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A Single Heteroclitic Epitope Determines Cancer Immunity After Xenogeneic DNA Immunization Against a Tumor Differentiation Antigen[1]

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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 D^b-restricted peptide, $hgp100_{25–33}$, were incapable of rejecting tumors. Furthermore, mice immunized with DNA constructs of hgp100 in which the $hgp100_{25–27}$ epitope was substituted with the weaker D^b-binding epitope from mgp100 ($mgp100_{25–27}$) or a mutated epitope unable to bind D^b 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-2^b 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 immunological 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)[3] (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 D^b-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-2^b mouse tumor model.

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[3] 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 $hgp100_{KVP\rightarrow EGS}$-pWRG1644 and $hgp100_{29(N\rightarrow W)}$-pWRG1644 using the primers 5′-GCTGTGGGGGCTACAGAAGGATCCAGAAACC AGGACTG-3′ and 5′-CAGTCCTGGTTTCTGGATCCTTCTGTAGCCC CCACAGC-3′ ($hgp100_{KVP\rightarrow EGS}$) as well as 5′-GGGGGCTACAAAA GTACCCAFATGGCAGGACTGGCTTGG-3′ and 5′-CCAAGCCAGTC CTGCCATCTGGGTACTTTTGTAGCCCCC-3′ ($hgp100_{29(N\rightarrow W)}$). The cDNAs coding for hgp100, $hgp100_{KVP\rightarrow EGS}$, and $hgp100_{29(N\rightarrow W)}$ were cleaved from the respective pWRG1644 plasmids using *Not*I, and each was cloned into the vector pWRG7077BEN (21). The inserts were then sequenced to assure that $hgp100_{KVP\rightarrow EGS}$ and $hgp100_{29(N\rightarrow W)}$ differed from hgp100 only at the intended sites. The oligonucleotides 5′-GATCCACC ATGAGATACATGATCCTGGGCCTGCTGGCCCTGGCTGCA-3′ and 5′-GCCAGGGCCAGCAGGCCCAGGATCATGTATCTCATGGTG-3′ were annealed and ligated into pCR3 and digested with *Bam*HI and *Pst*I to make an expression vector coding for MRYMILGLLALAA. This construct incorporates most of the endoplasmic reticulum insertion sequence from the adenovirus E3/19kDa gene product (MRYMILGLLALAAV CSA), with a *Pst*I site following the codon for the 13th amino acid, alanine. This vector was then expanded and sequenced. The remaining endoplasmic reticulum insertion sequence (VCSA) followed by minigenes coding for 1) $hgp100_{25–33}$ (KVPRNQDWL) or $mgp100_{25–33}$ (EGSRNQDWL), 2) the D^b restriction epitope from the influenza matrix protein (Flu) (ASNENMETM), or 3) the K^b epitope from OVA (SIINFEKL) followed by a stop codon were ligated by annealing the respective primer pairs (5′-GTGTG CAGCGCCAAAGTACCCAGAAACCAGGACTGGCTTTGAC-3′ and 5′-TCGAGTCAAAGCCAGTCCTGGTTTCTGGGTACTTTGGCGCTG CACACTGCA-3′ ($hgp100_{25–33}$), 5′-GTGTGCAGCGCCGCCTCCAAC GAGAACATGGAGACAATGTGAC-3′ and 5′-TCGAGTCACATTGTC TCCATGTTCTCGTTGGAGGCGGCGCTGCACACTGCA-3′ (*flu*), and 5′-GTGTGCAGCGCCAGCATCATCAACTTCGAGAAGCTGTGAC-3′ and 5′-TCGAGTCACAGCTTCTCGAAGTTGATGATGCTGGCGCTGC ACACTGCA-3′ (*ova*)) and cloning them into the minigene vector created above, after digestion with *Pst*I and *Xho*I. Sequence fidelity was confirmed by DNA sequencing. All primers and oligonucleotides were obtained from Life Technologies (Gaithersburg, MD).

DNA and peptide immunization

DNA immunization by helium-driven gold particle bombardment via the gene gun has been previously reported (21). In brief, plasmid DNA was coated on 1.0-μm-diameter gold particles (Bio-Rad, Hercules, CA) and precipitated on bullets of Teflon tubing (McMaster-Carr, East Rutherford, NJ). Gold particles containing 1 μg of plasmid DNA were delivered to each abdominal quadrant of a mouse using a helium-driven gene gun (Accell; PowderJect Vaccines, Madison, WI) for a total of four injections. Each mouse was immunized three times at weekly intervals. Peptide immunization was performed by footpad injection with TiterMax emulsion (CytRx, Norcross, GA) containing 5 μg of peptide (10 μl total volume).

Injection of high dose peptide

Mice were injected i.p. with 100 μg of either the $hgp100_{25–33}$ peptide (KVPRNQDWL) or an irrelevant D^b-binding peptide SQPKNEEEI (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 7 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 1×10^5 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. Tumors 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 D^b restriction epitope of Flu (ASNENMETM) served as a positive control for D^b. The A2.1-restricted HIV Nef peptide (VLEWRFDSRL) served as a negative control.

^{51}Cr release assay

Inguinal lymph nodes and spleens obtained from five immunized mice 5 days after the last immunization were pooled, and 1×10^8 cells were cultured for 5 days with 1 μM concentrations of the appropriate peptide, as previously described (22). Lysis of EL4 tumor cells pulsed for 1 h at 37°C with 6 μM peptide was determined by a standard 4-h ^{51}Cr release assay (23).

Depigmentation studies

Depigmentation was objectively scored by producing digital images of the abdomens 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 mice 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

Sera 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 $hgp100_{25–33}$ 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 D^b-restricted peptide epitope $hgp100_{25–33}$ and the corresponding syngeneic epitope $mgp100_{25–33}$.

To test the hypothesis that T cells specific for $hgp100_{25–33}$ are required for tumor protective immunity against B16, mice previously immunized with hgp100 were tolerized with high dose $hgp100_{25–33}$ 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 $hgp100_{25–33}$ 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 $hgp100_{25–33}$ epitope in tumor immunity after xenogeneic immunization.

The peptide epitope $hgp100_{25–33}$ has higher binding to D^b than $mgp100_{25–33}$, and binding of $hgp100_{25–33}$ 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 $hgp100_{29(N→W)}$, 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, $hgp100_{25–33}$ was a strong binder to D^b, which corroborates a previous report (11), whereas $mgp100_{25–33}$ was a weaker binder.

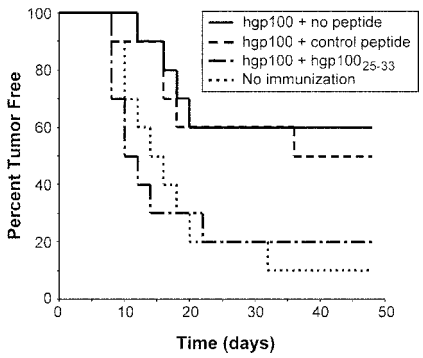

FIGURE 1. Mice immunized with hgp100 DNA after a tolerizing high dose $hgp100_{25–33}$ peptide are not protected against a subsequent challenge with B16 melanoma. Untreated mice or mice immunized three times weekly with hgp100 DNA were then injected i.p. with 100 μg of $hgp100_{25–33}$ peptide or an 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 $hgp100_{25–33}$ 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 $hgp100_{25–33}$ peptide).

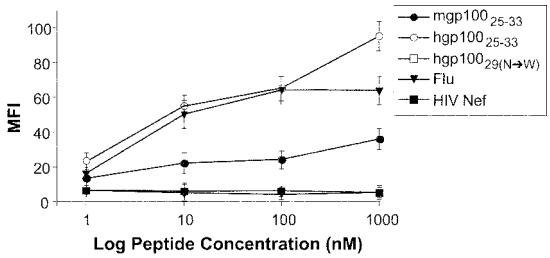

FIGURE 2. $hgp100_{25–33}$ is superior at stabilizing expression of the MHC class I molecule D^b on RMA-S cells compared with $mgp100_{25–33}$. A standard RMA-S stabilization assay was performed as described in *Materials and Methods*. Graph shows mean fluorescence intensity (MFI), which represents staining for D^b with a FITC-conjugated Ab, vs peptide concentration. The peptide encompassing residues 25 to 33 of $hgp100_{29(N→W)}$ is identical with $hgp100_{25–33}$ except for aa 29, which has been changed from asparagine (N), a major anchor residue for D^b, to tryptophan (W). The Flu matrix D^b epitope was used as a positive control, and the HIV Nef HLA-A2.1 epitope was the negative control. Experiments were repeated six times, and results were collated for analysis by two-tailed Student's *t* test with Bonferroni correction. Analysis shows that $hgp100_{25–33}$ binds to D^b significantly better than $mgp100_{25–33}$ ($p < 0.001$) at peptide concentrations of 10, 100, and 1000 nM.

The corresponding peptide (aa 25–33) from $hgp100_{29(N→W)}$ had no specific binding to D^b.

Requirement for the high affinity D^b-binding epitope of $hgp100_{25–33}$ 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 $hgp100_{25–33}$ is necessary for the tumor protection seen after xenogeneic immunization, site-specific mutants of hgp100 were constructed. The cDNA construct $hgp100_{KVP→EGS}$ 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, $hgp100_{KVP→EGS}$ comprises the $mgp100_{25–33}$ epitope embedded in the hgp100 framework, including hgp100-flanking residues (Table I). In contrast, $hgp100_{29(N→W)}$ 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 $hgp100_{KVP→EGS}$ nor immunization with $hgp100_{29(N→W)}$ 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 $hgp100_{KVP→EGS}$, 3 of 23 immunized with $hgp100_{29(N→W)}$, and none of the 29 unimmunized mice were tumor free.

The $hgp100_{25–33}$ epitope is not required for anti-gp100 Ab responses after hgp100 DNA immunization (Fig. 4). Immunization of mice with hgp100, $hgp100_{KVP→EGS}$, and $hgp100_{29(N→W)}$ DNA produced similar Ab reactivities when measured against the human $gp100^+$ human melanoma cell line GMEL ($p < 0.01$, compared with preimmune sera) (Fig. 4*A*). Immunization with mgp100 DNA produced lower Ab reactivity ($p = 0.02$ to <0.01 compared with hgp100, $hgp100_{KVP→EGS}$, and $hgp100_{29(N→W)}$) 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, $hgp100_{KVP→EGS}$, $hgp100_{29(N→W)}$, and

Table I. *Sequences of gp100 site-specific mutant constructs*[a]

Construct	Amino Acid Sequence 15–43		
hgp100	...IGALLAVGAT	*KVPRNQDWL*	GVSRQLRTKA...
$hgp100_{29(N \rightarrow W)}$	...IGALLAVGAT	*KVPR**W**QDWL*	GVSRQLRTKA...
$hgp100_{KVP \rightarrow EGS}$	...IGALLAVGAT	***EGS**RNQDWL*	GVSRQLRTKA...
mgp100	...LSALLAVGAL	***EGS**RNQDWL*	GVPRQLVTKT...

[a] Residues in italics and in the box represent the 25–33 epitopes. Residues in bold are those that differ from $hgp100_{25–33}$. Underlined residues, 29 and 33, are the predicted major anchors for D^b.

mgp100 ($p = 0.04$ to <0.01 compared with preimmune sera; $p < 0.15$ compared with each other) (Fig. 4*B*).

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 $hgp100_{25–33}$ epitope and that T cell help by $CD4^+$ T cells can further augment immunity.

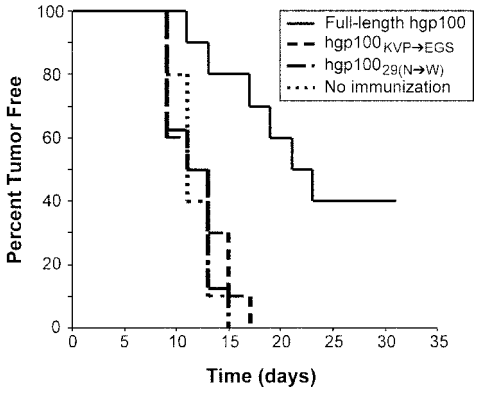

FIGURE 3. Presence of the $hgp100_{25–33}$ epitope is necessary for tumor protection after DNA immunization with full-length hgp100. Mice were immunized three times weekly with hgp100 DNA or the site-specific mutant DNA of hgp100, $hgp100_{KVP \rightarrow EGS}$, or $hgp100_{29(N \rightarrow W)}$. $hgp100_{KVP \rightarrow EGS}$ is identical with hgp100 except the sequence coding for aa 25–27 has been replaced with the corresponding mouse sequence. $hgp100_{29(N \rightarrow W)}$ 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 $hgp100_{KVP \rightarrow EGS}$ or $hgp100_{29(N \rightarrow W)}$ was not significantly different from that of the unimmunized mice.

Cytotoxic T cell responses against the self peptide $mgp100_{25–33}$ can be induced by immunization with a minigene encoding $hgp100_{25–33}$

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 $hgp100_{25–33}$ alone. A minigene containing an endoplasmic reticulum insertion signal followed by $hgp100_{25–33}$ was created and used to immunize mice. Lymphocytes were assessed in a standard 4-h ^{51}Cr release assay. Mice immunized either with the $hgp100_{25–33}$ minigene or with the full-length hgp100 cDNA had the same CTL activity against $mgp100_{25–33,}$ as measured by ^{51}Cr release (Fig. 6).

Tumor immunity against B16 melanoma is induced by immunization with $hgp100_{25–33}$ minigene

Mice immunized with the $hgp100_{25–33}$ 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 $hgp100_{25–33}$ minigene, 1 of 18 mice immunized with $mgp100_{25–33}$ minigene, and 1 of 40 mice left unimmunized were tumor free. Peptide immunization with the $hgp100_{25–33}$ 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 D^b epitope of the influenza matrix-binding protein developed hypopigmentation. Two of nine mice immunized with the $hgp100_{25–33}$ 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 $hgp100_{25–33}$ epitope is necessary and sufficient to induce tumor immunity against melanoma in an H-2^b mouse model, but this is accompanied by minimal autoimmunity.

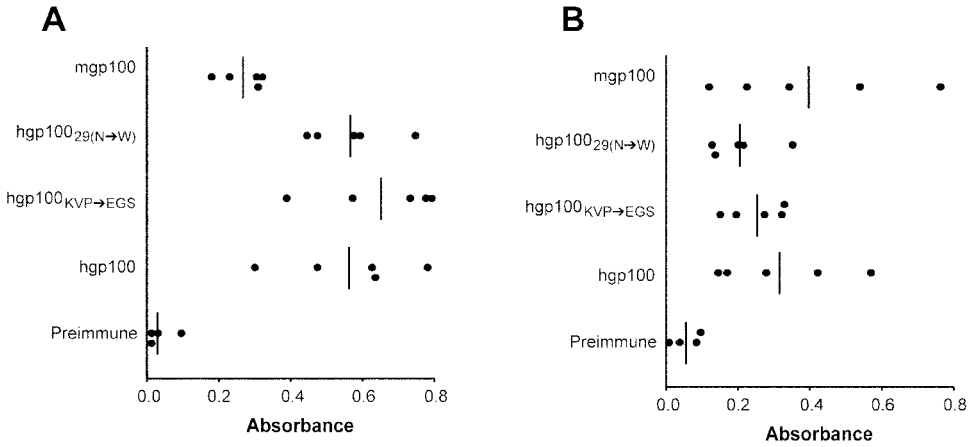

FIGURE 4. Induction of Abs against the syngeneic melanoma B16 and the human melanoma GMEL after immunization of mice with hgp100 DNA does not depend on the epitope hgp100$_{25–33}$. Mice were immunized with hgp100, hgp100$_{KVP\rightarrow EGS}$, hgp100$_{29(N\rightarrow W)}$, or mgp100 DNA. Serum was drawn ~6 wk after the last immunization. hgp100$_{KVP\rightarrow EGS}$ is identical with hgp100 except the sequence coding for aa 25–33 has been replaced with the corresponding mouse sequence. hgp100$_{29(N\rightarrow W)}$ is hgp100 with the codon for aa 29 changed from asparagine (N) to tryptophan (W). Each point represents the IgG Ab reactivity of serum from an individual mouse binding to cell lysates of hgp100-negative or mgp100-positive cells, when tested at a 1/50 serum dilution. The lines represent the mean absorbance for the groups. Whereas the ELISA against GMEL (hgp100-positive; *A*) showed that immunization with hgp100, hgp100$_{KVP\rightarrow EGS}$, hgp100$_{29(N\rightarrow W)}$, and mgp100 produced Ab reactivity greater than that seen with preimmune sera ($p < 0.01$), mgp100 immunization produced less Ab reactivity than the other groups ($p \leq 0.02$). Similarly, ELISA against B16 (mgp100-positive; *B*) showed that immunization with hgp100, hgp100$_{KVP\rightarrow EGS}$, hgp100$_{29(N\rightarrow W)}$, and mgp100 produced equivalent Ab reactivity that was greater than the one seen with preimmune sera ($p \leq 0.03$, compared with preimmune sera; $p < 0.15$, compared with each other).

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 hgp100 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 hgp100 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

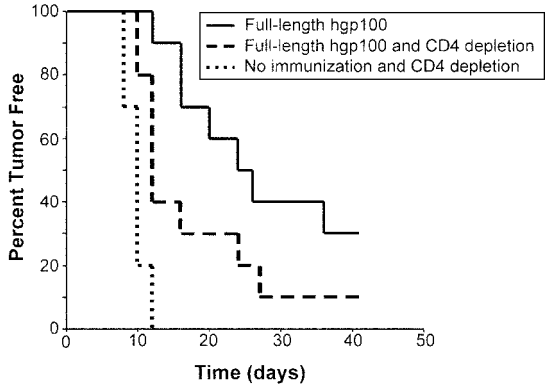

FIGURE 5. CD4$^+$ T cells are not necessary for tumor protection after xenogeneic immunization with hgp100 DNA. Mice depleted of CD4$^+$ T cells by i.p. injection of a CD4-specific mAb were immunized with full-length hgp100 DNA or were left untreated (10 mice per group). Another group of mice that was not depleted of CD4$^+$ T cells was simultaneously immunized with hgp100 (10 mice per group). Five days after the last immunization, mice were challenged intradermally with B16 melanoma. Tumor-free survival for CD4$^+$ T cell-depleted mice immunized with hgp100 DNA was greater than that of untreated depleted mice ($p < 0.01$). However, tumor-free survival for immunocompetent mice immunized with hgp100 DNA was not significantly greater than that of mice depleted of CD4$^+$ T cells and immunized with hgp100 ($p = 0.08$).

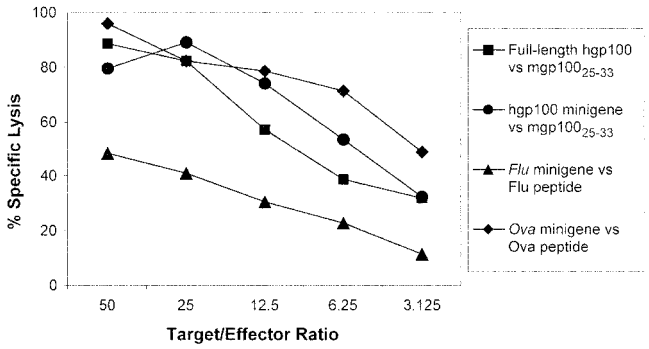

FIGURE 6. Immunization with a minigene construct, hgp100$_{25–33}$, induces CTL responses against the self peptide mgp100$_{25–33}$. Results of a standard 4-h ^{51}Cr release assay are shown. Immunization of mice with full-length hgp100 or the hgp100 minigene induced comparable lysis of syngeneic EL-4 target cells pulsed with the mgp100$_{25–33}$. Immunization of mice with the *flu* matrix protein gene or the *ova* gene induced lysis of target cells pulsed with the D^b-restricted Flu peptide or the K^b-restricted Ova peptide, respectively. Background lysis of target cells pulsed with an irrelevant peptide (Flu peptide for full-length hgp100- and hgp100 minigene-immunized mice, and mgp100$_{25–33}$ peptide for mice immunized with *flu* or *ova* minigenes) was <15% of total lysis.

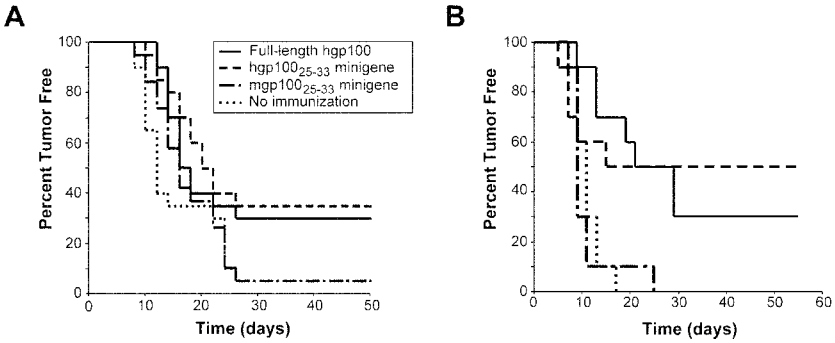

FIGURE 7. Immunization of mice with the hgp100$_{25–33}$ minigene construct induces tumor-protective immunity against a subsequent tumor challenge with B16 melanoma. Mice were immunized with full-length hgp100, hgp100 minigene$_{25–33}$, or mgp100$_{25–33}$ minigene DNA or were left unimmunized. Five days after the last immunization, mice were challenged with B16 melanoma intradermally. The experiment was performed twice with 20 mice per group (*A*) or 10 mice per group (*B*). Both immunizations with full-length hgp100 and with the hgp100$_{25–33}$ minigene produced significantly greater tumor protection than in unimmunized mice ($p = 0.02$ and $p = 0.01$, respectively), whereas immunization with the mgp100$_{25–33}$ minigene did not protect mice compared with no immunization. In a separate experiment (*B*), full-length hgp100 immunization provided significant tumor protection ($p < 0.01$), whereas hgp100$_{25–33}$ minigene provided a trend toward tumor protection ($p = 0.06$) compared with no immunization. Immunization with the mgp100$_{25–33}$ minigene provided no tumor protection compared with the control.

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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