The Density of Peptides Displayed by Dendritic Cells Affects Immune Responses to Human Tyrosinase and gp100 in HLA-A2 Transgenic Mice

Timothy N. J. Bullock, Teresa A. Colella and Victor H. Engelhard

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The Density of Peptides Displayed by Dendritic Cells Affects Immune Responses to Human Tyrosinase and gp100 in HLA-A2 Transgenic Mice

Timothy N. J. Bullock, Teresa A. Colella, and Victor H. Engelhard

Several HLA-A*0201-restricted peptide epitopes that can be used as targets for active immunotherapy have been identified within melanocyte differentiation proteins. However, uncertainty exists as to the most effective way to elicit CD8+ T cells with these epitopes in vivo. We report the use of transgenic mice expressing a derivative of HLA-A*0201, and dendritic cells, to enhance the activation of CD8+ T cells that recognize peptide epitopes derived from human tyrosinase and glycoprotein 100. We find that by altering the cell surface density of the immunizing peptide on the dendritic cells, either by pulsing with higher concentrations of peptide, or by changing the MHC-peptide-binding affinity by generating variants of the parent peptides, the size of the activated CD8+ T cell populations can be modulated in vivo. Significantly, the density of peptide that produced the largest response was less than the maximum density achievable through short-term peptide pulsing. We have also found, however, that while some variant peptides are effective at eliciting both primary and recall CD8+ T cell responses that can recognize the parental epitope, other variant epitopes lead to the outgrowth of CD8+ T cells that only recognize the variant. HLA-A*0201 transgenic mice provide an important model to define which peptide variants are most likely to stimulate CD8+ T cell populations that recognize the parental, melanoma-specific peptide. The Journal of Immunology, 2000, 164: 2354–2361.

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3 Abbreviations used in this paper: MDP, melanocyte differentiation protein; β2m, β2-microglobulin; DC, dendritic cell; gp, glycoprotein; iDC, immature DC; int, intermediate; mDC, mature DC.

insight into the use of these peptides for immunotherapy in melanoma patients, we have developed a preclinical model involving transgenic mice that express a recombinant MHC class I molecule in which the peptide binding domain of HLA-A*0201 is combined with the CD8 binding domain of the murine H-2D\(^{\beta}\) (27). CD8\(^{+}\) T cells from these mice have been shown to recognize HLA-A*0201-restricted Ags that are the same as those recognized by HLA-A*0201 human CTL (27–31). In addition, the strength of these responses is comparable with those restricted by endogenous murine H-2 molecules (27). In this study, we report the use of this model system to optimize immunization with DC that have been pulsed with therapeutically relevant HLA-A*0201-restricted melanoma Ags.

Materials and Methods

Peptides

Synthetic peptides were made by standard Fmoc chemistry using a model AMS422 peptide synthesizer (Gibson, Middleton, WI). All peptides were purified to >98% purity by reverse-phase HPLC on a C8 column (Vydac, Hesperia, CA). Purity and identity were confirmed using a triple quadrupole mass spectrometer (Finnigan, San Jose, CA).

Cell lines

ELA-A2/K\(^{\beta}\) is a transfectant of the EL4 thymoma that expresses the \(\alpha + \alpha\) domain of HLA-A*0201 in association with the \(\alpha\) domain of H2-K\(^{\beta}\) (a gift from Dr. Linda Sherman, The Scripps Institute, San Diego, CA). C1R-A2/A has been previously described (27). Both cell lines were maintained under selection in RPMI 1640 supplemented with 5% FBS/SerXtend (Irvine Scientific, Santa Ana, CA) and 300 \(\mu\)g/ml G418.

HLA-A*0201 peptide-binding assay

This was performed as previously described (32). Briefly, affinity-purified HLA-A*0201 molecules were incubated at room temperature with the indicated indicator peptide, FPLSDYFPPSV, and graded doses of test peptides in PBS, pH 7, containing 0.05% Nonidet P-40, 1 \(\mu\)M human \(\beta_{2}-\)microglobulin (\(\beta_{2}-\)m) (Calbiochem, La Jolla, CA), 1 mM PMSF, 1.3 mM 1,10-phenanthroline, 73 \(\mu\)M pepstatin A, 8 mM EDTA, and 200 \(\mu\)M \(N^{\gamma}\)-tosyl-l-lysine chloromethyl ketone (TLCK). After 48 h, class I peptide complexes were separated from free peptides by gel filtration, and the dose of individual test peptides that reduced the binding of indicator peptide by 50% (\(IC_{50}\)) was calculated.

Animals

Transgenic mice expressing a chimeric MHC class I composed of the \(\alpha\) and \(\alpha\)2 domains of HLA-A*0201 and the \(\alpha\)3 domain of H2-D\(^{\beta}\) (AAD) have been previously described (27) and were maintained in specific pathogen-free facilities at the University of Virginia (Charlottesville, VA).

Dendritic cells

DC were generated as described (33), with modifications. Bone marrow cells were excised from the femurs and tibias of AAD\(^{+}\) transgenic mice, depleted of RBC, and cultured in RPMI 1640 containing 10% FBS, 50 \(\mu\)g/ml gentamicin (Life Technologies, Grand Island, NY), 7.5 mM HEPES, 2 mM L-glutamine, 50 \(\mu\)M 2-ME, and 1000 U/ml of both IL-4 and GM-CSF (generous gifts of Schering-Plough, Kenilworth, NJ). Cultures were depleted of floating cells and refed with cytokine-supplemented media after 3 days, and one-half of the medium was replenished after 5 days. After an additional 2–4 days, immature DC (iDC) were isolated by centrifugation on a 14.5% metrizamide (Sigma, St. Louis, MO) gradient. Alternatively, iDC from 7-day cultures were then incubated at 5 \times 10^{6} cells/well of the above medium, except that 100 U/ml TNF-\(\alpha\) (Sigma) was substituted for 1000 U/ml IL-4. After 2 days, mature DC (mDC) were isolated on a StemSep column after incubation with a mixture of Abs for enrichment of mDC (StemCell, Vancouver, BC, Canada). By indirect immunofluorescence staining for HLA-A*0201 expression with an isotype-matched control, Flow cytometry was conducted on a FACScan using CellQuest software. Results are presented as percentage of positive cells after subtraction of isotype control values.

In vitro generation of peptide-specific HLA-A*0201-restricted CD8\(^{+}\) T cells

Spleens from primed mice were harvested at least 3 wk after immunization. A total of 1.5 \times 10^{6} responder cells and 7 \times 10^{5} autologous irradiated (2500 rad) spleen cells that had been pulsed with the indicated concentration of peptide for 3 h at 37°C were depleted of cytokine production using target/stimulator cells that had been pulsed overnight with the indicated concentration of peptide. For cytotoxicity assays, peptide-pulsed target cells were labeled with \(^{35}\)Cr and incubated with effector cells for 4 h at 37°C. To measure the production of intracellular cytokines, peptide-pulsed stimulator cells were incubated with enriched CD8\(^{+}\) T cells for 5 h at a ratio of 1:1 in media supplemented with 50 U/ml IL-2 and 5 \(\mu\)g/ml brefeldin A (Sigma). Stimulated cells were stained with PE-conjugated anti-CD8 (PharMingen, San Diego, CA), washed, and permeabilized in PerMwash/Fix (PharMingen), and then further stained with FITC-conjugated anti-IFN-\(\gamma\), anti-TNF-\(\alpha\), anti-IL-4, anti-IL-10 (all from PharMingen), or isotype-matched controls. Flow cytometry was conducted on a FACScan using CELLQuest software. Results are presented as percentage of positive cells after subtraction of isotype control values.

Quantitation of cell surface MHC-peptide complexes

HLA-A*0201 T2 cells were incubated overnight at 26°C in RPMI 1640 containing 5% FBS to generate a maximal amount of empty but peptide-receptive surface HLA-A*0201 molecules. To measure the relative amounts of HLA-A*0201-Tyr369 complexes formed during short-term peptide pulsing, the cells were incubated with the indicated amount of Tyr369 complexes from 3.5 h at 26°C in HBSS supplemented with 5% FBS, 5 \(\mu\)g/ml human \(\beta_{2}-\)m, and 5 \(\mu\)g/ml brefeldin A. The cells were washed twice and incubated for an additional 2 h at 37°C in HBSS supplemented with 5% FBS to allow determination of remaining peptide-free MHC molecules to denature. Alternatively, the relative amounts of HLA-A*0201 complexes formed during short-term peptide pulsing with gp100p26, gp100p26, or their variants, T2 cells that had been incubated at overnight 26°C were subjected to a mild acid treatment to denature cell surface HLA-A*0201 molecules (34). Acid-stripped cells were then incubated for 3 h at 37°C in 1 ml of RPMI supplemented with 5% FBS and 5 \(\mu\)g/ml \(\beta_{2}-\)m, and the indicated quantity of peptide, or no peptide. In both cases, cells were then washed twice before indirect immunofluorescence staining for HLA-A*0201 expression with the BB7.2 Ab and goat anti-mouse IgG (Fab\(^{\prime}\)) FITC (Jackson ImmunoResearch, West Grove, PA), fixed with 1% paraformaldehyde, and analyzed by flow cytometry. Results are expressed as percentage of HLA-A*0201 expression calculated using the formula: [(experimental HLA-A*0201 – no peptide HLA-A*0201)/(untreated T2 HLA-A*0201 at 37°C – no peptide HLA-A*0201)] \times 100.

To measure the dissociation rate of peptides from HLA-A*0201, T2 cells that had been incubated overnight at 26°C were loaded with 10 \(\mu\)g/ml of peptide for 20 h at 26°C in RPMI containing 5% FCS and 5 \(\mu\)g/ml \(\beta_{2}-\)m, washed twice, and then incubated at 37°C for the indicated time. Cells were stained and analyzed by flow cytometry, as described above. Results are expressed as geometric mean channel fluorescence after subtraction of the value obtained from cells that had not been incubated with peptide.

Results

DC stimulate CD8\(^{+}\) T cell responses to an HLA-A*0201-restricted human tyrosinase epitope in AAD transgenic mice

iDC are actively phagocytic and efficiently process exogenously supplied Ags. In contrast, although mDC are inefficient at Ag uptake, they express higher levels of class I and II MHC and costimulatory molecules (35). We were interested in whether these
two populations differed in their ability to activate CD8$^+$ T cells in vivo after they were pulsed with synthetic peptides. iDC were derived from bone marrow cells of AAD transgenic mice by culture in GM-CSF and IL-4, and mDC were derived by further culture of iDC in GM-CSF and TNF-α. These two populations were pulsed with an HLA-A*0201-restricted peptide, YMDGTMSQV, derived from residues 369–377 of human tyrosinase (Yty369), and used to immunize AAD transgenic mice. After 6 days, splenocytes were isolated, enriched for CD8$^+$ T cells, and tested immediately for Ag-specific cytolytic activity. Cells from mice primed with mDC showed significantly higher lytic activity toward targets pulsed with Yty369 than did cells from mice primed with iDC (Fig. 1A). This difference was maintained when splenocytes from mice primed at least 21 days previously with either iDC or mDC were restimulated in vitro for 1 wk in the presence of 1 μg/ml Yty369 (Fig. 1B). These results established that peptide-pulsed mDC are better than peptide-pulsed iDC in activating CD8$^+$ T cell responses. All additional experiments were thus conducted using mDC.

The dose of peptide used for pulsing influences the magnitude of the CD8$^+$ CTL responses following DC immunization

The density of peptide Ag presented on the surface of DC might be expected to influence the numbers of T cells activated. To address this, we first established the relationship between the concentration of peptide used during the pulsing step and the resulting level of peptide-MHC class I complexes. T2 cells were incubated overnight at 26°C to accumulate peptide-free HLA-A*0201 molecules at the cell surface and then incubated with increasing concentrations of Yty369 for 3.5 h at 26°C. Peptide was removed and the temperature shifted to 37°C to denature HLA-A*0201 molecules that had not bound peptide. Increasing the peptide concentration over the range of 0.1–100 μg/ml led to a stepwise increase in the number of stable HLA-A*0201 molecules, as determined by indirect immunofluorescence using an HLA-A*0201-specific Ab (Fig. 2A). This indicates that the epitope density on DC pulsed under comparable conditions should increase progressively over this concentration range of peptide. We next asked whether the density of Yty369 on the surface of DC used to immunize AAD transgenic mice influenced the number of activated CD8$^+$ T cells. CD8-enriched splenocytes were isolated from AAD mice 7 days after immunization with peptide-pulsed DC and used immediately for measurement of both Ag-specific cytolytic activity and Ag-induced accumulation of intracellular IFN-γ. As the density of Yty369-AAD complexes on the DC used for immunization was increased by changing the pulse concentration from 0.1 to 1 μg/ml, the cytolytic activity and number of IFN-γ-producing cells also increased (Fig. 2, B and C). Increasing the pulse concentration from 1 to 10 μg/ml Yty369 led to a modest decrease in both parameters. However, increasing the cell surface density still further by pulsing DC with 100 μg/ml Yty369 led to a significant decrease in both CD8$^+$ cytotoxic activity and the number of CD8$^+$ IFN-γ-producing cells. The dose dependence of activation was consistent over several days, and the highest level of response (as measured by accumulation of TNF-α) was observed on day 7 after immunization, regardless of peptide dose (Fig. 3). In addition, the reduction in the response observed at higher peptide doses was not associated with a shift to a type 2 cytotoxic T cell phenotype, based

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3 The sequence YMDGTMSQV from the tyrosinase protein was initially identified as residues 368–376, and this laboratory had previously used that numbering system (41). The correct numbering for the YMDGTMSQV peptide is 369–377.
on the lack of detectable secretion of either IL-4 or IL-10 (data not shown). Over the entire dose range, cytotoxic activity roughly paralleled the number of IFN-γ-producing cells, suggesting that the major effect was on T cell activation, rather than the nature of the activated phenotype. These data indicate that the cell surface density of an epitope presented to a naive T cell population can profoundly influence the number of CD8+ T cells that respond, and that the optimum density is less than the maximum achievable density.

Comparison of CD8+ T cell responses elicited by mDC and recombinant vaccinia virus

An important goal of developing a preclinical model is to obtain a system by which different vaccination protocols can be assessed. Therefore, we compared the ability of peptide-pulsed mDC and recombinant vaccinia virus to elicit primary CD8+ T cell responses. We determined that the activity of the primary CD8+ T cell response to recombinant vaccinia expressing a minigenie encoding Tyr369 (Tyr369-vac) peaked at day 7, and 5 × 10^7 PFU of recombinant virus was the optimal immunization dose (data not shown). Thus, AAD mice were primed with either mDC pulsed with 1 μg/ml Tyr369 or 5 × 10^7 PFU of a recombinant vaccinia virus expressing a minigenie encoding Tyr369 (Tyr369-vac). Splenocytes were either harvested and enriched for CD8+ cells after 7 days (A and B), or restimulated in vitro 21 days after immunization (C). A, Cytotoxic activity at an E:T ratio of 200:1 on EL4-AAK targets that had been either pulsed with 10 μg/ml Tyr369 (●) or left unpulsed (○). B, Accumulation of intracellular IFN-γ induced by incubation with EL4-AAK stimulator cells that had been pulsed with 100 μg/ml Tyr369 (■) or left unpulsed (□). C, Cytolytic activity of in vitro cultures (see Materials and Methods) 7 days after restimulation with 1 μg/ml YMDGTMQSV. Splenocytes from Tyr369-vac-primed mice in the absence of EL4-AAK (C, ●), or mDC (□)-primed mice were incubated with EL4-AAK targets that had been pulsed with 10 μg/ml YMDGTMQSV (filled) or left unpulsed (open). In each panel, data for each figure are representative of at least two independent assays.

Responses to other HLA-A*0201-restricted human MDP-derived Ags in AAD transgenic mice

We were interested in determining whether other known HLA-A*0201-restricted peptide Ags derived from MDP also activated CD8+ T cells in AAD mice, and whether the T cell responses correlated to peptide dose in a manner similar to Tyr369. Therefore, we immunized AAD transgenic mice with mDC that had been pulsed with 1 μg/ml of KTWGQYWQV (gp100154), ITDQPFSV (gp100154), YLEPGPVTVA (gp100236), LLDG TATLRL (gp100157), or VLRYRGFSFV (gp100476). We detected weak CD8+ T cell responses to gp100154 and gp100476, but were not able to detect responses to the remaining epitopes either directly ex vivo after 7 days, or after in vitro restimulation of splenocytes from animals primed 3 wk previously (Table I). Interestingly, the positive responses were associated with peptides that had high relative binding affinities for HLA-A*0201, suggesting that this parameter might be important.

It has been shown that some amino acid substitutions in a subset of MDP-derived peptide Ags increase their affinity for HLA-A*0201 and improve their ability to stimulate immune responses

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Amino acid substitutions into gp100-derived peptides decrease the dissociation rate from HLA-A*0201 without affecting the initial cell surface density achieved in short-term incubations. The off rates of the indicated peptides were evaluated by stabilization of HLA-A*0201 expression on T2 cells. T2 cells were loaded with peptide ( ), gp100209 parent; ( ), variant; ( ), gp100209 parent; ( ), variant), washed, and then incubated at 37°C for the indicated time to allow HLA-A*0201 denaturation. B, HLA-A*0201 stabilization on T2 cells by parent and variant peptides in short-term incubations. T2 cells were acid stripped, then allowed to reconstitute for 3.5 h in media containing 10 μg/ml the indicated concentration of the indicated peptide ( , gp100280 parent; , gp100280 variant; , gp100280 parent; , gp100280 variant), according to the protocol described in the Materials and Methods, before staining for HLA-A*0201.

from patient PBL (10, 36, 37). Accordingly, we synthesized variants of the two lowest affinity gp100-derived peptides tested in Table I: gp100209(M), in which Met was substituted for Thr at the P2 anchor position, and gp100280(V), in which Val was substituted for Ala at the P9 anchor position. HLA-A*0201-stabilization assays confirmed that these variant peptides dissociated more slowly from HLA-A*0201 than the parent peptides (Fig. 5A). Because differences in relative affinity are primarily a function of differences in off rate, this supports the idea that these substitutions increase the affinity of the variant peptides relative to the parents. We next determined how this increase in MHC-binding affinity affected the density of peptides on the cell surface after short-term peptide pulsing. In agreement with Fig. 2A, the number of HLA-A*0201 molecules stabilized was dependent upon the concentration of peptide used (Fig. 5B). However, at any given concentration, the parent and variant peptides were equivalent in their ability to stabilize HLA-A*0201 during short-term incubations of duration equivalent to those used to pulse DC (Fig. 5B). These results are consistent with the idea that the number of peptide MHC complexes presented by DC after short-term incubation with these exogenous peptides is not influenced by differences in their binding affinities. Instead, it is largely a function of the on rate, which should be similar or identical for these peptides. Thus, the effect of amino acid substitutions that increase binding affinity under these conditions is to prolong epitope expression on DC, rather than to change the initial level of expression.

AAD mice were immunized with mDC that had been pulsed with increasing concentrations of either the gp100280 or gp100280(V) peptides. Seven days after immunization, CD8+ T cells were isolated and their activation state was assessed by staining for the accumulation of intracellular IFN-γ after 5 h of in vitro culture with cells that had been pulsed with 100 μg/ml of either peptide. The responses in mice immunized with DC pulsed with parental gp100280 were not significantly different from responses in mice immunized with DC alone, regardless of which peptide was used for in vitro activation of IFN-γ accumulation (Fig. 6A). However, substantial responses were observed in mice immunized with DC that had been pulsed with gp100280(V). The number of activated CD8+ T cells increased with the dose of peptide pulsed onto DC in the range of 0.1–10 μg/ml, consistent with our earlier observations with Tyr369. In addition, the highest numbers of CD8+ T cells activated by gp100280(V) were similar to those observed in mice immunized with Tyr369-pulsed DC. Importantly, a fraction of the CD8+ T cells that were activated by the variant peptide in vivo could recognize the parent epitope in vitro (Fig. 6A). Similar results were obtained using DC-pulsed gp100280 or gp100280(M). Again, no significant response was observed in mice immunized with DC pulsed with the parent peptide, but a substantial, dose-dependent response was observed to DC pulsed with the variant (Fig. 6B). In this case, however, the peak number of activated CD8+ T cells was consistently lower than those seen in response to DC pulsed with Tyr369 or gp100280(V) (compare Fig. 6B with Figs. 6A and 2C). Again, a proportion of the activated CD8+ T cells could be stimulated by the parent peptide. These data confirmed a recent study that used a recombinant vaccinia virus expressing gp100280(M) to elicit CD8+ T cell responses to the gp100280 epitope (38). Collectively, these results indicate that the introduction of subtle amino acid substitutions in peptide epitopes, with the intent of decreasing the dissociation of peptide Ags from the surface of DC, enhances their immunogenicity.

Because only a fraction of the T cells activated with the variants recognized the parent peptides, we were concerned that continued stimulation with the variants might lead to the outgrowth of T cells that did not cross-react on the parents. To investigate this, CD8+ T cells from mice primed with gp100280(V)-pulsed DC were cultured with irradiated AAD splenocytes that had been pulsed with
ued stimulation with some, but not all, variants can lead to the full elicitation of primary immune responses. However, continuously variant peptides with enhanced MHC-binding affinity are used that variant was also lower than that seen in primary populations. HLA-A*0201 transgenic mice (27, 39, 40) provide an ideal model for these investigations, because they allow the systematic manipulation of these parameters as applied to peptides that are the subject of current clinical trials. In this study, we used AAD transgenic mice to identify strategies that augment immune responses to human melanoma peptide Ags presented by DC. Using this system, we determined that peptide-pulsed mDC were superior to iDC in their ability to activate cytolytic CD8+ T cells. This result contrasts with previous observations that iDC are better able to stimulate responses to either whole protein or recombinant nucleic acid (23, 41). This difference is likely to be due to the fact that iDC are better able to endocytose these materials via macropinocytosis than mDC (42, 43), but can mature thereafter. In contrast, the presentation of preprocessed Ags, such as synthetic peptides and acid extracts of tumors, is more likely to be augmented by the high level of MHC expression in mDC. Because presentation of these peptide Ags is also transient, their administration in the context of an already mature cell is also likely to be an advantage.

The use of peptide-pulsed DC also allowed us to evaluate how the surface density of an epitope, in combination with its MHC-binding affinity, influences the size of the primary CD8+ T cell response in vivo. This issue is not readily addressed using recombinant viral or bacterial delivery systems. Perhaps not surprisingly, we found that over a wide dose range, increasing the density of Ag on the DC resulted in a corresponding increase in the size of the peptide-specific CD8+ T cell response until a plateau was reached. It is not yet clear whether this reflects the preferential activation of only higher avidity T cells at lower Ag densities, or simply activation of a smaller number of T cells due to a lower occupancy rate on the DC. We were quite intrigued, however, to see a substantial decline in the size of the responding population at the highest Ag density examined. This was observed with several epitopes. It is possible that at a supraoptimal Ag density, peptide is shed from the surface of the DC and presented to naive T cells in a tolerogenic manner. This would be consistent with the observation that very high doses of peptide in IFA can cause T cell tolerance (44). On the other hand, at least in vitro, supraoptimal Ag density can also lead to the apoptosis of high avidity CD8+ effector T cells via TNF-α and down-regulation of Bcl2 (45). Although further work will be required to understand the mechanism, it is apparent that the density of Ag used to activate CD8+ T cells directly affects the effector T cell via a TNF-α and down-regulation of Bcl2 (45). Although further work will be required to understand the mechanism, it is apparent that the density of Ag used to activate CD8+ T cells directly affects the effector T cell via a

1 μg/ml of either the parent or variant peptides. After 1 wk, both T cell populations showed equivalent recognition of targets pulsed with either peptide (Fig. 7, A and B). This, repetitive stimulation with this particular variant peptide maintained a population of T cells with strong recognition of the parent peptide. In contrast, when a similar experiment was conducted using CD8+ T cells from mice primed with gp100280(M)-pulsed DC, only cells restimulated with the parent peptide maintained a high level of recognition (Fig. 7, C and D). Cells that were maintained on the variant peptide for even 1 wk in vitro showed substantially lower recognition of the parent. Furthermore, when we determined the number of peptide-specific T cells in each population based on accumulation of intracellular IFN-γ, it was apparent that only about 30% of cells restimulated with the variant peptide recognized the parent, as compared with 100% of the cells restimulated with the parent peptide. The percentage of cross-reactive cells after in vitro culture with the variant was also lower than that seen in primary populations (compare Figs. 6B and 8). Taken together, these data suggest that variant peptides with enhanced MHC-binding affinity are useful for elicitation of primary immune responses. However, continuous stimulation with some, but not all, variants can lead to the outgrowth of T cells that have lost reactivity with the parent peptide. The AAD transgenic mouse model can be used to distinguish these two categories of variant peptides.

**Discussion**

The use of DC as a vehicle to immunize with synthetic peptides representing tumor Ags is a promising immunotherapeutic approach for the treatment of cancer. However, because DC can be loaded with Ag in many different ways, it is important to define and optimize the parameters that induce the largest CD8+ T cell populations. HLA-A*0201 transgenic mice (27, 39, 40) provide an ideal model for these investigations, because they allow the systematic manipulation of these parameters as applied to peptides that are the subject of current clinical trials. In this study, we used AAD transgenic mice to identify strategies that augment immune responses to human melanoma peptide Ags presented by DC. Using this system, we determined that peptide-pulsed mDC were superior to iDC in their ability to activate cytolytic CD8+ T cells. This result contrasts with previous observations that iDC are better able to stimulate responses to either whole protein or recombinant nucleic acid (23, 41). This difference is likely to be due to the fact that iDC are better able to endocytose these materials via macropinocytosis than mDC (42, 43), but can mature thereafter. In contrast, the presentation of preprocessed Ags, such as synthetic peptides and acid extracts of tumors, is more likely to be augmented by the high level of MHC expression in mDC. Because presentation of these peptide Ags is also transient, their administration in the context of an already mature cell is also likely to be an advantage.

The use of peptide-pulsed DC also allowed us to evaluate how the surface density of an epitope, in combination with its MHC-binding affinity, influences the size of the primary CD8+ T cell response in vivo. This issue is not readily addressed using recombinant viral or bacterial delivery systems. Perhaps not surprisingly, we found that over a wide dose range, increasing the density of Ag on the DC resulted in a corresponding increase in the size of the peptide-specific CD8+ T cell response until a plateau was reached. It is not yet clear whether this reflects the preferential activation of only higher avidity T cells at lower Ag densities, or simply activation of a smaller number of T cells due to a lower occupancy rate on the DC. We were quite intrigued, however, to see a substantial decline in the size of the responding population at the highest Ag density examined. This was observed with several epitopes. It is possible that at a supraoptimal Ag density, peptide is shed from the surface of the DC and presented to naive T cells in a tolerogenic manner. This would be consistent with the observation that very high doses of peptide in IFA can cause T cell tolerance (44). On the other hand, at least in vitro, supraoptimal Ag density can also lead to the apoptosis of high avidity CD8+ effector T cells via TNF-α and down-regulation of Bcl2 (45). Although further work will be required to understand the mechanism, it is apparent that the density of Ag used to activate CD8+ T cells directly affects the size of the responding T cell population, and the highest cell surface peptide density is not necessarily the optimal way of stimulating these responses. This observation has direct bearing on the use of peptide-pulsed DC in clinical trial settings.

Our results are consistent with those obtained in a separate study in which recombinant vaccinia viruses were engineered to express different levels of Ag (46). These authors demonstrated that CTL responses increased with increasing Ag expression, but declined at the highest levels of expression. However, our results contrast with another report that demonstrated that the density of Ag above a minimum immunogenic threshold does not influence the size of...
the CD8\(^+\) T cell response (47). In this case, the authors used a recombinant *Listeria monocytogenes* delivery system and alterations in flanking residues to influence the efficiency of epitope processing and presentation. However, it is uncertain whether the activation of naive CD8\(^+\) T cells by microbial immunization is due to the direct infection of professional APC, or results from processing and presentation of Ag after the phagocytosis of infected cells by professional APC. Since Vijh et al. (47) measured the total available peptide on all infected cells, their observations could be a reflection of the amount of Ag that is present in an infected cell at the time it is endocytosed and presented by professional APC (48, 49).

We also used our model system to examine how the immunogenicity of a peptide Ag can be modulated by altering its MHC-binding affinity and dissociation rate. The gp100\(_{209}\) peptide, which has a relatively low HLA-A*0201-binding affinity and fast dissociation rate, was unable to activate CD8\(^+\) T cells when presented on DC, even after pulsing with very high concentrations of peptide. In contrast, gp100\(_{280}\) (V), which has a relatively higher affinity and slower off rate, activated a substantial CD8\(^+\) T cell response against both itself and the parental epitope. This observation is consistent with other studies that have used peptide variants derived from gp100 (50) or Mart-1 (51). However, by using peptide-pulsed DC, we extended this earlier work to establish that the inability of gp100\(_{280}\) to stimulate a response could not be overcome by increasing the pulsing concentration, and thereby the initial level of cell surface epitope expression. In fact, DC expressing a relatively low initial cell surface density of gp100\(_{280}\) (V) stimulated significant responses, whereas DC expressing a much higher initial density of gp100\(_{280}\) were nonimmunogenic. The explanation for this paradoxical result is that the short-term pulsing conditions used resulted in cell surface Ag densities that are largely a function of the peptide on rate, which is usually diffusion controlled and therefore similar or identical for ligands of identical size that are present in solution at the same concentration. However, once exogenous peptide is removed, the cell surface Ag density declines according to its off rate. In the case of gp100\(_{280}\), this decline is apparently so rapid as to render DC pulsed with a very high concentration of this peptide poorly immunogenic.

The mechanism described above may also account for the increased immunogenicity of gp100\(_{209}\) (M) compared with nonimmunogenic gp100\(_{209}\). However, immune responses to gp100\(_{209}\) are also likely to be influenced by the fact that this sequence, in contrast to gp100\(_{280}\), is identical in humans and mice. Thus, the gp100\(_{209}\)-specific response in AAD transgenic mice may be compromised by one or more mechanisms of self-tolerance. Indeed, gp100\(_{209}\) is nonimmunogenic, despite the fact that its affinity for HLA-A*0201 is similar to that of strongly immunogenic Tyr\(_{369}\). An intriguing possibility is that gp100\(_{209}\) as a self Ag, functions as a partial agonist, whereas gp100\(_{209}\) (M) has a sufficiently different conformation that enables it to function as a full agonist (52–54). It is clear that gp100\(_{209}\) (M) induces responses against gp100\(_{209}\) demonstrating that tolerance to the self peptide is partial at best. Again, further investigation into this possible mechanism of activation by variant peptides in the context of tumor Ags that are also self Ags is necessary.

Most immunotherapy protocols involve multiple rounds of immunization to generate large populations of activated tumor-specific CD8\(^+\) T cells. We found that CD8\(^+\) T cell populations primed and restimulated with gp100\(_{280}\) (V) maintained recognition of the parent peptide. A similar result was observed using peptide variants derived from Mart-1\(_{27–35}\) (51). Conversely, we found that T cell populations primed and restimulated with gp100\(_{209}\) (M) were substantially less effective at recognizing gp100\(_{209}\) than those primed with gp100\(_{209}\) (M) and restimulated with the parent epitope. Similar results have been obtained with human PBL from patients immunized with gp100\(_{209}\) (M) (55). Although the positions of the substitutions in both of these peptides point down into the HLA-A*0201 binding site, one possible explanation for this difference is that the substitution of Met for Thr at P2 in gp100\(_{209}\) induces a more significant change in the overall conformation of the peptide than the substitution of Val for Ala at P9 in gp100\(_{280}\) epitope. Alternatively, because the gp100\(_{209}\) sequence is identical in mice and humans, while the gp100\(_{209}\) sequences differ by two residues, endogenous expression of murine gp100\(_{209}\) may lead to more profound tolerance to the human variant peptides used for immunization.

If this tolerance is selective for the high avidity, putatively cross-reactive, T cell precursors (56), then the repertoire available for expansion upon immunization with gp100\(_{209}\) (M) will be skewed toward lower avidity T cells that are less likely to cross-recognize the parent. Further work will be required to distinguish between these two possibilities.

We have seen recognition of human melanoma cells by some of the T cells generated against Tyr\(_{369}\) and gp100\(_{209}\) (data not shown). However, the mice used for these experiments are transgenic for a chimeric HLA-A*0201, in which the α3 domain has been substituted with the α3 domain from H-2D\(^*\). Although this enhances the interaction between the murine CD8 and the chimeric MHC class I molecule, it results in a decreased efficiency in recognition of the normal HLA-A*0201 molecule expressed on human tumor cells. Therefore, even the failure to recognize a human tumor does not mean that these CTL would be ineffective against an appropriate AAD\(^+\) tumor. We are currently establishing a model system to evaluate this. Nonetheless, the results of this study have shown the value of using a human class I transgenic mouse model to study the variables involved in immunization with human peptide Ags. We have found that when peptide-pulsed DC are used for immunization, both the cell surface density and dissociation rate of the peptide used impact significantly on the size of the activated population. Although peptide variants with diminished dissociation rates provide a powerful means to enhance immunogenicity, our work also suggests that subtle conformational changes and/or self-tolerance are important issues to be considered in their use. Transgenic mouse models will be of great use in assessing such parameters before administration to humans.

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References


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