gp100/pmel 17 Is a Murine Tumor Rejection Antigen: Induction of "Self"-reactive, Tumoricidal T Cells Using High-affinity, Altered Peptide Ligand

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Summary

Many tumor-associated antigens are nonmutated, poorly immunogenic tissue differentiation antigens. Their weak immunogenicity may be due to "self"-tolerance. To induce autoreactive T cells, we studied immune responses to gp100/pmel 17, an antigen naturally expressed by both normal melanocytes and melanoma cells. Although a recombinant vaccinia virus (rVV) encoding the mouse homologue of gp100 was nonimmunogenic, immunization of normal C57BL/6 mice with the rVV encoding the human gp100 elicited a specific $CD8^+$ T cell response. These lymphocytes were cross-reactive with mgp100 in vitro and treated established B16 melanoma upon adoptive transfer. To understand the mechanism of the greater immunogenicity of the human version of gp100, we characterized a 9-amino acid (AA) epitope, restricted by H-$2D^b$, that was recognized by the T cells. The ability to induce specific T cells with human but not mouse gp100 resulted from differences within the major histocompatibility complex (MHC) class I–restricted epitope and not from differences elsewhere in the molecule, as was evidenced by experiments in which mice were immunized with rVV containing minigenes encoding these epitopes. Although the human ($hgp100_{25-33}$) and mouse ($mgp100_{25-33}$) epitopes were homologous, differences in the three NH_2-terminal AAs resulted in a 2-log increase in the ability of the human peptide to stabilize "empty" D^b on RMA-S cells and a 3-log increase in its ability to trigger interferon γ release by T cells. Thus, the fortuitous existence of a peptide homologue with significantly greater avidity for MHC class I resulted in the generation of self-reactive T cells. High-affinity, altered peptide ligands might be useful in the rational design of recombinant and synthetic vaccines that target tissue differentiation antigens expressed by tumors.

Key words: melanoma • tumor-associated antigen • gp100 • xenoimmunization • $CD8^+$ T lymphocyte

The recent cloning of tumor antigens recognized by T cells has caused considerable interest in the development of antigen-specific cancer vaccines (1–3). Some antigens are especially attractive candidates for use in vaccines due to their shared nature between individuals, including the melanocyte differentiation antigens (MDA)[1] gp100, melanoma antigen recognized by T lymphocytes (MART)-1, and tyrosinase (2), as well as several proteins in the MAGE family (1). However, as indicated by results from clinical trials thus far, inducing therapeutic T cells to these antigens has been difficult. One reason for the apparent hyporesponsiveness of the human immune system to many tumor antigens may be that they are normal, nonmutated "self"-proteins, expressed on normal tissues as well as on tumor cells. An incomplete understanding of the processes of central and peripheral tolerance has hampered the development of successful cancer vaccines targeting these autoantigens, limiting the use of the growing number of candidate tumor antigens.

[1] *Abbreviations used in this paper:* AA, amino acid; β-gal, β-galactosidase; CM, complete medium; DC, dendritic cell; ER, endoplasmic reticulum; FPV, fowlpox virus; h, human; m, mouse; MART, melanoma antigen recognized by T lymphocytes; MDA, melanocyte differentiation; NP, the nucleoprotein from influenza A; pDNA, plasmid DNA; rVV, recombinant vaccinia virus; TAP, the transporter associated with antigen processing; TRP, tyrosinase-related protein.

The absence of an immune response to a defined autoantigen can be due to negative selection of self-antigen–specific T cells during maturation in the thymus, termed "central" tolerance (4). A low level of autoreactivity is required for positive selection in the thymus (5, 6).

T cells with low reactivity to autoantigens thus persist. Mature T lymphocytes with reactivity to self-antigens may remain in a functionally tolerant state, termed "ignorance", if they do not traffic to antigen-bearing cells, or if the target antigen is not processed and presented to a level that can trigger the specific TCR. Mature self-reactive T cells that encounter antigen on normal tissues in the absence of an activating costimulatory microenvironment can be functionally eliminated by anergization or physically by deletion, thus effecting extrathymic or peripheral tolerance (7, 8).

The mechanisms of breaking tolerance to self-antigens may be relevant for the induction of immune responses to tissue differentiation antigens expressed by tumors. To study requirements for activation of self-reactive, tumor-specific T cells specific for a naturally expressed antigen, we targeted gp100, a normal, nonmutated MDA. In humans, gp100 is expressed both by normal melanocytes and the majority of malignant melanomas tested (9). $CD8^+$ T lymphocytes with reactivity to gp100 have been detected in patients with metastatic melanoma. The mouse homologue for gp100, also known as pmel 17, has been cloned previously (10, 11), and like its human counterpart is normally expressed in melanocytes in an unmanipulated C57BL/6 mouse as well as in mouse melanomas. We sought to determine the requirements to break T cell tolerance to a naturally expressed self-antigen. Furthermore, we evaluated the functional characteristics of autoreactive T cells in the recognition and destruction of a spontaneous mouse melanoma, B16, in vivo.

Materials and Methods

Animals and Cell Lines. Female C57BL/6 ($H-2^b$) mice, 6–10 wk old, obtained from Frederick Cancer Research Center (Frederick, MD) and maintained in a barrier facility, were used for all experiments. EL-4 thymoma ($H-2^b$) and the derived β-galactosidase (β-gal)–transfected clone E22 have been described previously (12). B16 ($H-2^b$), hereafter named B16.WT, is a spontaneous murine melanoma expressing gp100, MART-1, tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2 by FACS® and Western blot analysis (data not shown). B16.B7-1 is a hyperpigmented clone of B16.WT that was stably transfected using a Moloney mouse leukemia virus encoding the gene for B7-1 driven by a LTR promoter. JB/MS is a pigmented, chemically induced melanoma expressing gp100, provided by Dr. Vincent Hearing (National Cancer Institute, NIH, Bethesda, MD). $293K^b$ and $293K^bD^b$ are highly transfectable human renal 293 cells stably transfected with K^b and K^b plus D^b, respectively. RMA/S ($H-2^b$) is a cell line deficient in endogenous peptide loading (13). EL-4, B16.WT, RMA/S, MCA205, and MC38, a C57BL/6-derived colon carcinoma, were maintained in complete medium (CM; RPMI 1640 with 10% heat-inactivated fetal bovine serum [FBS; Biofluids, Rockville, MD], 0.03% l-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 50 μg/ml gentamycin sulfate [NIH Media Center, Bethesda, MD]). B16.B7-1 and E22 were maintained in CM with 400 μg/ml of bioactive G418. $293K^b$ and $293K^bD^b$ were maintained in DMEM with 10% heat-inactivated FBS, 0.03% l-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, 50 μg/ml gentamycin sulfate, and 400 μg/ml of bioactive G418.

Peptides. All synthetic peptides were synthesized using regular F-MOC chemistry. The following synthetic peptides were all synthesized by Peptide Technologies (Washington, D.C.) to a purity >99% by HPLC and amino acid (AA) analysis: KVPRNQDWL, spanning AAs 25–33 of human (h)gp100; EGSRNQDWL, spanning AAs 25–33 of mouse (m)gp100; ASNENMETM, spanning AAs 366–374 of the nucleoprotein of influenza A (NP); DAPIYTNV, spanning AAs 96–103 of *Escherichia coli* β-gal (12); and SIINFEKL, spanning AAs 257–264 of chicken OVA. The NP, hgp100, and mgp100 peptides are restricted by $H-2D^b$, and the β-gal and OVA peptides by $H-2K^b$.

Recombinant Viruses. All recombinant vaccinia viruses (rVVs) used in this study were generated by insertion of the foreign genes by homologous recombination and subsequent purification of recombinant progeny as described by Earl et al. (14). rVVs encoding the human and mouse gp100 epitopes as minigenes were constructed using a recombination plasmid, pKT1401, containing an early promoter driving the signal sequence of the adenovirus E3/19k protein followed by an additional Ala using code GCA, resulting in a putative signal peptide cleavage site, and fused to oligonucleotides encoding the epitope sequences for mgp100, hgp100, or β-galactosidase as described above. pKT1401 was recombined into the VV locus encoding the small subunit of viral ribonucleotide reductase. Correct integration was checked by PCR-based viral genome analysis using primers flanking the viral locus encoding the small subunit of ribonucleotide reductase (12). rVVmgp100, rVVmMART-1, rVVmTyr, rVVmTRP-1, and rVVmTRP-2 were based on the plasmid pSC65, in which the completely synthetic early/late promoter $pS_{E/liter}$ drives expression of the antigen and the early/late promoter $p7.5_{E/liter}$ drives expression of the LacZ gene (15). The $rVVS_{E/L}$β-gal has been described previously (15, 16). Cloning of the genes for mgp100 and mMART-1/Melan-A has been described previously (11), and the cDNAs for hgp100, mTyr, mTRP-1, and mTRP-2 were gifts from Dr. Y. Kawakami (Surgery Branch, NIH; reference 9), Dr. H. Yamamoto (Tohoku University, Sendai, Japan; reference 17), Dr. S. Shibahara (Friedrich Miescher Institut, Basel, Switzerland; reference 18), and Dr. V. Hearing (Laboratory of Cell Biology, NCI, NIH; reference 19), respectively. The rVVhgp100, recombinant fowlpox virus (rFPV)hgp100, rFPVmgp100, and rFPVβ-gal were provided by Therion Biologics Corp. (Boston, MA). Plasmid DNA constructs (pDNA) were based on the pCDNA3 backbone, and encoded hgp100, mgp100, or β-galactosidase under the control of the CMV promoter. Expression of rVV, rFPV, and pDNA were confirmed by immunostaining (20) as well as by Western blot of transfected and infected cells using antisera (21, 22) provided by Dr. V. Hearing. Recombinant adenoviruses were provided by Dr. Bruce Roberts (Genzyme Corp., Framingham, MA), and encoded the genes for hgp100, mgp100, or β-galactosidase under the control of the CMV promoter (11).

Generation of gp100-reactive T Cell Line. Mice were vaccinated twice at 3-wk intervals by a hand-held helium-driven device (Geniva Inc., Middletown, WI) with 1 μg of hgp100 DNA (23), and 3 wk after the second vaccination splenocytes were cultured with rVVhgp100-infected dendritic cells (DCs), generated as previously described (16) in CM with 30 IU/ml rhIL-2 (a gift from Chiron Corp., Emeryville, CA) for 7 d, and were subsequently restimulated every 7–10 d with 2×10^5 B16.B7-1

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(60,000 rad) and 5 × 10^6 irradiated splenocytes (1,500 rad) in CM with 30 IU/ml rhIL-2. T cell clones were derived by limiting dilution using 0.3 T cells per well cocultured with 4 × 10^5 irradiated splenocytes and 2 × 10^4 irradiated B16.B7-1 cells in U-bottomed 96-well plates. The generation of the β-gal reactive cell line has been described previously (12). All T cells were used between 5 and 10 d after restimulation. For peptide-induced T cells, mice were vaccinated as specified in figure legends. Spleens were harvested on day 21 after the last vaccination, separated into single cell suspension, and cultured in T-25 flasks in 15 ml CM at 5 × 10^7 cells per flask. Peptide was added to a final concentration of 1 μM. After 6 d of culture, cells were washed in CM and used in a cytokine release assay.

Cytokine Release Assay. To determine cytokine release, 5 × 10^4 target cells per well were added to effector T cells in round-bottomed 96-well plates at an E/T ratio of 1:1 for established T cell lines and 5:1 for secondary cultures, and were incubated for 24 h in CM. Supernatants were collected and tested using the mIFN-γ ELISA kit (Endogen, Cambridge, MA) or the mGM-CSF ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's protocol. rVV-, rFPV- and rAd-infected targets were infected at multiplicities of infection of >10, >10, and >100, respectively, incubated for 2 h, and then washed twice in CM. Peptide-pulsing was performed at 1 μM in CM for 2 h at 37°C and followed by two washes in CM. Transfected targets were prepared using Lipofectamine (GIBCO BRL, Gaithersburg, MD) following manufacturer's guidelines.

MHC Class I Stabilization Assay. RMA/S cells were incubated at room temperature for 24 h, pulsed with peptide at the indicated concentrations for 45 h at room temperature, and then incubated at 37°C for 4 h to allow turnover of "empty" MHC class I molecules (13). Cells were then stained with FITC-conjugated D^b-specific mAb KH95 (PharMingen, San Diego, CA) for 1 h at 4°C and staining was asserted using a FACScan® (Becton Dickinson, Sunnyvale, CA).

Adoptive Transfer Treatment. For adoptive transfer experiments, mice were injected with 2 × 10^5 B16 intravenously on day 0 followed by intravenous injection of the indicated number of T cells on day 3. rhIL-2 (3 × 10^5 IU) was given twice daily intraperitoneally for 5 d starting immediately after adoptive transfer.

Alternatively, mice were injected intravenously with the indicated number of T cells, followed 5 d later by 2 × 10^5 B16 intravenously. Mice were killed on day 17 after tumor injection and pulmonary nodules were enumerated. All in vivo experiments were performed in a blinded, randomized fashion.

Results

gp100 Is Recognized by Mouse T Cells. To generate gp100-specific T cells, mice were immunized with mgp100 encoded in plasmid DNA or rVV, two vectors with proven efficacy in the generation of specific T cells (3, 23, 24). Splenocytes from immunized mice were restimulated in vitro using syngeneic DCs infected with appropriate rVV, rFPV, or rAd, and subsequent restimulations were performed with irradiated splenocytes and irradiated B16.B7-1, a clone of B16 retrovirally transduced to express the costimulatory molecule CD80 (B7-1). None of the cultures exhibited gp100-specific reactivity when tested by IFN-γ release against gp100-positive targets (data not shown). Since xenogeneic antigen can, in some instances, induce immune reactivity where the autologous antigen failed (25–27), mice were immunized with pDNA encoding hgp100.

A $CD4^-CD8^+$ lytic T lymphocyte line was generated by gene-gun administration of pDNA encoding hgp100 followed by restimulation of splenocytes ex vivo with DCs that had been infected with rVVhgp100. After two subsequent in vitro restimulations with B16.B7-1 melanoma, the T cells recognized B16 melanoma, as well as the immortalized normal melanocyte line Melan-A, with a high degree of specificity (Fig. 1). The EL-4 thymoma and the MC38 sarcoma were not recognized. However, recognition could be conferred upon 293 K^bD^b cells after infection with rVV encoding either mgp100 or hgp100, but not after infection with rVV containing any of the other known MDAs, tyrosinase, TRP-1, or TRP-2.

Antitumor Activity of gp100-specific T Cells. We sought to evaluate the relevance of mgp100 as a tumor rejection antigen. To exclude the potential influence of T cells with a specificity other than gp100, the bulk T cell line was cloned by limiting dilution. 12 clones were evaluated and each had reactivities identical to the bulk T cell line shown in Fig. 1. The gp100-reactive T cell clone (clone no. 9) was tested in vitro (Fig. 2 *A*) and in vivo (Fig. 2 *B*). Mice bearing 3-d-old B16 pulmonary nodules were infused with clone no. 9 followed by rIL-2, resulting in a dramatic tumor destruction (P <0.0001, 4 × 10^6 β-gal–specific T cells + rhIL-2 versus 4 × 10^6 clone no. 9 T cells + rhIL-2), whereas treatment was ineffective using control β-gal–reactive T cells or rIL-2 alone (P >0.5 versus no treatment) (Fig. 2 *B*).

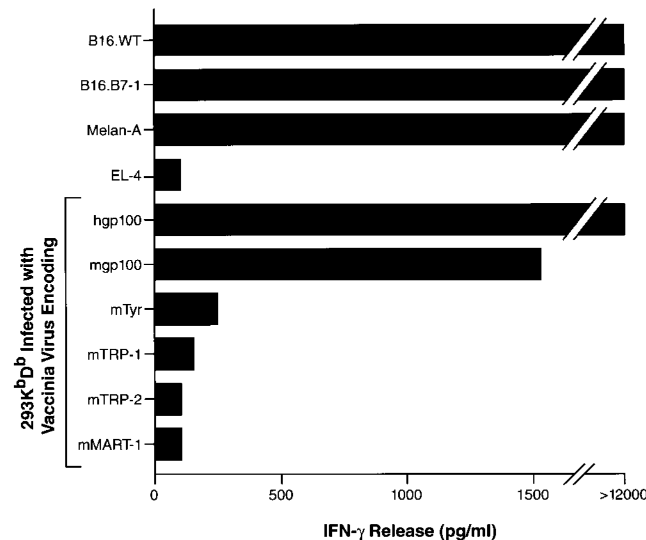

Figure 1. B16 melanoma-reactive T cells specifically recognize nonmutated gp100. Splenocytes from hgp100-immunized mice were cultured as described in Materials and Methods, and cocultured for 24 h with various targets shown on the ordinate, including B16 melanoma, the immortalized normal melanocyte line Melan-A, and human 293 kidney cells expressing the mouse restriction elements H-$2K^b$ and H-$2D^b$ infected with rVV encoding mouse melanocyte differentiation antigens. Supernatants were assayed for IFN-γ by ELISA. Specific IFN-γ release was detected against targets expressing mgp100 or hgp100.

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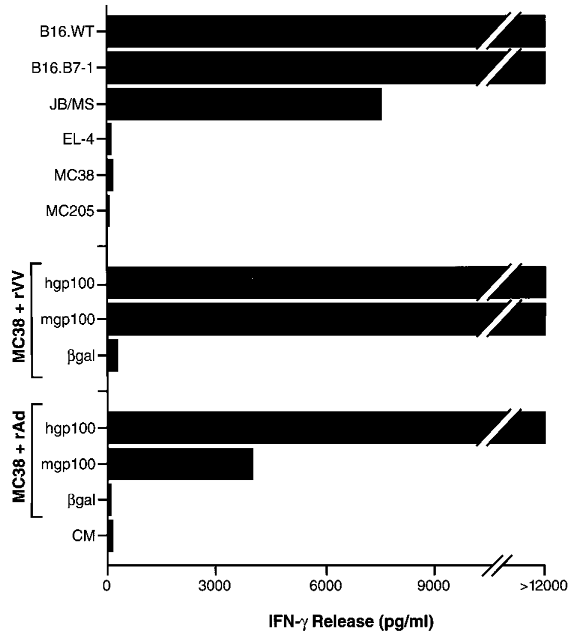

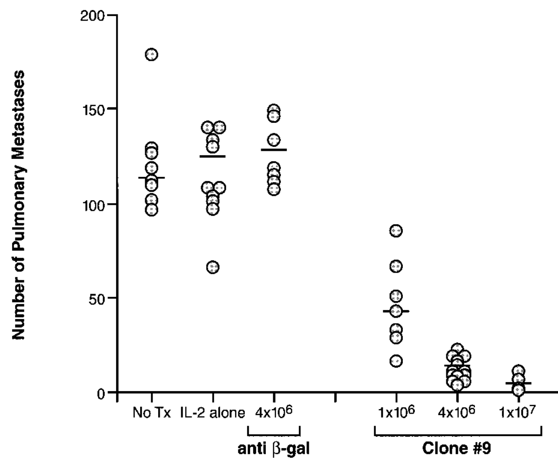

Figure 2. gp100 is a tumor rejection antigen. (*A*) A clone of bulk gp100-reactive T cells, clone no. 9, was established by limiting dilution at 0.3 cells/well. Upon coculture, clone no. 9 specifically recognized B16.WT, B16.B7-1, and JB/MS melanomas, but not control H-2^b tumors EL-4, MCA205, and MC38. Clone no. 9 also recognized MC38 sarcoma infected with either rVV or rAd encoding hgp100 or mgp100, but not control rVV or rAd. (*B*) Specific in vivo reactivity of gp100-specific T cell clone no. 9 was evaluated by injecting mice with 2×10^5 B16.WT cells intravenously. 3 d later, the specified numbers of clone no. 9 T cells were adoptively transferred. 3×10^5 IU rhIL-2 was then given intraperitoneally two times daily for 5 d. Mice were killed 18 d after tumor inoculation and pulmonary nodules were enumerated. Transfer of T cell clone no. 9, but not β-gal–specific T cells or rhIL-2 alone, dramatically reduced the number of pulmonary nodules in a dose-dependent manner, indicating that gp100 can function as a tumor rejection antigen in vivo. Additional independent clones yielded similar results in repeat experiments.

Separate gp100-reactive T cells clones were comparable in their ability to reject B16 in vivo (data not shown). These results suggest that gp100 functions as a true tumor rejection antigen in established murine melanoma.

Identification of a Cross-reactive, MHC Class I–restricted Epitope in gp100. We sought to understand why T cells could only be induced by the xenogeneic, human form of gp100 and not the self sequence. To determine whether the difference in immunogenicity resided in the actual peptides from gp100 that were recognized by T cells, or in differences in sequences outside of those recognized, we characterized the epitopes recognized by the T cell clone. To identify the MHC class I molecule that restricted gp100 recognition, we used the human renal cell carcinoma line, 293, stably transfected with the mouse restriction elements, K^b and D^b. Both lines were transiently transfected with plasmids encoding either mouse or human gp100, or with a control plasmid encoding NP. Only 293 K^bD^b cells (and not 293 K^b) cells expressing mgp100 or hgp100 triggered the release of IFN-γ from gp100-reactive T cells, suggesting that D^b was the dominant and perhaps only restriction element for gp100 recognition (Fig. 3 *A*).

At 626 AAs in length, many possible epitopes in gp100 could be recognized by gp100-reactive T cells. To reduce the number of candidate peptides, a set of progressively shorter versions of the gene encoding hgp100 was used (28). These shortened forms were generated by 3′ exonuclease digestion or PCR amplification of the original hgp100 cDNA. Fragments were then transfected into 293 K^bD^b cells that were used as targets for T cell recognition (Fig. 3 *B*). Even the shortest fragment of the hgp100 cDNA, with a length of 300 bp by gel electrophoresis, conferred a high degree of recognition upon the 293 transfectants, implying that a major epitope was located in the first 100 AAs of the molecule.

To identify the sequence of the epitope, 9-AA-long peptides were evaluated for their potential to bind to D^b using a computer-generated epitope forecast, based on previously published peptide binding data, that is designed to predict binding affinity for a variety of human and mouse MHC class I alleles (29, 30; and http://bimas.dcrt.nih.gov/molbio/hla_bind). Based on these predictions, sets of synthetic peptides were made from both hgp100 and mgp100, which although similar are not identical. When the peptides were added to cultures of gp100-reactive T cells, one peptide pair, $gp100_{25-33}$, was recognized with a high degree of specificity in a GM-CSF release assay (Fig. 3 *C*).

T Cell Reactivity to gp100 Can Be Induced by Xenoimmunization. With the identification of a defined peptide epitope, we explored the immunological mechanism underlying the disparate ability of mgp100 and hgp100 to induce gp100-specific T cells. Mice were vaccinated with rVVmgp100, encoding mgp100, or rVVhgp100, encoding hgp100, and splenocytes were isolated after 3 wk and stimulated for 6 d in vitro with synthetic peptides corresponding to $hgp100_{25-33}$, $mgp100_{25-33}$, or $OVA_{257-264}$, and then tested in a GM-CSF release assay (Table 1, *first two columns*). Splenocytes from

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A	
293 K^b	IFN-γ (pg/ml)
pcDNA3 mgp100	764
pcDNA3 hgp100	823
pcDNA3 control	704
293 K^bD^b	
pcDNA3 mgp100	>20,000
pcDNA3 hgp100	>20,000
pcDNA3 control	3074

B	
293 K^bD^b (bp)	IFN-γ (pg/ml)
pcDNA3 $hgp100_{1-1986}$	>12,000
pcDNA3 $hgp100_{1-1700}$	>12,000
pcDNA3 $hgp100_{1-1100}$	>12,000
pcDNA3 $hgp100_{1-800}$	>12,000
pcDNA3 $hgp100_{1-700}$	>12,000
pcDNA3 $hgp100_{1-300}$	>12,000
pCRIII $mgp100_{1-1878}$	3346
pcDNA3 control	100

C	
Peptide (aa)	GM-CSF (pg/ml)
$hgp100_{7-15}$	2
$mgp100_{7-15}$	0
$hgp100_{25-33}$	**119**
$mgp100_{25-33}$	**116**
$hgp100_{41-49}$	6
$mgp100_{41-49}$	0
$hgp100_{55-63}$	2
$mgp100_{55-63}$	0
$hgp100_{63-71}$	2
$mgp100_{63-71}$	2
$hgp100_{68-76}$	2
$mgp100_{68-76}$	4
$hgp100_{70-78}$	3
$mgp100_{70-78}$	7
$hgp100_{77-85}$	3
$mgp100_{77-85}$	0
$hgp100_{78-86}$	3
$mgp100_{78-86}$	0
$hgp100_{85-93}$	0
$mgp100_{85-93}$	1
control	3

Figure 3. B16-specific T cells recognize a D^b-restricted 9-AA peptide from gp100. In experiment 1, human 293 kidney cells stably transfected with K^b and D^b were transfected with human and mouse gp100 pDNA and cocultured for 24 h with gp100-specific T cells. Supernatants were assayed for IFN-γ by ELISA. Specific IFN-γ release was detected when T cells were cocultured with transfected 293 K^bD^b, but not 293 K^b, indicating that gp100 recognition was predominantly D^b-restricted. In experiment 2, human 293 kidney cells stably transfected with K^b and D^b were transfected with 3′ exonuclease truncated constructs of hgp100 pDNA and cocultured for 24 h with gp100-reactive T cells. Supernatants were assayed for IFN-γ by ELISA. Specific IFN-γ release was detected with each truncated construct, including the 300-bp cDNA fragment, indicating that a specific immunogenic peptide was located in the 100 NH_2-terminal AAs of the gp100 molecule. In experiment 3, peptides corresponding to the binding motif for D^b were identified in the first 100 residues of the hgp100 molecule and synthesized together with the corresponding sequences from mgp100. Individual peptides were added to gp100-reactive T cells, and supernatants were assayed for GM-CSF by ELISA. Specific GM-CSF release was detected with peptide pair $gp100_{25-33}$, suggesting it was the 9-AA epitope responsible for the gp100-reactivity of the T cells.

mice vaccinated with rVVmgp100 and restimulated with $mgp100_{25-33}$ or $hgp100_{25-33}$ peptide failed to develop peptide reactivity. Conversely, splenocytes from mice vaccinated with hgp100 and restimulated with either $mgp100_{25-33}$ or $hgp100_{25-33}$ peptide developed reactivity to both peptides. Similar results were obtained using splenocytes from mice vaccinated with recombinant fowlpoxvirus or plasmid DNA (data not shown). These observations indicated that T cells recognizing mgp100 could be induced exclusively by xenoimmunization with the hgp100 molecule.

Increased Immunogenicity of hgp100 Is Intrinsic to the MHC Class I–restricted Epitope. Several mechanisms could account for the apparent immunological unresponsiveness to mgp100 and the ability of hgp100 to break it. Nonhomologous regions of the full-length hgp100 (which is 76% identical to mgp100 at the AA level; reference 11) could result in intramolecular epitope-spreading (31, 32) or facilitate antibody-mediated antigen capture by APCs (26, 33–35). Alternatively, sequence differences in the relevant epitopes or their flanking sequences could result in differential proteolytic cleavage or transporter associated with antigen processing (TAP)–mediated transport across the endoplasmic reticulum (ER) membrane (36). To explore these possibilities, we constructed a series of rVV-containing minigenes encoding the relevant 9-AA T cell epitopes preceded by the E3/19K adenoviral ER-insertion signal sequence (ES), previously shown to result in TAP-independent transport of antigenic peptides (37), and followed by a double stop codon. These constructs eliminated differences in flanking sequences and other nonhomologous regions of the molecule.

Mice were vaccinated with rVVESmgp100, rVVEShgp-100_{25-33}, or rVVESβ-gal_{96-103}, encoding the 9-AA mgp-100_{25-33}, $hgp100_{25-33}$, and β-gal_{96-103} peptides, respectively (38). Splenocytes were cultured for 6 d with $mgp100_{25-33}$ or $hgp100_{25-33}$ peptides then tested for specificity in a cytokine release assay (Table 1). Reactivities were similar to those obtained with rVV encoding full-length gp100 molecules: immunization with the mouse minigene gp100 construct failed to induce T cells, whereas immunization with the hgp100 construct induced T cells that were cross-reactive with both $mgp100_{25-33}$ and $hgp100_{25-33}$ peptides. These results were not consistent with a major role for differential peptide processing and transport, antibody-facilitated antigen presentation, or intramolecular immunodominance in the differential in vivo immunogenicity of mgp100 and hgp100.

MHC Class I–Peptide Interactions and the Immunogenicity of gp100. To evaluate the relative avidity of the gp100-specific T cells for the $mgp100_{25-33}$ and $hgp100_{25-33}$ epitopes, we pulsed the peptides onto EL-4 cells (H-2^b) (Fig. 4). A striking difference in the relative T cell activities to the $mgp100_{25-33}$ and $hgp100_{25-33}$ peptides was observed. The $mgp100_{25-33}$ peptide was recognized at concentrations as low as 10^{-9} M, with half-maximal recognition occurring at about 10^{-8} M, whereas the $hgp100_{25-33}$ peptide was recognized at concentrations as low as 10^{-11} M. Alanine-substitutions at positions 1, 2, or 3 in either the mouse or human versions of the synthetic peptides did not substantially alter recognition by gp100-reactive T cells (Fig. 5 *A*). Indeed, a triple-substituted peptide, AAARNQDWL, fully retained the ability to sensitize EL-4 for recognition. (Fig. 5 *B*). The first three AAs could even be deleted and a substantial degree of recognition remained (Fig. 5 *B*). However, substitution of any of the AAs at positions 4 through 9 abrogated recognition of both the $hgp100_{25-33}$ and $mgp100_{25-33}$ pep-

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Table 1. *IFN-γ Release by Peptide-stimulated Splenocytes from gp100-immunized Mice*

Immunization / In vitro stimulation	rVVhgp100			rVVmgp100			rVVEShgp100$_{25-33}$			rVVESmgp100$_{25-33}$			Naive	
	hgp	mgp	OVA	hgp	mgp	OVA	hgp	mgp	OVA	hgp	mgp	OVA	hgp	mgp
Peptide targets														
None	264	584	236	137	0	51	279	428	5,297	862	1,082	1,324	215	456
hgp	**15,120**	**20,751**	250	137	1	0	**15,945**	**11,197**	1,267	1,295	1,580	819	229	428
mgp	**2,567**	**19,307**	257	94	58	37	**940**	**11,268**	1,438	911	847	1,629	215	527
OVA	165	343	144	137	37	208	172	449	300	648	698	2,141	257	478

Mice were vaccinated with indicated rVVs, and splenocytes were isolated 3 wk later and restimulated for 6 d with 1 μg/ml peptide. Subsequent stimulation of cultured cells with hgp100$_{25-33}$ or mgp100$_{25-33}$ peptide resulted in IFN-γ release only by splenocytes from mice immunized with rVVhgp100 or rVVEShgp100$_{25-33}$. *hgp*, hgp100 AA 25–33 (KVPRNQDWL); *mgp*, mgp100 AA 25–33 (EGSRNQDWL); *OVA*, ovalbumin, AA 257–264 (SIINFEKL). Four repeat experiments yielded similar results; numbers indicate IFN-γ (pg/ml) secreted by 2×10^5 CTLs in 24 h; numbers in bold indicate secretion >3-fold over control peptide.

tide variants. To determine the involvement of MHC class I binding affinity, an MHC class I stabilization assay was done on RMA/S cells, which lack activity of the TAP transporters (13). FACS® analysis revealed 50% stabilization of D^b by hgp100$_{25-33}$ peptide at a concentration ~100-fold lower than for mgp100$_{25-33}$ (Fig. 6). This indicated that the apparent avidity of gp100-reactive T cells for the hgp100$_{25-33}$ peptide could be largely attributed to its greater ability to stabilize the restricting MHC class I molecule, H-2D^b.

Self-reactive T Cells Induced with Altered Antigen Function In Vivo. One major potential use for self-reactive T cells is their application in cancer treatment. To evaluate the usefulness of altered antigen in the development of a synthetic cancer vaccine capable of eliciting therapeutic T cells, mice were vaccinated once with rVVhgp100 and restimulated for three rounds with splenocytes pulsed with 1 μM mgp100$_{25-33}$ peptide. $CD8^+$ T cells were tested for gp100 recognition by IFN-γ release (Fig. 7 *A*). T cells strongly recognized both mgp100$_{25-33}$ and hgp100$_{25-33}$ peptides, but not control OVA$_{257-264}$ peptide, and recognized as well melanoma B16.WT, B16.B7-1, and JB/MS, a chemically induced C57BL/6 melanoma. Control H-2^b, $gp100^-$ tumor lines were not recognized.

The function of T lymphocytes generated using purely recombinant and synthetic forms of gp100 were evaluated by adoptive transfer of T cells to mice bearing tumors established for 3 d in vivo. Mice receiving mgp100$_{25-33}$–specific T cells experienced a significant reduction in tumor burden compared with mice receiving control T cells (Fig. 7 *B*). The survival of these cells was evaluated in vivo by transfer 5 d before tumor challenge, with T cells retaining their antitumor activity for this period (Fig. 7 *C*). This indicated that tumor-specific T cells induced with an antigen-based cancer vaccine can survive in vivo and mediate efficient tumor destruction.

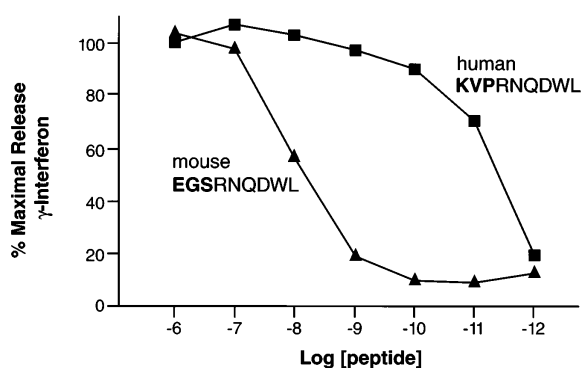

Figure 4. Recognition of mgp100$_{25-33}$ and hgp100$_{25-33}$ peptides at limiting concentrations. EL-4 thymoma cells were incubated with the mgp100$_{25-33}$ peptide EGSRNQDWL and hgp100$_{25-33}$ peptide KVPRNQDWL at the concentrations shown on the abscissa for 2 h at 37°C, washed twice, and cocultured for 24 h with gp100-reactive T cells. Supernatants were assayed for IFN-γ by ELISA, which was expressed as percentage of the maximal release (at 1 μM peptide, as shown on the abscissa). Half-maximal recognition of hgp100$_{25-33}$ was reached at a concentration ~1,000-fold lower than that needed for mgp100$_{25-33}$. Data shown is an average of two independent experiments.

Discussion

The nonhomologous sequences flanking a MHC-restricted epitope could influence the immunogenicity of the epitope through a variety of mechanisms. The full-length hgp100 and mgp100 molecules are 76% identical at the AA level (11). Xenoimmunization could induce antibodies to nonhomologous determinants on the xenoantigen. When expressed on the surface of B cells that produce them, these antibodies could capture the xenoantigen and make it available for B cell processing and presentation on MHC class II to activate $CD4^+$ T cells (35). This mechanism has been postulated to play a role in the initiation of human autoimmune diseases such as SLE, which is largely mediated by $CD4^+$ T cells and autoreactive antibodies (25, 34). Recent data suggest that B cells may cross-present antigen on MHC class I after capture (39). However, there is a vig-

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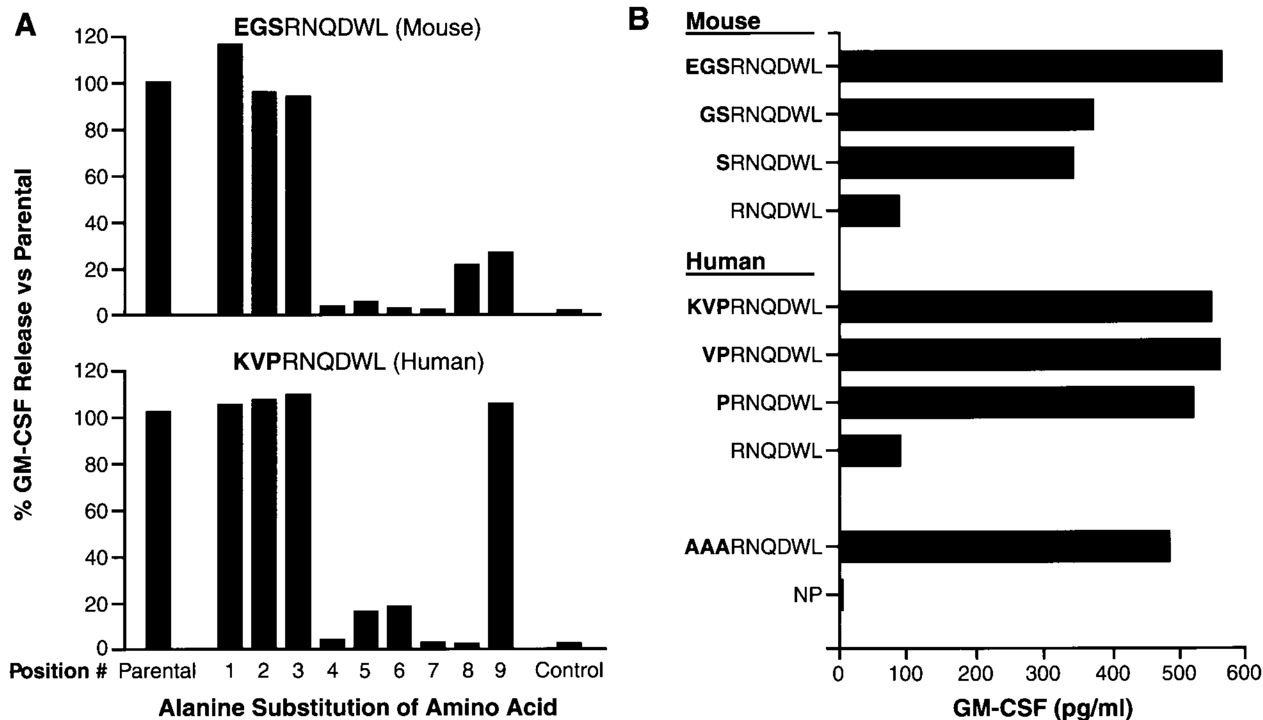

Figure 5. Fine specificity of gp100-reactive T cells: EL-4 thymoma cells were incubated with either alanine-substituted peptide variants (*A*) or NH_2-terminally deleted peptide variants (*B*) for 2 h at 37°C, washed twice, and cocultured for 24 h with gp100-reactive T cells. Supernatants were assayed for GM-CSF by ELISA.

orous debate over the ability of B cells to activate "virgin" T cells (i.e., T cells that have not previously been activated by antigen) (33, 40). DCs can also capture immune complexes containing xenoantigen through Fc receptors and present it through MHC class I and class II pathways, inducing de novo activation of autoreactive T cells (41). In this scenario, the completely autologous mgp100 would not induce such antibodies and thus would fail to be captured and presented. The involvement of extra-epitope sequences in the immunogenicity of the human gp100 molecules is not consistent with the results shown in Table 1 where the immunogenicity of human full-length and minimal determinant constructs are compared. In fact, the two constructs elicit comparable $CD8^+$ T cell responses to the 25–33 epitope.

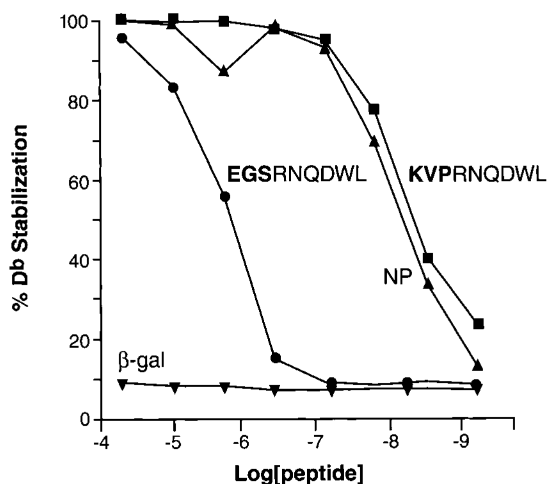

Figure 6. The TAP-deficient cell line RMA-S, expressing "empty" MHC class I molecules, was incubated with the concentrations shown on the abscissa with the $mgp100_{25-33}$ and $hgp100_{25-33}$ peptides, as well as control peptides, for 45 min at 25°C, then incubated for 4 h at 37°C. Cells were then stained with FITC-conjugated H-$2D^b$–specific mAb KH95 for 1 h at 4°C, and staining was assessed using flow cytometric analysis and expressed as percentage of the maximal stabilization at 100 μM. The $hgp100_{25-33}$ peptide stabilized H-$2D^b$ molecules at a 100-fold lower concentration than did the $mgp100_{25-33}$ peptide.

Another mechanism by which xenoimmunization could enhance immunogenicity is by the processing of a given epitope. For example, the $hgp100_{25-33}$ peptide might be processed more efficiently than the $mgp100_{25-33}$ peptide, since the two differ in the three NH_2-terminal AAs, as well as in the AA sequences surrounding the peptides in the full-length molecule (36, 42). The differences between the processing of the human and mouse sequences is minimized and possibly eliminated by the use of minigene constructs preceded by ER-insertion signal sequences that bypass proteasome-mediated peptide liberation as well as TAP-mediated peptide transport (Table 1).

Indeed, the increased immunogenicity of human gp100 appeared to reside completely within the 9-mer peptide. The $hgp100_{25-33}$ peptide differed from its mouse counterpart in three NH_2-terminal AAs. In fact, these three differences were rather dramatic, with a positively charged lysine (K) replacing a negatively charged glutamic acid (E), a me-

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Figure 7. rVVhgp100-induced T cells are tumor specific and can treat established melanoma. (*A*) Mice were vaccinated with 10^7 rVVhgp100 intravenously 3 wk before splenocytes were isolated and cultured with 1 μM of mgp100$_{25-33}$ peptide for 6 d, after which the cells underwent two more cycles of restimulation with mgp100$_{25-33}$-pulsed splenocytes. The resulting $CD4^-CD8^+$ T cells were cocultured for 24 h with various tumor targets, as well as MC38 sarcoma infected with indicated rVV or MC38 sarcoma pulsed with 1 μM of peptide, at an E/T ratio of 1:1, and IFN-γ release was measured by ELISA. rVVhgp100-induced T cells were mgp100$_{25-33}$– and hgp100$_{25-33}$–specific, and specifically recognized mgp100$^+$ melanomas, as well as MC38 sarcoma infected with rVVhgp100 or rVVmgp100 but not rVVβ-gal, and MC38 sarcoma pulsed with hgp100$_{25-33}$ or mgp100$_{25-33}$ peptide, but not OVA$_{257-264}$ peptide. (*B*) Mice were injected intravenously with 2×10^5 B16.WT and treated 3 d later with 5×10^6 of the T cells that are shown in *A*, or with 5×10^6 β-gal–specific T cells, followed by 3×10^5 IU rhIL-2 intraperitoneally twice daily for 5 d. 18 d after tumor inoculation, the number of pulmonary nodules was specifically reduced by mgp 100$_{25-33}$–specific T cells. (*C*) Mice were injected intravenously with 4×10^6 of the T cells that are shown in *A*, or 4×10^6 β-gal–specific T cells. 5 d later, mice were challenged intravenously with 2×10^5 B16.WT. 18 d after tumor inoculation, the number of pulmonary nodules was dramatically lower in mice receiving mgp 100$_{25-33}$–specific T cells, indicating in vivo survival of functional, autoreactive T cells. Two repeat experiments yielded similar results.

dium-sized valine (V) replacing a small glycine (G), and proline (P), which is a cyclic residue and known to reduce the number of possible conformations due to impaired hydrogen bonding, replacing serine (S), which may not induce such a structural distortion. Despite the presence in both peptides of optimal anchor residues at both dominant anchor positions, 5 (N) and 9 (L), the difference in the three NH_2-terminal residues resulted in a dramatically increased affinity of the hgp100$_{25-33}$ peptide for the mouse MHC class I allele, H-2D^{b,} compared with the mgp100$_{25-33}$ peptide (Fig. 6). Clearly, the ability of the peptide to stabilize D^b molecules on the surface of RMA-S cells could reflect the amount of peptide presented on the surface of APCs, which in turn could determine the activation of T cell precursors. In retrospect, it was fortuitous that the interspecies differences in the gp100 sequences created such a high binder, whereas the similarities preserved sufficient T cell receptor contact residues to allow cross-recognition.

The efficacy of the mechanism described here through which xenoimmunization induced autoreactive T cells required that a number of criteria be satisfied. There must be an MHC-binding epitope in the autologous protein which is naturally processed and presented that can be recognized by the available T cell repertoire. Most importantly, a homologous epitope from the xenogeneic protein must also be naturally processed and presented, but must be presented in the context of MHC at a higher density on the surface of the APCs. The chance that such an epitope will be found in any give xenoantigen may be small. However, an understanding of this mechanism clearly points the way to the rational design of immunogens based on the enhancement of the stability of peptide–MHC complexes.

The data presented here suggest that the main difference between the mgp100$_{25-33}$ peptide and its hgp100$_{25-33}$ analogue resides in binding to MHC class I. In human in vitro studies, modification of the second AA of the hgp100 pep-

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tide epitope hgp100$_{209-217}$ to methionine has been shown to significantly increase its affinity to HLA-A2, leading to dramatically increased ability to raise gp100-specific T cells from patient PBLs in vitro (43). The higher affinity peptide is also more effective in vaccinating patients in vivo, increasing gp100-specific T cell precursor levels, and possibly resulting in higher treatment response rates (43a). Similarly, other groups have reported increased immunogenicity of peptide epitopes with enhanced MHC class I binding (26, 44, 45).

Taken together, these data suggest that there is indeed unresponsiveness to gp100 in mice. However, the unresponsiveness is relative and can be broken by using a peptide homologue with higher affinity for MHC class I. One mechanism through which a peptide with higher MHC class I binding can break tolerance is based on the assumption that T cell tolerance exists to a level of antigen rather than to the identity of the antigen (44, 46, 47). In the case of gp100, $CD8^+$ T cell precursors with the ability to recognize a certain amount of peptide in the context of MHC class I are inactivated, through either thymic and/or peripheral deletion or anergization. The remaining T cells have TCRs with an affinity that is too low to be triggered by the levels of gp100 peptide present on melanocytes or APCs in lymph nodes draining the skin. Therefore, these T cells are never activated, deleted, or anergized. Instead, they remain "ignorant" of MHC class I with mgp100$_{25-33}$ peptide. A vaccination with autologous mgp100 will not trigger these T cells unless the vaccine is able to significantly raise the amount of peptide–MHC class I complex on professional APCs to a level high enough to surpass the TCR threshold. Only then do the T cells become activated. We are currently evaluating the clinical efficacy of antitumor effects of gp100-based cancer vaccines that contain epitopes with enhanced stability of peptide–MHC complexes.

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