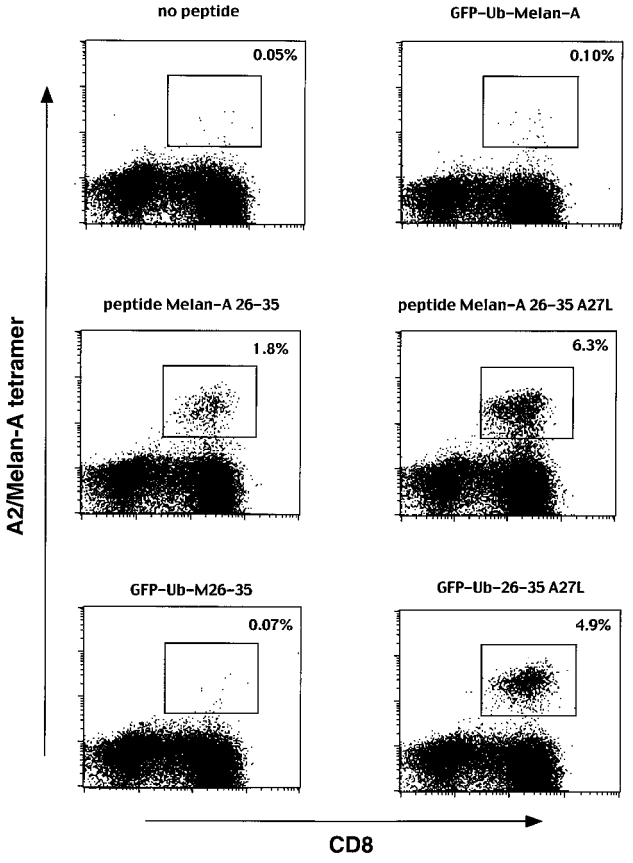

FIGURE 3. Induction of A2/Melan-A tetramer$^+$ CD8$^+$ T cells by stimulation with rVV-infected APC. Highly enriched CD8$^+$ lymphocytes from melanoma patient LAU 56 were stimulated as detailed in *Materials and Methods*. Where indicated, synthetic peptides were added to the culture medium at a final concentration of 1 μM. Cultures were stained with anti-CD8^{FITC} mAb and A2/Melan-A^{PE} tetramers at 14 days after stimulation. Similar results were consistently obtained in several independent experiments.

Fig. 2*B*, prolonged infection with the various inactivated rVV did not result in improved CTL recognition.

Melan-A-specific tumor-reactive CTL are efficiently induced upon stimulation of PBMC from melanoma patients with autologous APCs infected with rVV encoding the mutated, but not the parental, Melan-A$_{26-35}$ minigene

Highly enriched CD8$^+$ T cells isolated from PBMC of melanoma patient LAU 56 were stimulated with autologous APCs infected with rVV encoding either parental or analogue Melan-A sequences. As a control, CD8$^+$ T cells were also stimulated with uninfected autologous APCs alone or in the presence of synthetic parental Melan-A nona- or decapeptides. Cultures were tested 14 days after stimulation for the presence of CD8$^+$ A2/Melan-A tetramer$^+$ cells. A2/Melan-A tetramer$^+$ T cells were detected in freshly isolated CD8$^+$ T cells from patient LAU 56 at a frequency of 0.08% (not shown). This frequency did not significantly vary after the 2-wk culture period in the absence of Ag-specific stimulation (Fig. 3). The frequency of A2/Melan-A tetramer$^+$ CD8$^+$ T cells was not increased over pre-existing values upon infection with rVV encoding parental Melan-A sequences as either a full gene or a minigene. In contrast, infection with rVV encoding the Melan-A$_{26-35}$ A27L analogue sequence resulted in a potent expansion of A2/Melan-A tetramer$^+$ CD8$^+$ T cells (98-fold increase), superior to the expansion obtained upon stimulation with 1 μM of the parental peptide Melan-A$_{26-35}$ (36-fold increase) and similar to that obtained upon stimulation with the peptide Melan-A$_{26-35}$ A27L (126-fold increase). Similar experiments performed with CD8$^+$-enriched PBMC from three additional melanoma patients yielded similar results. Nevertheless, some variation was also found, as patient LAU 97 responded to rVV encoding the Melan-A A27L analogue sequence less efficiently than after stimulation with the corresponding synthetic peptide (7- vs 48-fold expansion). Patient LAU 203 also responded to stimulation with rVV containing the parental minigene, although weakly compared with the response obtained after stimulation with the rVV containing the mutated minigene (5.7- vs 130-fold expansion). We have recently documented the functional specificity of A2/Melan-A tetramer$^+$ T cells found in PBMC cultures from melanoma patients (20). To further document the ability of Melan-A-specific CTL obtained by stimulation with rVVs to specifically recognize Melan-A-expressing melanoma cells we tested IFN-γ release upon stimulation with Melan-A-expressing and nonexpressing melanoma cell lines. Background levels of IFN-γ were released by the cultures following stimulation with the Melan-A-negative cell line NA8-MEL. However, significant amounts of IFN-γ were produced by the cultures containing increased numbers of A2/Melan-A tetramer$^+$ cells upon stimulation with the Melan-A-positive melanoma cell line Me 290. These levels directly correlated with the percentage of A2/Melan-A tetramer$^+$ cells present in the culture (Table I).

Injection of rVV encoding the mutated, but not the parental, Melan-A$_{26-35}$ minigene elicits Melan-A-specific CTLs in A2/K^b transgenic mice

To evaluate the impact of the enhanced stimulatory capacity of rVV carrying the Melan-A$_{26-35}$ A27L mutated minigene on the in vivo immunogenicity of our recombinant viral vectors, we immunized A2/K^b transgenic mice with rVV carrying either the parental or the A27L mutated Melan-A$_{26-35}$ minigene. Highly enriched T cells obtained from the spleen of A2/Kb transgenic mice immunized with rVV encoding the parental Melan-A$_{26-35}$ sequence exhibited low to undetectable peptide-specific CTL activity after two rounds of in vitro stimulation. This specific CTL activity was similar to that obtained from A2/Kb transgenic mice immunized with an irrelevant rVV (rVV MAGE-3) and to that of naive mice included as internal controls (Fig. 4). In contrast, high levels of peptide-specific lysis were detected for splenic T cells obtained from mice immunized with rVV carrying the A27L mutated 26–35 minigene.

Introduction of the A27L mutation into rVV encoding the Melan-A full gene results in specific lysis of infected target cells but inefficient induction of Melan-A-specific CTL

The introduction of a single mutation was critical in determining the immunogenicity of rVV vectors carrying the Melan-A decapeptide minigene. To determine the impact of introducing the same mutation in the full gene, we constructed and analyzed the corresponding rVV (Fig. 1, construct V). Infection of NA8-MEL cells with this rVV resulted in efficient CTL recognition (Table II). However stimulation of CD8$^+$ T cells from patient LAU 56 with rVV carrying the Melan-A full gene A27L did not result in expansion of A2/Melan-A tetramer$^+$ CD8$^+$ T cells significantly above background levels. In addition, only low levels of TNF-α were produced by the CTL line upon stimulation with NA8-MEL cells transiently transfected with the corresponding UPR/Melan-A construct. These results suggest that infection with rVV carrying the Melan-A mutated full gene may lead to Ag expression, but only at lower levels than rVV carrying the mutated minigene and

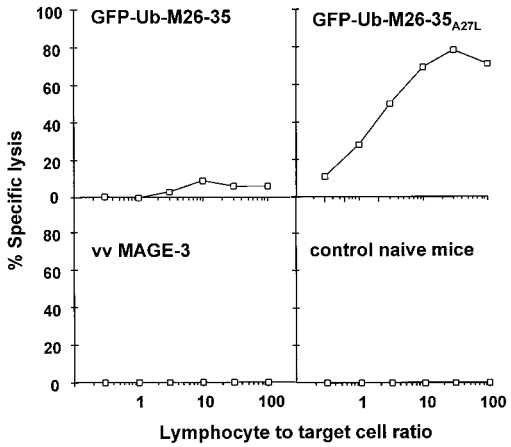

FIGURE 4. In vivo induction of Melan-A-specific CTL by immunization of A2/K^b transgenic mice with rVV. HLA-A*0201/K^b transgenic mice (two mice per group) were immunized by i.p. injection with the indicated rVV. Three weeks later, splenic T cells were stimulated with EL-4 A2/K^b cells previously pulsed with peptide Melan-A_{26-35} A27L (1 μM). Naive splenic T cells were also stimulated as a control. Cultures were tested for peptide-specific cytolytic activity after two rounds of weekly stimulation. The values represent the percent specific lysis of Melan-A_{26-35} A27L peptide (1 μM)-loaded target cells minus the percent specific lysis of control EL-4 A2/K^b target cells.

at levels not sufficient to stimulate the expansion of specific CTL precursors.

Discussion

This study reports two major findings. First, infection of melanoma cells with rVV carrying a Melan-A_{26-35} A27L mutated minigene results in a much more efficient target cell recognition by Melan-A-specific CTL than with cells infected with rVV encoding Melan-A parental sequences. Importantly, only the rVV carrying the Melan-A_{26-35} A27L analogue was highly immunogenic both in vitro, leading to the expansion of Melan-A-specific CTL precursors, and in vivo, when used to immunize HLA-A2/K^b transgenic mice. Second, when the mutation encoding the amino acid substitution A27L was introduced into a rVV carrying the Melan-A full gene insert, its positive effect (clearly detectable at the level of infected target cell recognition; Table II) was not sufficient to overcome the low immunogenicity of the rVV encoding the Melan-A full gene. These results suggest that although optimization of antigenic sequences can dramatically modify the immunogenic properties of recombinant viral vectors encoding minigenes, additional factors other than the antigenic sequence itself can influence the immunogenicity of recombinant viral vectors encoding full-length genes.

Recombinant viral vectors encoding full-length melanocyte differentiation Ags represent, in principle, attractive vaccines. Indeed, this vaccination strategy is based on the endogenous processing and presentation of the whole Ag containing many potential T cell epitopes that could be presented in the context of each individual HLA allele, thus overcoming the need to characterize the patient's HLA phenotype and immunogenic epitopes. One potential limitation for the use of recombinant viral vectors is the presence of neutralizing Abs either as a result of previous infection with a wild-type form of the vector or postimmunization. However, the availability of numerous vectors may now allow highly effective prime/boosting immunization protocols employing non-cross-reactive vectors. In addition, immunization with recombinant viral vectors could be alternated with other immunization strategies using recombinant viral vector-infected dendritic cells, synthetic peptides, or naked DNA. Unfortunately, vaccination attempts based on in vivo expression, processing, and presentation of whole differentiation Ags (including Melan-A), using recombinant technology, have to date failed to fulfill these expectations (16, 29).

One explanation for this failure could be the existence of a high degree of tolerance to self sequences derived from melanocyte differentiation Ags. The observation that immunization of C57BL/6 mice with recombinant adenovirus encoding human, but not murine, gp100 could protect mice from challenge with murine B16 melanoma (11), favors this hypothesis. However, in humans tolerance to melanocyte differentiation Ags may operate mainly by deleting high affinity specific T cells (30, 31). Indeed, CTL specific for melanocyte-derived differentiation Ags can often be found in both normal donors and melanoma patients. However, they generally recognize the antigenic peptide at concentrations much higher than those required for efficient recognition by T cells specific for virally derived Ags (20, 32). Nevertheless, because target cell lysis generally occurs at Ag densities lower than those required for full T cell activation (including cytokine production and proliferation), relatively low affinity T cells could still be instrumental in destroying Ag-expressing tumor cells, provided that they are efficiently activated and expanded through appropriate immunization protocols.

In this context, the use of modified antigenic peptides exhibiting improved binding and antigenicity could be fundamental. Indeed, enhanced peptide analogues of HLA-A2-restricted CTL epitopes derived from gp100 have recently been shown to be more immunogenic that parental sequences in HLA-A2 melanoma patients (8, 33). Although the antigenic Melan-A peptide originally described by Kawakami et al. (34) was indeed the nonapeptide Melan-A_{27-35}, we have shown that the decapeptide Melan-A_{26-35}

Table I. *Assessment of Ag specificity and tumor reactivity of A2/Melan-A tetramer$^+$ CD8$^+$ T cells elicited by stimulation with rVV*

	LAU 233			LAU 97			LAU 203		
	% A2/Melan-A tetramer$^+$ cells[a]	IFN-γ (pg/ml)[b]		% A2/Melan-A tetramer$^+$ cells	IFN-γ (pg/ml)		% A2/Melan-A tetramer$^+$ cells	IFN-γ (pg/ml)	
		Me 290	NA 8-MEL		Me 290	NA 8-MEL		Me 290	NA 8-MEL
No infection	0.13	142	178	0.07	178	276	0.04	141	134
No infection + peptide ELAGIGILTV	2.2	1193	178	3.4	1782	276	3.7	1230	141
GFP-Ub-M26-35	0.07	146	161	0.08	178	216	0.23	172	141
GFP-Ub-M26-35 A27L	3.7	1632	172	0.5	390	264	5.2	1020	145

[a] Highly enriched CD8$^+$ T cells from A2$^+$ melanoma patients were stimulated and stained 2 wk later as detailed in *Materials and Methods*.
[b] At day 14, CD8$^+$-stimulated lymphocytes were cocultured overnight at 37°C with either NA8-MEL or Me 290 melanoma cells. IFN-γ production was measured in the culture supernatant using an ELISA assay.

Table II. *Impact of A27L mutation on rVV encoding Melan-A A27L mini and full-length genes*

	% Specific Lysis[a]	% A2/Melan-A Tetramer$^+$ Cells[b]	TNF-α (pg/ml)[c]
No infection	4	0.05	112
No infection + peptide EAAGIGILTV	86	1.8	9,216
No infection + peptide ELAGIGILTV	86	6.3	21,504
GFP-Ub-M26-35	18	0.07	224
GFP-Ub-M26-35 A27L	84	4.9	5,376
GFP-Ub-Melan-A A27L	68	0.13	554

[a] Chromium release assay was performed as detailed in *Materials and Methods*. The E:T cell ratio used was 10:1. Similar results were consistently obtained in several independent experiments.

[b] CD8$^+$ highly enriched T cells from melanoma patient LAU 56 were stimulated as described in *Materials and Methods.* Where indicated, synthetic peptides were added to the culture medium at a final concentration of 1 μM. Cultures were stained with anti-CD8^{FITC} mAb and A2/Melan-A^{PE} tetramers 14 days after stimulation.

[c] NA8-MEL melanoma cells transiently transfected with the indicated constructs were tested for their ability to stimulate TNF-α release by the Melan-A-specific CTL line LAU 203 EAA. CTL were added at the lymphocyte to stimulator cell ratio of 10, and TNF-α release was measured as described (24).

is recognized more efficiently than the nonapeptide Melan-$A_{27–35}$ by the majority of Melan-A-specific CTL (20, 35). Based on these results, we designed the Melan-$A_{26–35}$ A27L analogue (ELAGIGILTV), which has a higher binding affinity and stability to HLA-A*0201 as well as a higher T cell antigenicity and immunogenicity than the natural Melan-A decapeptide EAAGIGILTV or the nonapeptide AAGIGILTV (19). We have extensively investigated the CTL response elicited both in vitro and in vivo by the Melan-$A_{26–35}$ A27L analogue (19, 21, 22) and found complete cross-reactivity of Melan-$A_{26–35}$ A27L analogue elicited CTL with Melan-A parental peptide sequences. More importantly, analogue-induced CTL could specifically and efficiently recognize endogenously expressed Ag on Melan-A-expressing tumor cells. In the present study we further assessed specific tumor Ag recognition by CTL lines obtained from A2 melanoma patients upon stimulation with either Melan-$A_{26–35}$ A27L analogue or autologous APC infected with rVV encoding Melan-$A_{26–35}$ A27L (Table I). Thus, even though the Melan-$A_{26–35}$ A27L analogue is obviously not present at the surface of Melan-A-expressing tumors, the immune response elicited by stimulation with Melan-$A_{26–35}$ A27L efficiently cross-recognize endogenously expressed Melan-A sequence(s).

We and others (19, 36) have also recently described analogues of the Melan-$A_{27–35}$ nonapeptide. Substitution of A at position 2 of the nonapeptide with L resulted in highly increased binding to HLA-A2, but loss of recognition by specific CTL. We also designed a nonapeptide analogue bearing an L to A substitution at position 27. This peptide did not exhibit improved binding to A2, but was recognized by specific CTL more efficiently than Melan-$A_{27–35}$ parental sequence. Thus, in this case the enhanced recognition can be exclusively attributed to an enhanced efficiency of recognition by the TCR (superagonist peptide) that might be expected to vary considerably between TCRs. For these reasons we decided not to use this analogue and choose the Melan-$A_{26–35}$ A27L for further investigations.

In the present study we have shown that enhanced immunogenicity is also obtained when the optimized sequence is endogenously expressed by APC from a minigene construct. It is not immediately clear why introduction of the same mutation in the context of the Melan-A full-length gene does not lead to the same result. Previous studies had already suggested that generation of Ag upon infection with recombinant viral vectors carrying the Melan-A full-length gene was suboptimal and could be improved by treatment of infected cells with sodium butyrate and TNF-α (11). In addition, in vitro induction of Melan-A-specific CTL expansion from PBMC stimulated with recombinant viral vectors carrying the Melan-A full-length gene has been obtained only when potent professional APC, such as dendritic cells, were used (15). In the present study only low levels of specific target cell lysis and no induction of specific CTL expansion were observed upon infection with rVV encoding the Melan-A parental full-length gene.

Melan-A is a membrane protein expressed in melanosomes and endoplasmic reticulum (ER) (37). In the classical pathway for processing of MHC class I-associated peptides, proteins are degraded in the cytosol by cytosolic proteases such as the proteasome to generate peptides that are then transported into the ER where they bind nascent MHC class I molecules (38, 39). Because membrane proteins are normally cotranslationally translocated in the ER, they would, in principle, bypass the cytosolic proteases of this pathway of Ag processing. Thus, degradation of membrane proteins and, consequently, processing and presentation of membrane protein-derived epitopes most likely occurs through alternative degradation pathways that may involve partial or complete proteolysis in the ER (40, 41), such as mistranslation in the cytosol (42, 43) or, as recently shown for tyrosinase, reverse translocation of the intact protein from the ER to the cytosol for proteolysis (44). Although the degradation pathway of Melan-A has not yet been elucidated, it could be of relatively low efficiency. Alternatively, the suboptimal efficiency of Melan-A processing could be inherent to the Melan-A sequence itself. Indeed, the Melan-A immunodominant epitope is located in the putative transmembrane region of the protein, that because of its high hydrophobicity might be not easily accessible to proteases. In favor of this last hypothesis, we have recently observed that reciprocal transplantation of the Melan-$A_{26–35}$ sequence into the region 270–278 of the MAGE-3 protein resulted in improved Ag recognition of transiently transfected target cells by Melan-A-specific CTL (D. Valmori and F. Lévy, unpublished observations).

In conclusion, the results illustrated in the present study suggest that recombinant viral vectors encoding the Melan-$A_{26–35}$ A27L analogue sequence can be highly immunogenic and may represent promising tools for immunotherapy of melanoma. In addition, the elucidation of the molecular mechanisms that govern Ag processing of Melan-A protein could in the future contribute to improve the effectiveness of vaccines based on recombinant viral vectors encoding Melan-A full protein.

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