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A Single Heteroclitic Epitope Determines Cancer Immunity After Xenogeneic DNA Immunization Against a Tumor Differentiation Antigen

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Successful active immunization against cancer requires induction of immunity against self or mutated self Ags. However, immunization against self Ags is difficult. Xenogeneic immunization with orthologous Ags induces cancer immunity. The present study evaluated the basis for immunity induced by active immunization against a melanoma differentiation Ag, gp100. Tumor rejection of melanoma was assessed after immunization with human gp100 (hgp100) DNA compared with mouse gp100 (mgp100). C57BL/6 mice immunized with xenogeneic full-length hgp100 DNA were protected against syngeneic melanoma challenge. In contrast, mice immunized with hgp100 DNA and given i.p. tolerizing doses of the hgp100 Dβ-restricted peptide, hgp100 25–33, were incapable of rejecting tumors. Furthermore, mice immunized with DNA constructs of hgp100 in which the hgp100 25–33 epitope was substituted with the weaker Dβ-binding epitope from mgp100 (mgp100 25–27) or a mutated epitope unable to bind Dβ did not reject B16 melanoma. Mice immunized with a minigene construct of hgp100 25–33 rejected B16 melanoma, whereas mice immunized with the mgp100 25–33 minigene did not develop protective tumor immunity. In this model of xenogeneic DNA immunization, the presence of an hgp100 heteroclitic epitope with a higher affinity for MHC created by three amino acid (25 to 27) substitutions at predicted minor anchor residues was necessary and sufficient to induce protective tumor immunity in H-2b mice with melanoma. The Journal of Immunology, 2003, 170: 5188–5194.

Induction of cancer immunity against self Ags can be difficult because of immune tolerance or ignorance. Immunity against a major class of self Ags, the differentiation Ags, can be induced by xenogeneic immunization, which is vaccination of a host from one species with DNA encoding an orthologous gene from another species (1–4). Differentiation Ags are shared between cancer cells and their normal cell counterparts (5). The strategy of xenogeneic immunization has been successful, whereas immunization with the syngeneic differentiation Ag failed to induce immunity. Although it has been presumed that individual amino acid sequence differences in the xenogeneic protein create heteroclitic epitopes, which trigger immunity against the homologous self protein, this hypothesis has not been directly demonstrated.

Prototypical examples of differentiation Ags are glycoproteins shared by melanomas and melanocytes, categorized as the melanosomal differentiation Ags (6). Immunity against differentiation Ags is a form of autoimmunity that can mediate destruction of tumors and normal tissues (1–4, 7–11). In fact, tumor-infiltrating lymphocytes from patients with melanoma most often recognize melanocyte differentiation Ags (12). Previous studies have shown that xenogeneic immunization against the melanosomal differentiation Ags tyrosinase-related protein-1 TRP-1/gp75 (3, 4), dopachrome tautomerase (1), and gp100/pmel 17 (2), either with DNA encoding the Ag or with recombinant protein, can result in the rejection of transplanted syngeneic melanomas in mice.

One mechanism that could mediate xenogeneic immunization is the creation of heteroclitic epitopes. A heteroclitic epitope describes an altered peptide that is a better agonist for inducing T cell responses than the native, unaltered peptide (13). This terminology has been applied to other altered peptides with a higher immunologic potency than their unaltered counterparts (14–16). Heteroclitic peptides have increased potency either due to increased binding to MHC molecules (15, 17–19) or to increased agonist properties to stimulate TCRs (16).

We recently described xenogeneic DNA immunization of mice with the gene encoding human gp100 (hgp100) (2). The gp100 Ag is a melanosomal differentiation Ag expressed by melanomas and melanocytes (20). In this model, mice are protected against melanoma challenge, and this immunity correlates with T cell reactivity against the MHC class I Dβ-restricted epitope gp100 25–33. Immunity after xenogeneic immunization against gp100 was achieved in mice deficient in MHC class II molecules, suggesting that immunity directed against MHC class I epitopes was sufficient without T cell help. In the present study, we assessed the role of hgp100 25–33 after xenogeneic DNA immunization. We show that hgp100 25–33 is a heteroclitic epitope that is both necessary and sufficient to induce autoimmunity and melanoma immunity in an H-2b mouse tumor model.

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2 Abbreviations used in this paper: hgp100, human gp100; mgp100, mouse gp100.

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Materials and Methods

Mice

C57BL/6 female mice were acquired from the National Cancer Institute (Bethesda, MD) or The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in a pathogen-free vivarium according to National Institutes of Health Animal Care guidelines under an institutional protocol reviewed and approved by the Animal Care Committee. All mice entered in the study were between 7 and 10 wk of age.

Cell lines and tissue culture

B16F10 (B16) is a pigmented mouse melanoma cell line of C57BL/6 origin provided by Dr. I. Fidler (M. D. Anderson Cancer Center, Houston, TX). This cell line has been maintained in our laboratory for tumor challenges by in vivo passage through mice, followed by in vitro expansion and storage at −70°C. The EL4 cell line is a C57BL/6 mouse lymphoma. Tumor cell lines were cultured as previously described (8).

Plasmid constructs

hgp100-pWRG1644 was a gift of Dr. N. P. Restifo (National Cancer Institute). PCR-based site-specific mutagenesis was performed on hgp100 to create hgp100KVP→E29-gpWRG1644 and hgp100N20→W-pWRG1644 using the primers 5′-GCTGGGGGCTCACGAGATCGACCCAGGATC-3′ and 5′-CAGTCTGGTGTGGACTTCTGTGAGCC-3′ (hgp100KVP→E29) as well as 5′-GGGGGCTACAAAACTGCGCATCC-3′ and 5′-CAGAAGACGCTCATGCGATTGAC-3′ (hgp100N20→W). The cDNAs coding for hgp100, hgp100KVP→E29, and hgp100N20→W were cleaved from the respective pWRG1644 plasmids using NotI and each was cloned into the vector pWR7077BEN (21). The fragments were then sequenced to assure that hgp100KVP→E29 and hgp100N20→W differed from hgp100 only at the intended sites. The oligonucleotides 5′-GATCCACCATGAGATACATGATCCTGGGCCTGCTGGCCCTGGCTGCA-3′ and 5′-GCCAGGCGCAGCAGCAGGATCATGTATCTCATGGTG-3′ (EGSRNQDWL), 2) the Dβ33 peptide differed from 3) the Dα33 peptide (VLEWRFDSRL) served as a negative control. The A2.1-restricted HIV Nef peptide (VLEWRFDSRL) served as a positive control for Dβ.

Injection of high dose peptide

Mice were injected i.p. with 100 µg of either the hgp100KVP→E29 peptide (KVPNRQDWL) or an irrelevant Dβ-binding peptide SQPKNEEER (R. Dyall, unpublished observations) every 2 days starting 30 days after the third full-length hgp100 DNA immunization, for a total of 7 injections. Mice were then boosted with full-length hgp100 DNA by gene gun 3 days after the final peptide injection. Peptides were provided by Research Genetics (Huntsville, AL).

CD4+ cell depletion

Mice were injected with the mAb GK1.5 from the American Type Culture Collection (Manassas, VA) produced by the Monoclonal Antibody Core Facility at Memorial Sloan-Kettering Cancer Center to deplete CD4+ cells. Mice were injected i.p. with 500 µg of GK1.5 on days 0 and 2, and then every 2 days until the end of the experiment. Depletion of >95% of CD4+ cells was confirmed by FACS of peripheral blood for all mice used in these studies. Mice began DNA immunization on day 4.

Tumor challenge

Mice were injected intradermally with 1 × 106 B16 melanoma cells on the right flank 5 days after the final DNA immunization. The mice were then followed for tumor onset by palpation every other day. Tumor sizes were scored as present once they reached a 2-mm diameter and continued to grow. Mice were sacrificed once it was assured the tumors were progressing (usually at a size of ~1 cm). Kaplan-Meier tumor-free survival curves were constructed and log rank analysis was performed to determine statistical significance.

Peptide-MHC binding assay

Peptide-MHC binding was quantified by determining relative expression of stable MHC molecules on the TAP-deficient cell line RMA-S in the presence of the indicated peptides (22). The Dβ restriction epitope of Flu (ASNENMETM) served as a positive control for DP. The A2.1-restricted HIV Nef peptide (VLEWRFDSRL) served as a negative control.

Depigmentation studies

Depigmentation was objectively scored by producing digital images of the abdomen of immunized and control mice −6 wk after the final immunization (M. E. Engelhorn, unpublished method). The images were obtained by scanning mice on a flatbed document scanner (Hewlett-Packard, Palo Alto, CA). Mean and SD of the luminosity of the pixels for the unimmunized control mouse were obtained by opening the image in Adobe Photoshop 5.5 (Adobe, San Jose, CA), adjusting the contrast to +20, selecting the area for all the mouse abdomens, and constructing a histogram. The threshold for depigmentation was considered to be 2 SD greater than the mean. The mean luminosity of the pixels for the abdomen of each immunized mouse was then similarly obtained and scored as either depigmented or not.

ELISA for detection of Ab responses

Serum from immunized mice were collected −6 wk after the last immunization and tested against cell lysates from both a human (GMEL) and a mouse (B16) melanoma cell line in a modification of a previously described ELISA (24). Both cell lines express the gp100 protein.

Results

Tumor immunity induced by xenogeneic DNA immunization with hgp100 is abolished by high dose hgp100KVP→E29 peptide

We have previously shown that immunization of C57BL/6 mice with full-length hgp100 cDNA, but not syngeneic mouse mgp100, induces tumor protection against challenge with the syngeneic, poorly immunogenic B16 melanoma, which expresses mgp100 (2). We also showed that hgp100 DNA immunization induces T cell immunity against the Dβ-restricted peptide epitope hgp100KVP→E29 and the corresponding syngeneic epitope mgp100KVP→E29.

To test the hypothesis that T cells specific for hgp100KVP→E29 are required for tumor protective immunity against B16, mice previously immunized with hgp100 were tolerized with high dose hgp100KVP→E29 peptide administered without adjuvant. Injection of
mice with a regimen of high dose peptide has been demonstrated to cause rapid expansion, followed by disappearance of peptide-specific T cells (25, 26). No cytotoxic T cell responses were detected in mice tolerized with peptide compared with nontolerized mice (data not shown). As shown in Fig. 1, mice immunized with full-length hgp100 DNA followed by high dose hgp10025–33 peptide are not protected against a subsequent challenge with B16 melanoma compared with nontolerized or naive mice (p = 0.91) (representative of two experiments). Mice immunized with hgp100 DNA followed by a similar treatment with a control peptide were provided the same degree of tumor protection as mice immunized with hgp100 DNA alone (p = 0.66 for control peptide vs no peptide after hgp100 immunization, p = 0.03 for hgp100 immunization plus control peptide vs no immunization). These results are consistent with a necessary role for the hgp10025–33 epitope in tumor immunity after xenogeneic immunization.

The peptide epitope hgp10025–33 has higher binding to D\(^{b}\) than mgp10025–33, and binding of hgp10025–33 to D\(^{b}\) is abrogated by an amino acid change (N→W) at anchor position 29.

One mechanism for a heteroclitic response to a peptide epitope is through increased binding of the heteroclitic peptide to MHC molecules, leading to higher affinity (15, 17–19). The RMA-S stabilization assay was performed to determine relative binding of the D\(^{b}\) epitopes used in this study (Fig. 2).

Table I shows the amino acid sequences of the relevant epitopes from hgp100 and mgp100, including flanking residues. Also shown is the corresponding amino acid sequence for the mutant hgp10029(N→W), in which the asparagine (N) residue at position 29 is changed to tryptophan (W). The N residue at this specific position is the major anchor for hgp100 binding to D\(^{b}\). As shown in Fig. 2, hgp10025–33 was a strong binder to D\(^{b}\), which corroborates a previous report (11), whereas mgp10025–33 was a weaker binder.

The hgp100 25–33 epitope in tumor protection induced by immunization with full-length hgp100 DNA.

![FIGURE 1. Mice immunized with hgp100 DNA after a tolerizing high dose hgp10025–33 peptide are not protected against a subsequent challenge with B16 melanoma. Untreated mice or mice immunized three times weekly with hgp100 DNA were then injected i.p. with 100 \(\mu\)g of hgp10025–33 peptide or an irrelevant D\(^{b}\) binding peptide every 2 days, starting 30 days after immunization for a total of seven injections, where appropriate. Immunized mice were then boosted with hgp100 DNA 3 days after the final peptide injection, and 5 days later they were challenged cutaneously with B16 melanoma. Mice immunized with hgp100 DNA and treated with high dose hgp10025–33 peptide had tumor-free survival that was not significantly different from that of unimmunized mice (p = 0.91) but was worse than that of immunized mice not given the peptide (p = 0.03). Mice immunized with hgp100 DNA and given control peptide or no peptide had equivalent tumor protection (p = 0.66 for hgp100 plus control peptide vs hgp100 alone, p = 0.03 for hgp100 plus control peptide vs no immunization, p = 0.06 for hgp100 plus control peptide vs hgp100 plus hgp10025–33 peptide).](http://www.jimmunol.org/)

For further experiments, the corresponding peptide (aa 25–33) from hgp10029(N→W) had no specific binding to D\(^{b}\).

**Requirement for the high affinity D\(^{b}\)-binding epitope of hgp10025–33 in tumor protection induced by immunization with full-length hgp100 DNA.**

To further test the hypothesis that the presence of the single higher affinity epitope hgp10025–33 is necessary for the tumor protection seen after xenogeneic immunization, site-specific mutants of hgp100 were constructed. The cDNA construct hgp100KVP→EGS was created from hgp100 by replacing the sequence coding for aa 25–27 (KVP) with the corresponding mgp100 sequence (EGS). These three amino acids include predicted minor anchors for binding to D\(^{b}\). Thus, hgp100KVP→EGS comprises the mgp10025–33 epitope embedded in the hgp100 framework, including hgp100-flanking residues (Table I). In contrast, hgp10029(N→W) contains hgp100 wild-type sequences with a single mutation at aa 29 (N→W), the D\(^{b}\) anchor residue (Table I). As seen in Fig. 3, neither immunization with hgp100KVP→EGS nor immunization with hgp10029(N→W) full-length DNA significantly protected against tumor challenge with B16 melanoma, whereas immunization with hgp100 was protective (p < 0.01) (representative of three experiments). Combined results from all 3 experiments show that at 30 days after tumor challenge 19 of 30 mice immunized with full-length hgp100, 4 of 30 mice immunized with hgp100KVP→EGS, 3 of 23 immunized with hgp10029(N→W), and none of the 29 immunized mice were tumor free.

The hgp10025–33 epitope is not required for anti-gp100 Ab responses after hgp100 DNA immunization (Fig. 4). Immunization of mice with hgp100, hgp100KVP→EGS, and hgp10029(N→W) DNA produced similar Ab reactivities when measured against the human gp100\(^{+}\) human melanoma cell line GMEL (p < 0.01, compared with preimmune sera) (Fig. 4A). Immunization with hgp100 DNA produced lower Ab reactivity (p = 0.02 to <0.01 compared with hgp100, hgp100KVP→EGS, and hgp10029(N→W)), but higher Ab reactivity compared with preimmune sera (p < 0.01). The same weaker Ab response against mgp100 (measured against the syngeneic melanoma B16 cell line) was observed in sera of mice immunized with hgp100, hgp100KVP→EGS, and hgp10029(N→W).
mgp100 (p = 0.04 to <0.01 compared with preimmune sera; p < 0.15 compared with each other) (Fig. 4B).

Tumor protection after hgp100 DNA immunization does not require CD4⁺ cells

We have previously shown that hgp100 DNA immunization can elicit tumor protection in MHC class II knockout mice (2). To confirm that CD4⁺ T cells are not required for tumor protection in mice with immune systems that developed under normal physiological conditions, CD4⁺ cells were depleted from adult mice immediately before hgp100 immunization and depletion was maintained throughout the tumor challenge (10 mice per group). As shown in Fig. 5, tumor protection was seen after xenogeneic hgp100 DNA immunization in CD4⁺ cell-depleted mice (p < 0.01). Although greater protection was observed in CD4⁺-competent mice, the difference did not reach statistical significance (p = 0.08). Further studies have shown that CD4⁺ T cells enhance immunity in this model, and we are presently searching for class II MHC-restricted epitopes within hgp100 (data not shown). In summary, these results confirm that the predominant immune mechanism in this model is through CD8⁺ T cells recognizing the class I MHC hgp100₂₅–₃₃ epitope and that T cell help by CD4⁺ T cells can further augment immunity.

Cytoxic T cell responses against the self peptide mgp100₂₅–₃₃ can be induced by immunization with a minigene encoding hgp100₂₅–₃₃

Because immunity induced by full-length hgp100 DNA does not require CD4⁺ T cells or MHC class II, it is possible that immunity against mgp100 could be induced by the MHC class I peptide epitope hgp100₂₅–₃₃ alone. A minigene containing an endoplasmic reticulum insertion signal followed by hgp100₂₅–₃₃ was created and used to immunize mice. Lymphocytes were assessed in a standard 4-h ⁵¹Cr release assay. Mice immunized either with the hgp100₂₅–₃₃ minigene or with the full-length hgp100 cDNA had the same CTL activity against mgp100₂₅–₃₃, as measured by ⁵¹Cr release (Fig. 6).

Tumor immunity against B16 melanoma is induced by immunization with hgp100₂₅–₃₃ minigene

Mice immunized with the hgp100₂₅–₃₃ minigene and challenged with B16 melanoma demonstrated tumor protection that was comparable with protection observed in mice immunized with full-length hgp100 (Fig. 7) (representative of three experiments). In all experiments combined, at 30 days after tumor challenge, 10 of 40 mice immunized with full-length hgp100, 15 of 40 mice immunized with hgp100₂₅–₃₃ minigene, 1 of 18 mice immunized with mgp100₂₅–₃₃ minigene, and 1 of 40 mice left unimmunized were tumor free. Peptide immunization with the hgp100₂₅–₃₃ peptide with the TiterMax adjuvant did not elicit any tumor protection in 2 separate experiments with 10 mice per group (0 of 20 mice, data not shown). These results suggest that there are other unaltered epitopes within gp100 that have not yet been identified and that participate in immunity possibly through epitope spreading, although they are insufficient to induce tumor rejection.

Another measure of immunity against melanosomal differentiation Ags is autoimmunity, manifested as coat color depigmentation (1–4, 7–9). Because melanosomal differentiation Ags are present in normal melanocytes, immunity can result in the destruction of pigment cells in hair follicles, and the degree of coat color hypopigmentation can be easily assessed in live animals. None of the 10 mice immunized with full-length hgp100, as well as none of the 10 mice immunized with a minigene construct based on the D₁ epitope of the influenza matrix-binding protein developed hypopigmentation. Two of nine mice immunized with the hgp100₂₅–₃₃ minigene were depigmented (J. S. Gold, unpublished observations). We believe that hypopigmentation is a real but infrequent consequence of minigene immunization. These results are consistent with a threshold for tumor immunity that is lower than that for autoimmunity after minigene vaccination.

The xenogeneic DNA immunization with hgp100₂₅–₃₃ epitope is necessary and sufficient to induce tumor immunity against melanoma in an H-2b mouse model, but this is accompanied by minimal autoimmunity.

### Table I. Sequences of gp100 site-specific mutant constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>Amino Acid Sequence 15–43</th>
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<tbody>
<tr>
<td>hgp100</td>
<td>...IGALLAVGAT KVPRNQDWL GVSQRLRTKA ...</td>
</tr>
<tr>
<td>hgp100₂₅(N→W)</td>
<td>...IGALLAVGAT KVPRNQDWL GVSQRLRTKA ...</td>
</tr>
<tr>
<td>hgp100₂₇(V→E)</td>
<td>...IGALLAVGAT EGSFRNQDWL GVSQRLRTKA ...</td>
</tr>
<tr>
<td>mgp100</td>
<td>...LSALLAVGAL ...GVPRQVLTVKT...</td>
</tr>
</tbody>
</table>

* Residues in italics and in the box represent the 25–33 epitope. Residues in bold are those that differ from hgp100₂₅–₃₃. Underlined residues, 29 and 33, are the predicted major anchors for D₁.

### FIGURE 3. Presence of the hgp100₂₅–₃₃ epitope is necessary for tumor protection after DNA immunization with full-length hgp100. Mice were immunized three times weekly with hgp100 DNA or the site-specific mutant DNA of hgp100, hgp100₂₇(V→E), or hgp100₂₅(N→W). hgp100₂₇(V→E) is identical with hgp100 except the sequence coding for aa 25–27 has been replaced with the corresponding mouse sequence. hgp100₂₅(N→W) is hgp100 with the codon for aa 29 changed from an asparagine (N) to a tryptophan (W). Five days after immunization, mice were tumor challenged s.c. with B16 melanoma. Tumor-free survival for mice immunized with full-length hgp100 was greater than that for unimmunized mice (p < 0.01). In contrast, tumor-free survival for mice immunized with hgp100₂₇(V→E) or hgp100₂₅(N→W) was not significantly different from that of the unimmunized mice.
compared with preimmune sera; wk after the last immunization. hgp100 KVP xenogeneic immunization with hgp100 DNA. Mice depleted of CD4+/H11001 group of mice that was not depleted of CD4+ T cells by i.p. injection of a CD4-specific mAb were immunized with full-length hgp100 DNA or were left untreated (10 mice per group). Another peptide epitopes, but to our knowledge this has never been previously demonstrated for tumor immunity directly in vivo. It has been postulated that the mechanism underlying xenogeneic immunization for tumor immunity is the creation of heteroclitic epitopes, but to our knowledge this has never been previously demonstrated for tumor immunity directly in vivo. The gp100 Ag was originally defined by mouse mAb and subsequently shown to be recognized by autologous T cells of melanoma patients (20, 27). Previous studies by Overwijk et al. (11) showed that gp100 contained an altered peptide ligand that could be used to activate T cells for adoptive therapy in a syngeneic mouse melanoma model. We have previously shown that active immunization with gp100 was able to induce CD8+ T cell-mediated immunity independently of MHC class II and CD4+ T cells, providing a model to study the requirement of individual MHC class I epitopes (2). We have shown that the induction of tumor immunity depends on the presence of a single heteroclitic MHC class I epitope. Furthermore, this heteroclitic epitope is sufficient to induce tumor-protective immunity when delivered as a minigene construct, and this tumor immunity is associated with only minimal autoimmunity. These results provide support for a strategy to

Discussion
Immunization with syngeneic melanosomal differentiation Ags in the form of recombinant proteins or naked DNA has generally been unsuccessful, whereas xenogeneic vaccination has proved to be a method of inducing immunity against these shared Ags (1–4). It has been postulated that the mechanism underlying xenogeneic immunization for tumor immunity is the creation of heteroclitic peptide epitopes, but to our knowledge this has never been previously demonstrated for tumor immunity directly in vivo. The gp100 Ag was originally defined by mouse mAb and subsequently shown to be recognized by autologous T cells of melanoma patients (20, 27). Previous studies by Overwijk et al. (11) showed that gp100 contained an altered peptide ligand that could be used to activate T cells for adoptive therapy in a syngeneic mouse melanoma model. We have previously shown that active immunization with gp100 was able to induce CD8+ T cell-mediated immunity independently of MHC class II and CD4+ T cells, providing a model to study the requirement of individual MHC class I epitopes (2). We have shown that the induction of tumor immunity depends on the presence of a single heteroclitic MHC class I epitope. Furthermore, this heteroclitic epitope is sufficient to induce tumor-protective immunity when delivered as a minigene construct, and this tumor immunity is associated with only minimal autoimmunity. These results provide support for a strategy to
immunize against tumor Ags using minigenes containing defined heteroclitic epitopes that induce cross-reactivity to tumor Ags.

Delivery of this heteroclitic epitope as a minigene was effective in protecting against a subsequent tumor challenge, whereas vaccination with synthetic peptide in a strong adjuvant was not. The minigene construct presumably leads to more efficient processing and presentation through translation into the endoplasmic reticulum than uptake of peptide through an exogenous pathway, even in the presence of a potent immune adjuvant that presumably induces cytokines, heat shock proteins, and other components that could enhance immunity. Also, the prokaryotic unmethylated CpG motifs in plasmid DNA may enhance the potency of the minigene immunization (28).

Further elucidation of the mechanisms of tumor protection after xenogeneic immunization may allow the creation of more rational vaccines. The design of heteroclitic epitopes for differentiation Ags and their delivery to patients as minigenes may also be a valuable strategy for the immunotherapy of cancer.

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References


