Poor immunogenicity of a self/tumor antigen derives from peptide–MHC-I instability and is independent of tolerance

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Understanding the mechanisms underlying the poor immunogenicity of human self/tumor antigens is challenging because of experimental limitations in humans. Here, we developed a human-mouse chimeric model that allows us to investigate the roles of the frequency and self-reactivity of antigen-specific T cells in determination of the immunogenicity of an epitope (amino acids 209–217) derived from a human melanoma antigen, gp100. In these transgenic mice, $CD8^+$ T cells express the variable regions of a human T cell receptor (hTCR) specific for an HLA-A*0201–restricted $gp100_{209–217}$. Immunization of hTCR-transgenic mice with $gp100_{209–217}$ peptide elicited minimal T cell responses, even in mice in which the epitope was knocked out. Conversely, a modified epitope, $gp100_{209–217(2M)}$, was significantly more immunogenic. Both biological and physical assays revealed a fast rate of dissociation of the native peptide from the HLA-A*0201 molecule and a considerably slower rate of dissociation of the modified peptide. In vivo, the time allowed for dissociation of peptide-MHC complexes on APCs prior to their exposure to T cells significantly affected the induction of immune responses. These findings indicate that the poor immunogenicity of some self/tumor antigens is due to the instability of the peptide-MHC complex rather than to the continual deletion or tolerization of self-reactive T cells.

Introduction

Significant progress has been made in the past decade in the identification of tumor-associated antigens. More than 170 antigenic peptides derived from 60 human tumor antigens are expressed in the context of MHC molecules and are recognizable by cells in the available T cell repertoire (1). It is surprising that so many of these are nonmutated "self" antigens (2). Many candidate peptides have been used in clinical trials in efforts to develop therapeutic cancer vaccines, but most of these have failed to elicit measurable immune responses in the majority of the patients immunized, even when highly sensitive techniques for measuring these responses are used. One example of this phenomenon is illustrated in studies using a native peptide epitope derived from the human melanoma differentiation antigen, gp100. The epitope corresponding to amino acids 209–217 (ITDQVPFSV) of gp100 protein ($gp100_{209–217}$) is restricted by HLA-A*0201 and has been studied extensively in tumors from patients with metastatic malignant melanoma (3–6).

The gp100 protein is abundantly expressed in most human melanomas. High-avidity T cell precursors specific to $gp100_{209–217}$ can often be identified in circulating blood and within the tumor bed (3, 4). Recognition of the $gp100_{209–217}$ epitope by adoptively transferred tumor-infiltrating lymphocytes has been correlated with tumor regression (3, 4). The nonamer $gp100_{209–217}$ peptide binds to HLA-A*0201, the most commonly expressed MHC class I molecule in patients with melanoma, with an intermediate affinity of approximately 100 nM (5, 7). Hence, this peptide was a very attractive candidate for active immunization in melanoma patients. However, attempts at immunization of HLA-A*0201–positive melanoma patients using peptide emulsified in Montanide (Seppic, Paris, France) or recombinant adenoviruses or poxviruses encoding gp100 had a minimal effect on activating the immune system to recognize the tumor antigen (6, 8, 9). We have previously shown that native $gp100_{209–217}$ peptide is also a poor immunogen in mice transgenic for a chimeric HLA-A2/K^b molecule (10).

The reasons underlying the poor immunogenicity of many candidate antigens used in clinical trials, including the $gp100_{209–217}$ epitope, remain unelucidated. Nevertheless, it is clear that a modified version of this epitope (IMDQVPFSV), $gp100_{209–217(2M)}$, in which the threonine at position 2 has been changed to a methionine, is more immunogenic in melanoma patients and in mice transgenic for the HLA-A*0201/K^b molecule (6, 8, 10–12). The molecular bases for why modifications to a tumor-associated epitope improve immunogenicity have been the subject of some speculation. One hypothesis is that $gp100_{209–217}$–specific T cells are present in at very low frequencies and that immunization using the altered peptide ligand $gp100_{209–217(2M)}$ enables the activation of a subset of T cells that are cross-reactive to the native epitope, as shown in autoimmune diseases (13) as well as in tumor immunity (12). A second possibility is that the immunogenicity of a self antigen is dampened because of reduction in the number of high-affinity self-reactive T cells, which result from tolerating mechanisms such as clonal deletion, ignorance, anergy, or suppression in the host (14). A third theory stems from the recent understanding of the role of immunological synapses in T cell activation (15), in which a stable peptide-MHC complex may facilitate the formation of the synapses between T cells and APCs

Nonstandard abbreviations used: human TCR (hTCR); human Vβ8 (hVβ8); incomplete Freund's adjuvant (IFA); *JR209* transgenic (*JR209*-Tg); T cell receptor (TCR).

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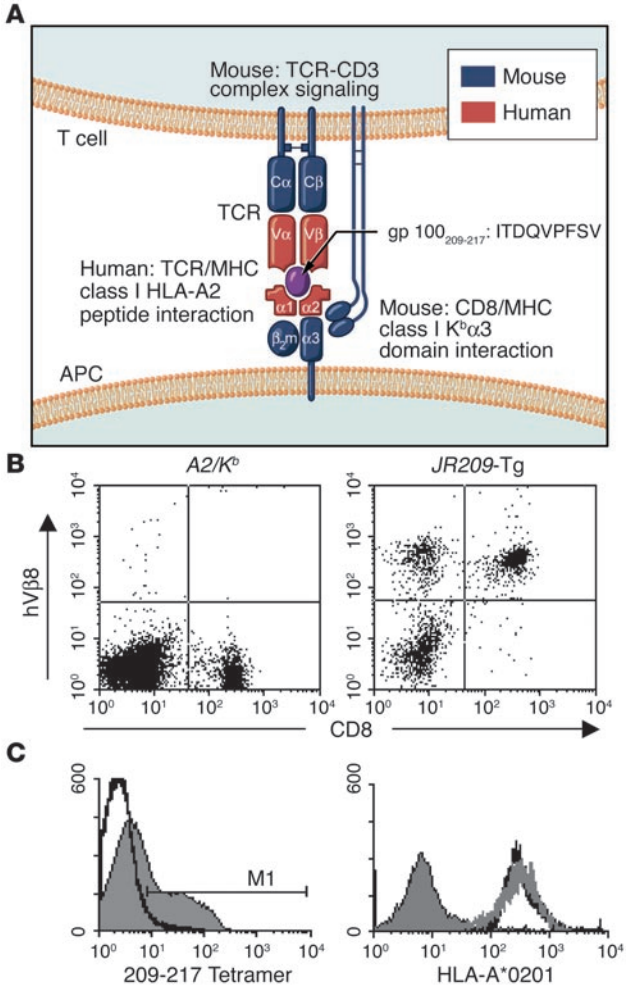

Figure 1
Characterization of the human-mouse TCR–HLA-A2/K^b transgenic mouse model. (**A**) Illustration of a hTCR–HLA-A*0201–gp100$_{209–217}$ complex in the *JR209*-Tg mouse model. (**B**) hTCR Vβ8 and CD8 expression on lymphocyte-gated population from spleens of non–TCR-transgenic *A2/K^b* (*A2/K^b*) and *JR209*-Tg littermate mice. The percentage of hVβ8$^+$CD8$^+$ lymphocytes was approximately 0 in *A2/K^b* mice and 41 in *JR209*-Tg littermates. (**C**) Binding of HLA-A*0201–gp100$_{209–217}$ tetramer to the lymphocyte-gated population from spleens of *A2/K^b* (area under thick black line) and *JR209*-Tg (gray area) mice. M1 (tetramer positive gate) represented 42% of the gated population. (**D**) HLA-A*0201 expression on lymphocyte-gated population from splenocytes of C57BL/6 (gray area), *A2/K^b* (area under gray line), and *JR209*-Tg (area under thick black line) mice.

(16). The altered peptide may thus allow full T cell activation through sustained signaling and therefore an increased peptide immunogenicity (17), while the native peptide forms an unstable complex that could fail to fully sustain signaling. Recent studies have suggested that the immunogenicity of MHC-I–binding viral and tumor peptides were dependent on MHC-peptide complex stability (18–21). Unfortunately, without a defined T cell receptor (TCR) specificity, these studies had to assume that the frequencies and affinity of T cell precursors with different antigen specificities were equal in the open TCR repertoires.

Because of the clinical importance of the gp100$_{209–217}$ epitope, we sought to precisely study the molecular interaction of this relevant TCR–MHC-I–peptide complex in vivo. Numerous gp100$_{209–217}$–specific CD8$^+$ CTL clones have been successfully raised from melanoma patients. One of the representative clones, R6C12, was selected for this study because it was highly reactive to both native and modified gp100$_{209–217}$ peptide–pulsed T2 cells and gp100$^+$HLA-A*0201$^+$ melanoma cells. We genetically chimerized the variable regions of human TCR (hTCR) with the constant regions of mouse TCR. In addition, the peptide-binding domains of the human HLA-A*0201 molecule were combined with mouse K^b α3 domains, which allow interactions with mouse CD8 coreceptors in these mice. Because the gp100$_{209–217}$ sequence is identical in mouse and man, we were able to study the in vivo activities of the transgenic T cells with a defined specificity to a true, "noninduced" self/melanoma antigen. This also provided us a unique opportunity to investigate the self-tolerance mechanisms that regulate the transgenic T cells in both target epitope–expressing and knockout mice. Furthermore, our transgenic model allowed us to compare the immunogenicity of the native and HLA-A*0201 anchor–modified gp100$_{209–217}$ peptides, which are both recognized by the parental human T cell clone. In this study, our goal was to understand the nature of the poor immunogenicity of the gp100$_{209–217}$ epitope and the precise reason why this poor immunogenicity is reversed when using the modified peptide.

Results

T cells from the chimeric TCR-transgenic mouse are functional. "Humanized" TCR-transgenic mice have been previously reported only to model human CD4$^+$ T cells specific to myelin basic protein in the context of HLA-DR2 (22). Our model is the first to graft human self/tumor–reactive, HLA-A2–restricted CD8$^+$ T cells into mice (Figure 1A). To validate the bioimmunological functions of the transgenic T cells in *JR209* transgenic (*JR209*-Tg) mice, we examined the number, antigen specificity, and MHC restriction of the transgenic T cells.

Like conventional TCR-transgenic mice, the human-mouse chimeras had an increased percentage of CD8$^+$ lymphocytes (40%) in the spleen compared with their TCR transgene–negative littermates (20%) (Figure 1B), and had a similar number of total lymphocytes (not shown). More than 95% of the CD8$^+$ T cells from *JR209*-Tg mice expressed human Vβ8 (hVβ8) (Figure 1B). To confirm the proper TCR Vα and Vβ configurations on the transgenic T cells, we used HLA-A*0201–gp100$_{209–217}$ tetramer to label the lymphocytes. We found that approximately 40% of lymphocytes stained positive with the tetramer (Figure 1C), which was similar to the percentage of CD8$^+$hVβ8$^+$ lymphocytes (Figure 1B). The expression of HLA-A*0201 in mice with and without the transgenic TCR was similar (Figure 1D).

CD8$^+$ T cells from naive *JR209*-Tg mice were in an inactivated state, expressing low levels of CD69 and CD44 and a high level of CD62L, but no CD25 (Figure 2A). gp100$_{209–217}$ peptide–pulsed APCs induced IFN-γ production by naive *JR209*-Tg T cells at the concentration of 10^{-8} M (Figure 2B). After culturing in media containing 1 μM of gp100$_{209–217}$ peptide and 30 IU/ml of IL-2 for 7 days, *JR209*-Tg T cells were fully activated. They not only phenotypically expressed high levels of CD25, CD69, and CD44 and a low level of CD62L (Figure 2A), but also secreted more IFN-γ upon antigen stimulation (Figure 2B). These activities were peptide-specific (Figure 2C) and could be blocked by mAb's against HLA-A2 and mouse CD8 (Figure 2D).

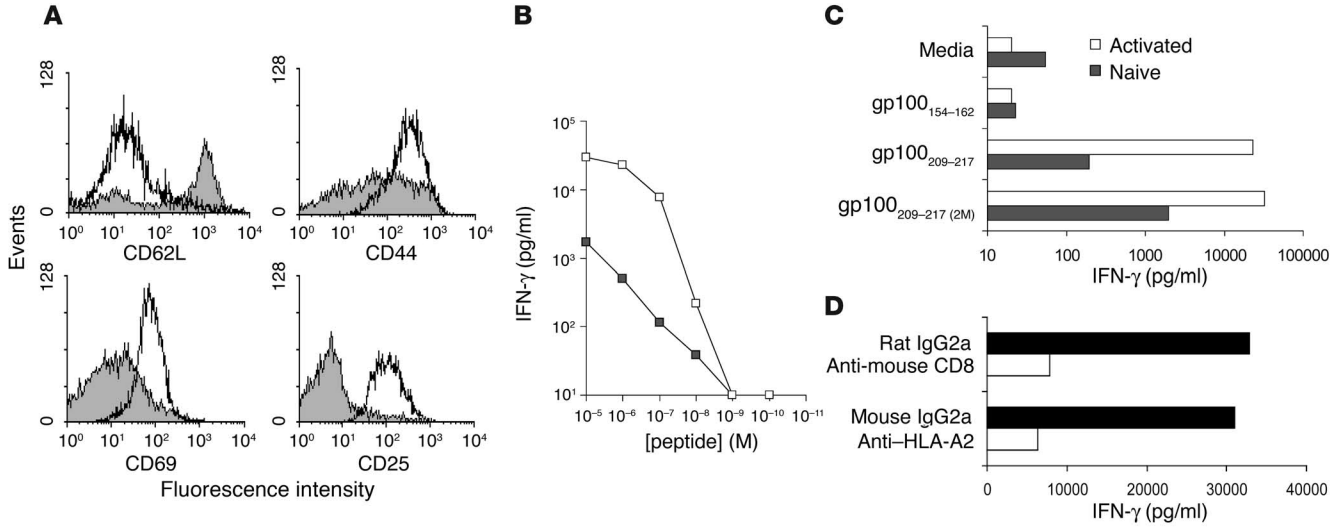

Figure 2
T cells from *JR209*-Tg mice are functional. (**A**) Expression of CD62L, CD44, CD69, and CD25 on freshly isolated (gray area) and ex vivo peptide-stimulated *JR209*-Tg (area under thick black line) $CD8^+$ T cells. The florescence intensities of cells labeled with isotype Ab's were less than 10^2 (not shown). (**B**) IFN-γ release in 24-hour coculture of 1×10^5 naive (filled squares) or $gp100_{209–217}$ peptide–activated (open squares) $CD8^+$ *JR209*-Tg T cells and 1×10^5 *A2/K*^{b} splenocytes pulsed with titrated $gp100_{209–217}$ peptides. Data represented the mean of duplicate testing samples. (**C**) IFN-γ release in 24-hour coculture of 1×10^5 freshly isolated (gray bars) or ex vivo peptide-stimulated (white bars) $CD8^+$ *JR209*-Tg T cells and 1×10^5 *A2/K*^{b} splenocytes pulsed with 1 μM of $gp100_{209–217(2M)}$, $gp100_{209–217}$, and $gp100_{154–162}$ (irrelevant control) peptide. (**D**) IFN-γ release in 24-hour coculture of 1 μM $gp100_{209–217}$ peptide–pulsed *A2/K*^{b} splenocytes and activated *JR209*-Tg T cells and their blockade by anti–HLA-A2 and anti-mCD8 mAb's. Data represent the mean of duplicate testing samples.

We noticed that about 20% of lymphocytes in the spleen of the *JR209*-Tg mice were $CD4^+$ T cells that also expressed human *Vβ8* transgene. These $CD4^+$ T cells did not proliferate in response to in vitro stimulation with $gp100_{209–217}$ peptide (data not shown), and their functions were not known.

Tolerance is observed in JR209-Tg mice with the gp100$_{209–217}$ epitope knockout. Despite large numbers of circulating antigen-specific $CD8^+$ T cells and the expression of target antigens in normal melanocytes and other pigmented cells in eyes and brain, no significant changes in the color or appearance of hair, skin, or eyes were observed in *JR209*-Tg mice compared with nontransgenic littermates. To test whether these self-reactive T cells were tolerized, we compared the lymphocytes in *JR209*-Tg mice and *JR209*-Tg mice in which the $gp100_{209–217}$ epitope was disrupted by insertion of the *neomycin* gene into exon 4 (corresponding to cDNA sequence 636–902 bp) of the *gp100* gene. This resulted a truncation of gp100 protein from amino acid 212.

Although the numbers of thymocytes and splenocytes in both types of *JR209*-Tg mice were similar, naive splenocytes from *JR209-Tg–gp100$_{209–217}^{KO}$* mice produced more IFN-γ within 24 hours of peptide–APC stimulation than did those from *JR209-Tg–gp100$_{209–217}^{WT}$* mice expressing the epitope (Table 1). The differences between the naive self and non-self *JR209*-Tg T cells seemed to be more obvious when the antigen-specific proliferative responses were examined. In the absence of exogenous IL-2 in the culture media, more than 70% of *JR209*-Tg T cells from naive non–epitope-expressing mice had divided at least once within 48 hours of $gp100_{209–217}$ stimulation. In comparison, less than 20% of *JR209*-Tg T cells from epitope-expressing mice had divided in the same period of time (Figure 3A). The sensitivity of *JR209*-Tg T cells to $gp100_{209–217}$ epitope concentrations in ex vivo assays was similar regardless of the expression of the epitope (Table 1), which indicated unchanged TCR affinity in both types of mice. When *JR209*-Tg T cells were cultured ex vivo with antigen and IL-2 (known to reverse anergy of self-reactive T cells; see ref. 23), the differences in IFN-γ production and proliferation between the cells from epitope-expressing and nonexpressing mice diminished (data not shown). Our experimental results indicated that self-tolerance mechanisms partially abrogate the early T cell activation events.

Native gp100$_{209–217}$ antigen fails to immunize JR209-Tg T cells in the epitope-expressing and knockout mice. To investigate whether the poor immunogenicity of the $gp100_{209–217}$ epitope was due to the low frequency of self-reactive T cells, we compared immune responses induced by peptides in *JR209* TCR-transgenic and non–TCR-transgenic *A2/K*^{b} mice (or *A2/K*^{b} mice). In *A2/K*^{b} mice, immunization with neither the native nor the modified $gp100_{209–217}$ peptide could elicit antigen-specific $CD8^+$ T cell responses, even at a 100-μg dose (data not shown). However, immunization of *JR209*-Tg mice with as little as 10 μg of the modified peptide enhanced $gp100_{209–217}$ peptide–specific IFN-γ production in draining lymph nodes compared with PBS controls (Figure 3B). Clearly, in the case of the modified $gp100_{209–217}$ peptide, there was a positive correlation between the frequency of antigen-specific T cells and peptide immunogenicity. When we examined this correlation in *JR209*-Tg mice immunized with the native peptide, we found that none of the tested doses activated *JR209*-Tg T cells in draining lymph nodes compared with PBS controls (Figure 3B). Our data indicated that the quantity of tumor-specific T cells was not a determining factor for the failure of the native $gp100_{209–217}$ peptide to immunize.

To investigate whether self-tolerance was responsible for the inferior immunogenicity of the native $gp100_{209–217}$ epitope, we immunized *JR209-Tg–gp100$_{209–217}^{KO}$* mice with the peptides. Seven days after immunization, none of the tested doses activated

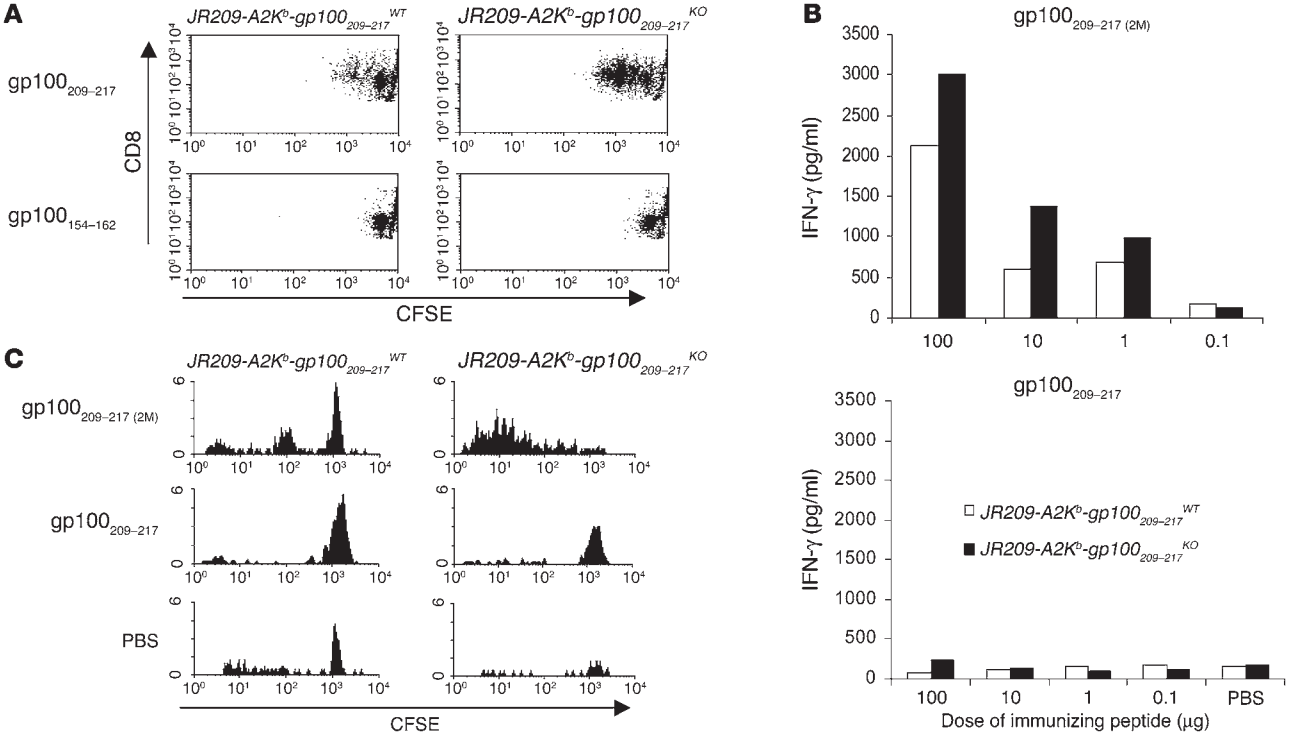

Figure 3
Native gp100$_{209–217}$ peptide fails to activate *JR209*-Tg T cells in both *gp100$_{209–217}^{WT}$* and *gp100$_{209–217}^{KO}$* mice. (**A**) Ex vivo antigen-specific proliferative responses of freshly isolated splenocytes from *JR209*-Tg mice with and without gp100$_{209–217}$ epitope expression. CFSE-labeled splenocytes were cultured in media containing 1 μM of gp100$_{209–217}$ or gp100$_{154–162}$ peptide for 48 hours before FACS. The dot plots represent 10,000 total events in each sample. (**B**) gp100$_{209–217}$ peptide–specific IFN-γ release in cells from draining lymph nodes (pooled from two mice in each group) after various doses of gp100$_{209–217(2M)}$ and gp100$_{209–217}$ peptide immunization in *JR209*-Tg mice with (white bars) and without (black bars) the epitope expression. Draining lymph nodes were collected 7 days after immunization. One micromole of gp100$_{209–217}$ peptide was added to 1 × 10^5 cells in 200 μl of culture media and incubated for 24 hours. IFN-γ concentrations in the supernatant were determined by ELISA. (**C**) In vivo antigen-specific proliferative responses of adoptively transferred freshly isolated splenocytes from *JR209*-Tg mice with and without gp100$_{209–217}$ epitope expression. CFSE-labeled splenocytes (1 × 10^7) from *JR209-Tg–gp100$_{209–217}^{WT}$* or *JR209-Tg–gp100$_{209–217}^{KO}$* mice were intravenously injected into *A2/K^b* recipient mice, followed by immunization (into the footpad) with 100 μg of gp100$_{209–217(2M)}$, gp100$_{209–217}$ peptide, or PBS (in IFA). Four days after immunization, the cells of draining lymph nodes were pooled from two mice in each group and gated on hVβ8$^+$CD8$^+$ T cells for FACS analysis.

JR209-Tg T cells in draining lymph nodes to produce antigen-specific IFN-γ (Figure 3B). In contrast, the *JR209-Tg–gp100$_{209–217}^{KO}$* mice immunized with the modified gp100$_{209–217(2M)}$ peptide responded better than *JR209-Tg–gp100$_{209–217}^{WT}$* mice did (Figure 3B). When CFSE-labeled naive *JR209*-Tg T cells from *gp100$_{209–217}^{WT}$* or *gp100$_{209–217}^{KO}$* mice were adoptively transferred into *HLA-A2/K^b* recipient mice and immunized with gp100$_{209–217(2M)}$ peptide, significant numbers of the transferred *JR209*-Tg T cells were proliferative 4 days after immunization. In addition, *JR209*-Tg T cells from *gp100$_{209–217}^{KO}$* mice seemed to proliferate more than those from *gp100$_{209–217}^{WT}$* mice (Figure 3C). However, 4 days after recipient mice were immunized with the native peptide, neither *JR209*-Tg T cells from *gp100$_{209–217}^{WT}$* mice nor those from *gp100$_{209–217}^{KO}$* mice had divided (Figure 3C). These results led us to consider factors other than self-tolerance that might contribute to the poor immunogenicity of the native epitope.

Native gp100$_{209–217}$–MHC-I complexes are metastable. Initiation and maintenance of immunological synapses and TCR signaling following interaction with peptide-MHC complexes are essential for full activation of T cells (15, 17, 24). Crystal structures of both native and modified HLA-A*0201–gp100$_{209–217}$ complexes have been recently resolved (O.Y. Borbulevych and B.M. Baker, unpublished data). Preliminary analyses indicated minimal differences in peptide binding and TCR interface structure between the native and modified gp100$_{209–217}$ peptides. This data was consistent with the observations that human T cell clones induced by either the native or modified peptide had no fine specificity enabling them to distinguish between the two forms of the peptide. Therefore, it is unlikely that two peptide–MHC-I complexes would result in different interactions with the TCR.

We decided to address whether the stability of the peptide–MHC-I complexes correlated to peptide immunogenicity. We determined the peptide–MHC-I dissociation rate by measuring the ability of a gp100$_{209–217}$–specific T cell clone to recognize peptide-pulsed target cells at different time points after the peptide–MHC-I binding occurred. A human T cell clone specific for gp100$_{209–217}$ (CK3H6) recognized gp100$_{209–217(2M)}$ peptide–pulsed T2 cells 24 hours after binding. In contrast, recognition of gp100$_{209–217}$ peptide–pulsed T2 cells diminished 4 hours after binding. The calculated dissociation rate of the modified peptide–HLA-A2 complexes was significantly slower ($P < 0.05$) than that of native peptide–HLA-A2 (Figure 4A). Using cultured *JR209*-Tg T cells in which the TCR–peptide–MHC-I

Table 1
Comparison of lymphocytes in $gp100_{209–217}^{WT}$ and $gp100_{209–217}^{KO}$ *JR209*-Tg mice

	$gp100_{209–217}^{WT}$	$gp100_{209–217}^{KO}$	*P* value
Thymocytes	$3.4 \times 10^7 \pm 0.6 \times 10^7$	$4.3 \times 10^7 \pm 0.8 \times 10^7$	0.3667
Splenocytes	$4.6 \times 10^7 \pm 0.9 \times 10^7$	$5.1 \times 10^7 \pm 1.0 \times 10^7$	0.7020
% of $CD8^+CD3^+$ T cells in splenocytes	38% ± 4%	37% ± 5%	0.9522
% of $hV\beta8^+CD8^+CD3^+$ T cells in $CD8^+CD3^+$ T cells	93% ± 2%	92% ± 2%	0.6674
IFN-γ production (pg/ml) per 10^6 splenocytes			
1 μM $gp100_{209–217}$	730 ± 75	1612 ± 202	0.0149[A]
0.1 μM $gp100_{209–217}$	310 ± 31	736 ± 86	0.0096[A]
0.01 μM $gp100_{209–217}$	91 ± 19	236 ± 134	0.3457
0.001 μM $gp100_{209–217}$	18 ± 8	55 ± 33	0.3296
1 μM $gp100_{154–162}$	12 ± 8	8 ± 4	0.3486

n = 5–8 mice in each group. [A]Significantly different ($P < 0.05$).

interaction was independent of CD8 coreceptors, we found similar results as when we used human T cell clones (Figure 4B). Therefore, the native peptide dissociated from HLA-A2 molecules at a much faster rate than the modified peptide, which was likely to be detrimental to activation of naive $CD8^+$ T cells.

The above experiments report biologically on peptide dissociation and provide apparent dissociation rates unique to the T cell–APC pair, yet do not directly measure the effect of modification on peptide dissociation from the HLA-A2 molecule. To investigate this, we measured peptide dissociation directly from purified, recombinant HLA-A2 using an in vitro fluorescence assay (Figure 4C). The results clearly indicated that position 2 modification resulted in slower peptide dissociation, with a dissociation rate (off-rate, or k_{off}) at 37°C of 0.18 h^{-1} for $gp100_{209–217}$ and 0.03 h^{-1} for $gp100_{209–217(2M)}$ (i.e., the modified peptide dissociates 6-fold slower). The difference in these rates was especially clear when we considered the $t_{1/2}$ of the peptide-MHC complexes, or the time required for 50% of the complex to decay. The $t_{1/2}$ for the parental peptide was 3.7 hours, whereas the $t_{1/2}$ for the modified peptide was considerably greater at 27 hours. The rates from in vitro assays were slower than those reported for these biological measurements, as expected given the time required to form an immunological synapse, initiate downstream signaling events, and release IFN-γ. However, the relative differences between the rates were consistent with the biological measurements.

The affinity of the modified $gp100_{209–217}$ peptide for HLA-A2 has been reported to be approximately 9-fold stronger than that of the parental peptide (5, 7). As K_d is proportional to k_{off}/k_{on}, the k_{off} measurements predict an approximate 2-fold increase in the peptide association rate for the modified peptide. Thus the increased affinity resulting from the position 2 modification was predominantly due to slower peptide dissociation. As peptide binding to HLA-A2 is rate-limited by a transition in the heavy chain from a "peptide-inaccessible" to a "peptide-accessible" conformation (25–27), direct calculation of k_{on} values from the current data are not possible. However, preliminary measurements of association rates for the two peptides supported the conclusion that the association rate for the modified peptide was slightly faster than that of the parental peptide (T.K. Baxter and B.M. Baker, unpublished data).

Stable peptide–MHC-I complexes are required for successful induction of antitumor responses in vivo. Mouse melanoma cells expressing HLA-$A2/K^b$ molecules were not recognized by naive *JR209*-Tg T cells, but were recognized by activated *JR209*-Tg T cells (Figure 5A) due to the increased avidity for sensing the low-density antigen–MHC complexes on tumor cells (28). In *JR209*-Tg mice, growth of cutaneously implanted B16-$A2/K^b$ tumor was comparable to growth in TCR transgene–negative, *A2/K^b* mice. Only when *JR209*-Tg mice were immunized with both $gp100_{209–217(2M)}$ peptide and IL-2 was

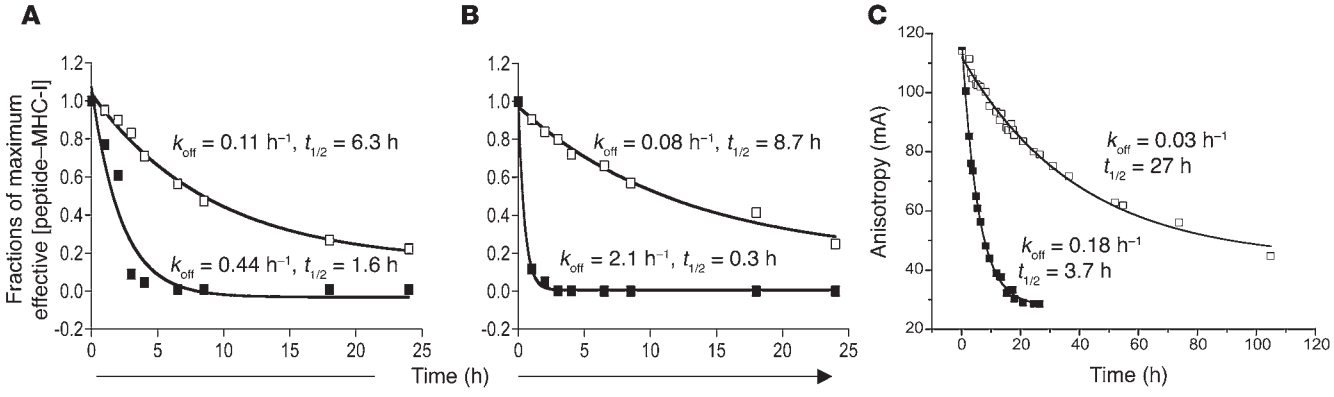

Figure 4
Native $gp100_{209–217}$–MHC-I complex is metastable. (**A** and **B**) Determination of peptide dissociation rates from target cell surface. Calculated amounts of $gp100_{209–217}$ (filled squares) and $gp100_{209–217(2M)}$ (open squares) peptide bound to HLA-A2 molecules were plotted over time. Apparent k_{off} values were determined by fitting to a single exponential decay of all points above an undetectable concentration of peptide (1×10^{-10} M using human T cell clone for **A** and 1×10^{-9} M using *JR209*-Tg T cells for **B**). $t_{1/2}$ was determined from the relationship $t_{1/2} = 0.693/k_{off}$. (**C**) Direct assay of peptide dissociation from purified peptide-MHC complexes using fluorescence anisotropy. Dissociation rates and $t_{1/2}$ of $gp100_{209–217}$ and $gp100_{209–217(2M)}$ peptides are indicated. Experiments were repeated in triplicate; experimental errors for the reported parameters were 3% for $gp100_{209–217}$ and 7% for $gp100_{209–217(2M)}$. mA, millianisotropy.

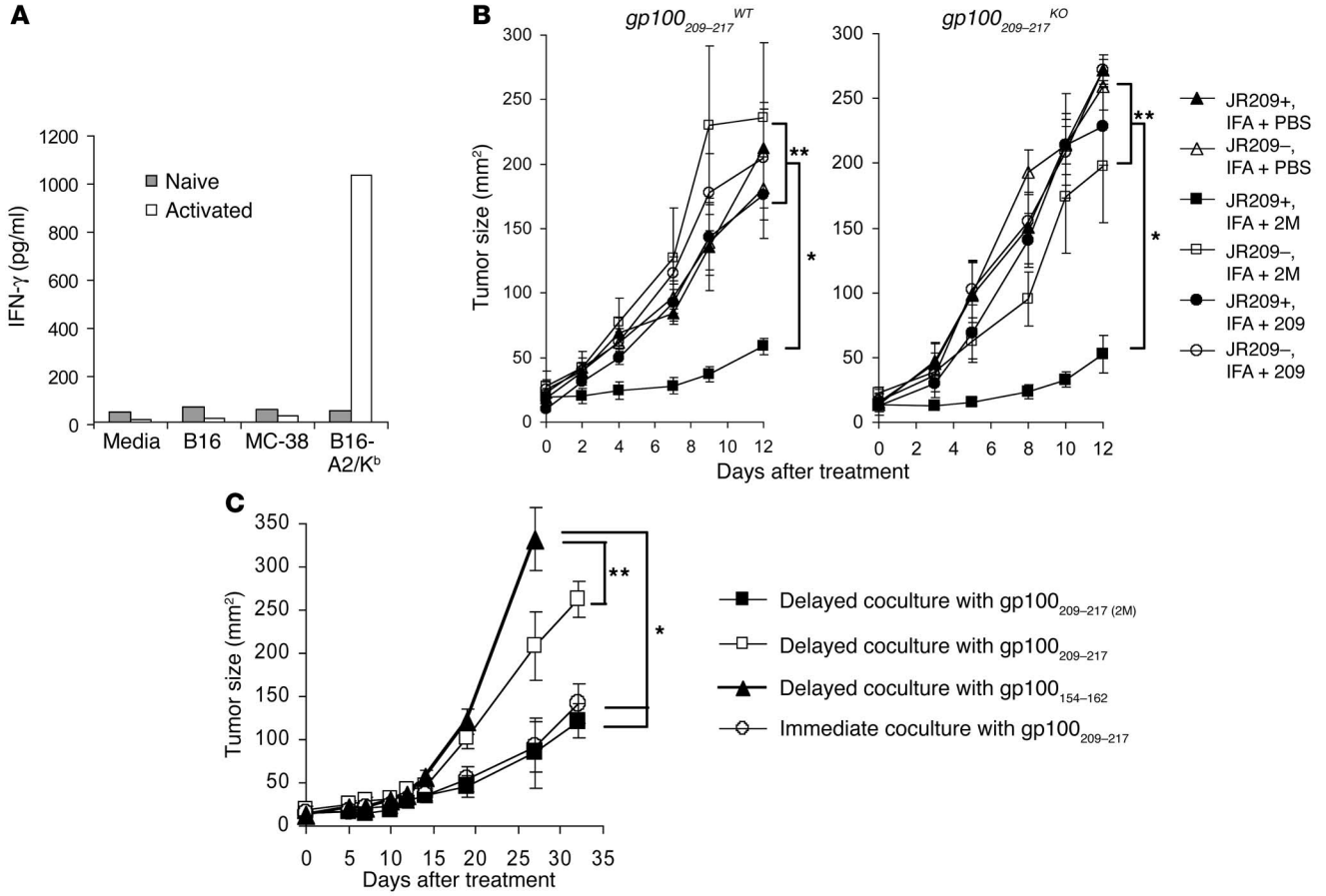

Figure 5
Stable peptide–MHC-I complex is required for in vivo induction of antitumor responses in *JR209*-Tg mice. (**A**) Tumor-specific IFN-γ production by freshly isolated (gray bars) or ex vivo peptide-stimulated (white bars) $CD8^+$ *JR209*-Tg T cells in 24-hour coculture with target cells from B16-A2/K^b, its parental B16 melanoma, and MC-38 murine adenocarcinoma. Data represent the mean of duplicate testing samples. (**B**) Treatment of B16-A2/K^b tumor in *A2/K*^{b} (open symbols) and *JR209*-Tg (filled symbols) mice by peptide immunization. One hundred micrograms of $gp100_{209–217}$ (circles), $gp100_{209–217(2M)}$ (squares) peptides or PBS (triangles) in IFA was subcutaneously injected into mice 13 days after tumor inoculation. Five mice were in each group. *Significantly different ($P < 0.05$). **Not significantly different ($P > 0.05$). (**C**) Treatment of B16-A2/K^b tumor in *A2/K*^{b} transgenic mice receiving adoptive transfer of ex vivo immunized *JR209*-Tg splenocytes. Peptide-pulsed splenocytes were separated from *JR209*-Tg T cells for 24 hours before being cocultured overnight and then injected into tumor-bearing mice. In a control group, $gp100_{209–217}$ peptide–pulsed splenocytes were directly cocultured with *JR209*-Tg T cells overnight and injected into tumor-bearing mice. Seven mice were in each group.

B16-A2/K^b tumor growth inhibited (Figure 5B). As shown in an early experiment, the native peptide failed to activate *JR209*-Tg T cells in vivo, and immunization of *JR209*-Tg mice with the native peptide and IL-2 had no impact on B16-A2/K^b tumors (Figure 5B). In *JR209-Tg–gp100*$_{209–217}^{KO}$ mice, B16-A2/K^b tumor also had a similar growth rate to that in TCR transgene–negative littermates. Immunizing these mice with native peptide and IL-2 did not result in the significant tumor reduction seen with the modified peptide.

Because in vivo activation of naive tumor-specific $CD8^+$ T cells is known to occur in the draining lymph nodes where APCs cross-present tumor antigens to T cells (29), we hypothesized that after peptide immunization, the stability of peptide–MHC-I complexes during the migration from immunization sites to draining lymph nodes could determine the efficiency of the induction of immune responses. To test this, we used an ex vivo model in which naive *JR209*-Tg T cells and peptide-pulsed *A2/K*^{b} splenocytes were separated for 24 hours before they were mixed in culture and transferred into B16-A2/K^b tumor–bearing *HLA-A2/K*^{b} mice. Tumor growth was significantly inhibited only by ex vivo immunization with the modified peptide, not the native peptide (Figure 5C). In contrast, adoptively transferred *JR209*-Tg T cells that were stimulated ex vivo with *HLA-A2/K*^{b} splenocytes immediately after native peptide pulsing inhibited tumor growth (Figure 5C). Therefore, the level of available $gp100_{209–217}$–MHC complexes on APCs at the time they encounter $CD8^+$ T cells could have a dramatic impact on an antigen's ability to sensitize T cells.

Discussion

The *JR209*-Tg mouse model presents a magnified picture of the interactions between a human self/tumor antigen–specific $CD8^+$ T cell and its cognate peptide–MHC-I ligand. This model clearly shows that a stable peptide–MHC-I complex is the first requirement for activating self-reactive $CD8^+$ T cells during peptide immunization. It not only explains the poor immunogenicity of

vaccines based on nonmodified tumor antigens, but also elucidates a plausible escape mechanism for autoreactive T cells with intermediate affinity to self/tumor antigens.

One mechanism by which autoreactive T cells escape negative selection is the lack of tissue-specific antigen expression in the thymus (30). This is unlikely in the case of *JR209*-Tg T cells because gp100 is highly expressed in mouse thymic epithelial and dendritic cells (31). We have shown that the number of *JR209*-Tg T cells in $gp100_{209–217}^{WT}$ mice is comparable to the number found in $gp100_{209–217}^{KO}$ mice. Therefore, the majority of *JR209*-Tg T cells escape thymic selection regardless of antigen expression. A possible explanation is that with a very fast rate of dissociation, the $gp100_{209–217}$ epitope–MHC-I complex is not stable enough to initiate TCR signaling in the thymus and thus fails to clonally delete the self-reactive T cells.

However, in the periphery, *JR209*-Tg T cells have reduced responsiveness to self-antigen stimulation compared with those that mature in a self antigen–free environment. If the self-reactive T cells do not "see" the antigen, there is no need to downregulate their response to it. Therefore, ignorance cannot explain the reduced responsiveness of tolerized *JR209*-Tg T cells. There are fewer $CD4^+$ T cells in *JR209*-Tg mice than in $A2/K^b$ mice, but it is not clear whether there are suppressive $CD4^+$ T cells in the *JR209*-Tg mice. Anergy can result in tolerization of T cells. Although it is not known how anergy is induced in vivo, the lack of costimulatory signals during TCR-antigen recognition is the common speculation (32). Because gp100 is abundantly expressed in melanoma and normal melanocytes, there are apparently still enough peptide-MHC complexes on the cell surface at any given time, even with a fast dissociation rate from MHC complexes. This could provide the cognate T cell signal 1. In addition, we have evidence that IL-2, which has been shown to reverse T cell anergy (23), can restore the responsiveness of *JR209*-Tg T cells from $gp100_{209–217}^{WT}$ mice to self-antigen stimulation to the same level as that of *JR209*-Tg T cells from $gp100_{209–217}^{KO}$ mice. If this mouse model mimics the situation in man and many of the tumor-reactive T cells in the host are anergized, it is important to combine de-anergizing solutions such as common γ-chain cytokines or costimulatory factors with immunization protocols to achieve effective antitumor immune responses.

The key to understanding the poor immunogenicity of the $gp100_{209–217}$ epitope becomes apparent following analysis of the dissociation rate of peptide–MHC-I complexes. The "anchor-modified" $gp100_{209–217}$ peptide, in which a methionine replaces the natural threonine at position 2 of the nanomer, was predicted to fit well into the HLA-A2 binding pocket based on resolved peptide–HLA-A2 crystal structures. This prediction also indicated minimal differences of the peptide surface structures facing the TCR, which agreed with the clinical findings that human T cell clones do not distinguish the two peptides in the context of HLA-A2 molecules (5, 6). Thus, it is unlikely that the $gp100_{209–217(2M)}$ peptide could stimulate a subset of T cells that cross-react to the native antigen. We are in the process of resolving the crystal structure of the TCR–peptide–MHC-I complex to further test this prediction. The parental peptide has a 9-fold weaker binding affinity than the modified peptide (5, 7), due primarily to faster peptide dissociation. Thus, at any given time after initial presentation on the cell surface, a greater amount of the modified peptide remains bound to MHC-I than does its parental peptide. The correlation between this difference in dissociation rate and immunological potency was clearly demonstrated when peptide-pulsed APCs were separated for 24 hours before mixing; in this experiment only those cells pulsed with the modified peptide remained active. The expected 2-fold difference in association rates was not likely to influence these results, since no exogenous peptide was present during the 24-hour incubation, ensuring that the peptide concentration outside the cell remained extremely low. Note that the 24-hour time period is not even a single half-life for the modified peptide ($t_{1/2}$ of 27 hours), yet is over six half-lives for the parental peptide ($t_{1/2}$ of 3.7 hours). Thus, the 100-fold greater potency of the modified peptide can best be attributed to slower peptide dissociation.

It has been recently demonstrated by Spiotto et al. and Nguyen et al. that tumor antigens are cross-presented to $CD8^+$ T cells in draining lymph nodes by professional APCs (29, 33). Using real-time two-photon microscopy, Bousso et al. elegantly illustrated the dynamics of $CD8^+$ T cell priming by dendritic cells in intact lymph nodes. Contrary to conventional wisdom, it takes hours, not minutes, for APCs to activate naive $CD8^+$ T cells (34). Antigen uptake by an APC either at the tumor site or at the peptide immunization site, migration to draining lymph nodes, and stable contact with an antigen-specific T cell could take hours and possibly days. Therefore, the stability of peptide–MHC-I complexes becomes an important determinant of the immunogenicity of a given antigen.

In our transgenic mouse model, because the variable regions of the TCR were fixed, we could not address the issue brought by cross-reactive T cells. However, clinically it has become evident that T cells reactive to self/melanoma antigens with high avidity (capable of recognizing 1 nM or less peptide) do exist in humans (18, 35–37), which perhaps explains why immunotherapy is more efficient in treating patients with melanoma than those with other cancers. We have successfully isolated and cloned a $CD8^+$ T cell specific for the gp100 mouse homologue Pmel-17, residues 25–33 from normal C57BL/6 mice that were immunized with recombinant vaccinia virus expressing human gp100. This clone was able to recognize target cells pulsed with self-peptide at a level as low as 1 pM (38). Transgenic T cells derived from this clone, antigenic stimulation, and the administration of IL-2 have been demonstrated to be three inseparable necessities to successfully destroy cutaneously implanted solid syngeneic murine melanoma (39). Clearly, there are mechanisms regulating the function of these high-avidity autoreactive T cells in the host to prevent autoimmune diseases; however, in cancer immunotherapy, the goal is the opposite. In our study, we demonstrated that the strength of the stable peptide–MHC-I complexes could determine an antigen's ability to break the self-tolerance in the host. As numerous approaches are undergoing investigation in the field, we strongly recommend modifying the selected epitope to achieve the maximum stability of peptide–MHC-I complexes for effective immunization against self/tumor antigens.

Methods

Peptide synthesis. Native and modified $gp100_{209–217}$ peptides (ITDQVPFSV and IMDQVPFSV, respectively) were synthesized by Macromolecular Resources (Colorado State University, Fort Collins, Colorado, USA). They were more than 95% pure by HPLC analysis. All the peptides were dissolved in DMSO at 10 mg/ml as stock for ex vivo use. Fluorescently labeled peptides, modified at position 5 with a fluorescein-derivatized lysine [ITDQ(K-Flc)PFSV and IMDQ(K-Flc)PFSV], were synthesized by SynPep Corp. (Dublin, California, USA) and were HPLC-purified to greater than 95% purity.

Production of transgenic mice expressing human gp100$_{209–217}$–specific TCRs. R6C12, an HLA-A*0201–restricted, gp100$_{209–217}$–specific CD8$^+$ T cell clone derived from a melanoma patient was selected for hTCR cloning. The basic procedures used to identify and clone the TCR were adopted from the methods described by Kouskoff et al. (40). Briefly, the VJ fragments of the α chain and the VDJ fragments of the β chain were cloned by 5′ rapid amplification of cDNA ends and identified by DNA sequencing as *Vα41S1*, *Jα54*, *Vβ8S1*, *Dβ2*, and *Jβ2S1*. Genomic DNA of the TCR α and β chain, from the 10–12 bp upstream of the start codon and up to the 200-bp intronic sequences downstream of the junction regions, were PCR amplified and inserted into pTα and pTβ cassette vectors, respectively (kindly provided by Diane Mathis of Harvard University School of Medicine, Boston, Massachusetts, USA). The inserted genomic DNA fragments were verified by restriction enzyme digestion. After removing the prokaryote DNA sequences, the linearized pTα and pTβ cassettes were coinjected into fertilized eggs from C57BL/6 mice. Founder mice carrying both the α and β chains of the transgenic TCR were identified by Southern blot analysis. To positively select the hTCR in mice, the TCR-transgenic mice were bred to mice expressing HLA-A2/K^b MHC molecules (41) (purchased from The Jackson Laboratory, Bar Harbor, Maine, USA). Transgenic mice expressing both TCR and *HLA-A2/K^b* transgenes were named *JR209*-Tg mice.

Expression of human gp100$_{209–217}$–specific TCR in epitope-knockout mice. *JR209*-Tg mice were crossed with C56BL/6 mice lacking expression of the gp100$_{209–217}$ epitope (provided by Byoung Kwon, Indiana University, Indianapolis, Indiana, USA). Epitope-knockout mice were generated by insertion of a *neomycin* gene into exon 4 (corresponding to cDNA sequence 636–902 bp) of the *gp100* gene. This genetic interruption resulted in a truncation of gp100 protein amino acid residue 212 and therefore eliminated the target epitope. Mice were housed at the NIH-10A animal facility and at Biocon Inc. (Rockville, Maryland, USA). All animal study protocols were approved by the NIH institutional review board.

Murine melanoma cell line engineered to express HLA-A2/K^b molecules. B16 murine melanoma cells were stably transfected with plasmid DNA encoding HLA-A2/K^b as described previously (10). The expression of the transgene on the B16-A2/K^b tumor cells was monitored by FACS analysis of HLA-A2 on the cell surface.

Evaluation of JR209-Tg T cells. Freshly isolated splenocytes (5 × 10^6 cells) from *JR209*-Tg mice were cultured at 37°C with 5% CO_2 in 2 ml of RPMI culture media as described previously (10), containing 30 IU/ml of human recombinant IL-2 (Chiron Corp., Emeryville, California, USA) and 1 μM of gp100$_{209–217}$ native or modified peptide in a 24-well plate. Once the cells grew confluent, they were subdivided in culture media containing IL-2.

To detect activation markers on *JR209*-Tg T cells upon antigen stimulation, 1 × 10^5 to 1 × 10^6 splenocytes or cultured T cells were stained with fluorescence-labeled mAb against hVβ8 (clone JR-2), CD25 (clone PC-61), CD62L (clone MEL-14), CD69 (clone H1.2F3), CD44 (clone TM-1), or their isotype controls (BD Biosciences – Pharmingen, San Diego, California, USA) and analyzed by FACS. In antigen-specific IFN-γ release assays, 1 × 10^5 naive or peptide-stimulated *JR209*-Tg T cells were cocultured with 1 × 10^5 peptide-pulsed, irradiated (30 Gy) *HLA-A2/K^b* splenocytes or tumor cells from B16-A2/K^b, its parental B16 murine melanoma, and MC-38 murine colon adenocarcinoma (Southern Research Institute, Birmingham, Alabama, USA) in 200 μl of culture media/well of a 96-well U-bottom plate for 24 hours.

To test MHC restriction, 10 μg mAb against HLA-A2 (clone KS-21; Surgery Branch, National Cancer Institute, Bethesda, Maryland, USA), CD8 (clone 53-6.7, BD Biosciences – Pharmingen), and their isotype controls were added to target cells 30 minutes before coculture. The coculture supernatants were collected 24 hours later and assayed for IFN-γ by ELISA.

To measure the proliferation of *JR209*-Tg T cells in response to antigen stimulation, 1 × 10^6 freshly isolated splenocytes from naive *JR209*-Tg mice were labeled with 1 nM of CFSE (Molecular Probes Inc., Eugene, Oregon, USA) and cultured in culture media with 1 μM of peptide for 1–5 days before FACS for CFSE on CD8$^+$hVβ8$^+$ populations.

To examine the antigen-specific proliferation in vivo of naive *JR209*-Tg T cells, 1 × 10^7 CFSE-labeled naive *JR209*-Tg splenocytes were injected intravenously into each recipient mouse, which was then immunized with 100 μg of peptide in incomplete Freund's adjuvant (IFA; Sigma-Aldrich, St. Louis, Missouri, USA) in the footpad. Three to five days later, the draining lymph nodes of immunized mice were harvested and FACS for CFSE on CD8$^+$hVβ8$^+$ populations was performed.

Tumor treatment. B16-A2/K^b melanoma cells (2 × 10^5 cells in 100 μl of PBS) were injected intradermally into the flank region of mice. Seven to 14 days after tumor injection, mice were immunized (by injection into the footpads) with various doses of peptides emulsified in 100 μl of IFA. In adoptive-transfer experiments, tumor-bearing recipient mice were given whole-body sublethal irradiation (5 Gy) followed by intravenous injection of 1 × 10^7 cultured *JR209*-Tg T cells. All mice in the tumor treatment experiments were given recombinant human IL-2 (Chiron Corp.) starting the day of treatment, at 600,000 IU/dose twice daily for 3 days. Tumor size was measured in a randomized, blinded fashion. Vitiligo was determined by shaving the coat hair on the abdominal area of the mice and examining the return of depigmented hair at the site.

Ex vivo modeling of peptide immunization. Splenocytes from *HLA-A2/K^b* transgenic mice were pulsed with 1 μM of peptide for 3 hours at 37°C, washed three times with media, plated in 24-well plates, and incubated at 37°C overnight. Equal numbers of freshly isolated *JR209*-Tg splenocytes were then added to the wells and cocultured in media containing 30 IU/ml of IL-2 for 24 hours before adoptive transfer. As a control, freshly *JR209*-Tg splenocytes were cocultured with APCs immediately after peptide pulsing.

Determination of peptide dissociation rates from APCs. T2 cells, derived from human lymphoma and lacking transporter associated with antigen processing, were pulsed with various concentrations of peptide for 3 hours at 37°C and washed three times. Peptide-pulsed T2 cells were then suspended in culture media and incubated at 37°C for various time periods (see Figure 4 for time points) before they were cocultured for 24 hours with a human HLA-A2–restricted CD8$^+$ T cell clone specific for gp100$_{209–217}$ (CK3H6; Surgery Branch, National Cancer Institute) or *JR209*-Tg T cells stimulated with peptide for 7 days. The supernatants from the cocultures were assayed for IFN-γ by ELISA. The correlation between the concentration of peptide-MHC complexes and IFN-γ levels was calculated in the following manner. A regression analysis of peptide concentrations and IFN-γ production was determined immediately after peptide pulsing. Using this correlation, the relative amount of peptide–MHC-I complexes remaining on the cell surface at each time point after peptide pulsing was determined. Data were fit to a first-order rate equation of the form $y = A_0 + \exp(-1/k_{off}t) + y_0$, where A_0 is the initial amplitude, k_{off} is the dissociation rate, t is time, and y_0 is a baseline offset.

In vitro determination of peptide dissociation rates. Recombinant, soluble HLA-A2 and β$_2$m were refolded from bacterially expressed inclusion bodies as previously described (42) in the presence of fluorescently labeled native or position 2–modified gp100$_{209–217}$ peptide. Refolded peptide–MHC-I was purified chromatographically. Dissociation rates were measured using a fluorescence anisotropy assay as previously described (26). Briefly, about 7 nM HLA-A2 loaded with fluorescent peptide was incubated at 37°C with a 1,000-fold excess of unlabeled peptide, and the anisotropy was measured as a function of time using a Beacon 2000 fluorescence polarization instrument (Invitrogen Corp., Carlsbad, California, USA). The decay in anisotropy was fit to the first-order rate equation $y = A_0 + \exp(-1/k_{off}t) + y_0$, described above. The assay solution consisted of

10 mM HEPES and 150 mM NaCl, pH 7.4. A second kinetic phase, attributed in other reports to initial dissociation of β_2m prior to dissociation of the peptide (26), was not observed for dissociation of the gp100-based peptides. Measurements were performed in triplicate.

Statistical analysis. Statistical differences in tumor growth for different treatment groups were analyzed by Wilcoxon rank sum test. A Student's *t* test was used to compare the average cell numbers or IFN-γ concentrations in different groups.

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