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Manipulation of Avidity to Improve Effectiveness of Adoptively Transferred CD8\(^+\) T Cells for Melanoma Immunotherapy in Human MHC Class I-Transgenic Mice\(^1\)

Timothy N. J. Bullock, David W. Mullins, Teresa A. Colella, and Victor H. Engelhard\(^2\)

The adoptive transfer of tumor-reactive CD8\(^+\) T cells into tumor-bearing hosts provides an attractive alternative to vaccination-based active immunotherapy of melanoma. The development of techniques that result in the preferential expansion of tumor-reactive T cells is therefore of great importance. In this study, we report the generation of HLA-A*0201-restricted CD8\(^+\) T cell populations that recognize either tyrosinase\(_{369-376}\) or gp100\(_{209-217}\) from tolerant human class I MHC-transgenic mice by using single amino acid-substituted variant peptides. Low peptide concentration or restimulation with the parent peptide was used to enhance the functional avidity, defined by stimulation of IFN-\(\gamma\) accumulation, and cross-reactivity of the resulting T cell populations. We found a direct correlation between the ability of a T cell population to respond in vitro to low concentrations of the precise peptide expressed on the tumor and its ability to delay the outgrowth of B16 melanoma after adoptive transfer. Surprisingly, we found that some T cells that exhibited high functional avidity and were effective in controlling tumor outgrowth exhibited low structural avidity, as judged by MHC-tetramer staining. Our results establish strategies for the development and selection of CD8\(^+\) T cell populations that persist despite peripheral tolerance, and that can control melanoma outgrowth. Furthermore, they support the use of human MHC class I-transgenic mice as a preclinical model for developing effective immunotherapies that can be rapidly extended into therapeutic settings. The Journal of Immunology, 2001, 167: 5824–5831.
single amino acid-substituted variant peptides, it had not been determined whether the cross-reactive T cells could be manipulated to recognize tumors. In this study, we have extended this work by making use of cumulative in vitro selection approaches, based on the manipulation of peptide concentration and specificity, to engineer high-avidity, tumor-reactive CD8⁺ T cell populations that are effective in controlling the outgrowth of the aggressive B16 melanoma after adoptive transfer.

Materials and Methods

Animals

Transgenic mice expressing a chimeric MHC class I composed of the α1 and α2 domains of HLA-A*0201 and the α3 domain of H2-DⅡ (AAD) have been described previously (20). Mice were maintained in specific pathogen-free facilities at the University of Virginia.

Cell lines

C1R-AAD has been previously described (20). The C57BL/6-derived melanoma cell lines B16-F10 (CRL-6475) and B16-F1 (CRL-6323) were obtained from the American Type Culture Collection (Manassas, VA). The generation of AAD⁺ transfectants of B16-F10 and B16-F1 is described in detail elsewhere (47). Transfectants were cultured in RPMI 1640 containing 5% FBS supplemented with SerXtend (Irvine Scientific, Santa Ana, CA) and 300 μg/ml G418 (Life Technologies, Grand Island, NY), and screened regularly by flow cytometric analysis for AAD expression using the HLA-A2-binding Abs CR11-351 or BB7.2.

Peptides

Synthetic peptides were made by standard F-moc chemistry using a model AMS422 peptide synthesizer (Gilion, Middleton, WI) in the Biomedical Core Facility at the University of Virginia. All peptides were purified to >98% purity by reverse-phase HPLC on a C-8 column (Vydac, Hesperia, CA). Purity and identity were confirmed using a triple quadrupole mass spectrometer (Finnigan, San Jose, CA).

Dendritic cells (DC)

DC were generated as described (21), with modifications (6). Immature DC were isolated on a StemSep column after incubation with a mixture of Abs that enrich for DC, for then incubated at a 2:1 ratio overnight with irradiated CD40 ligand (CD40L)-transfected NIH-3T3 fibroblasts (a kind gift from R. Lapointe, National Cancer Institute, Bethesda, MD). These activated DC expressed high levels of MHC class I, MHC class II, CD80, CD86, and CD40, and demonstrated intracellular accumulation of IL-12 after a 5-h incubation in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO).

Immunization

DC were pulsed with the indicated concentration of peptide for 4 h at 37°C in HBSS containing 5% FBS and 5 μg/ml human β₂-microglobulin (Calbiochem, La Jolla, CA), washed twice, and resuspended in HBSS. Mice were injected in tail veins with 10⁵ DC.

Generation of peptide-specific HLA-A*0201-restricted CD8⁺ T cells

Splenies from primed mice were harvested at least 3 wk after immunization. A total of 1.5 × 10⁵ responder cells was incubated in upright 25-mm tissue culture flasks (Costar, Cambridge, MA) with 7 × 10⁶ autologous irradiated (2500 rad) spleen cells that had been pulsed with the indicated concentration of peptide for 3 h at 37°C. After culture for 6–7 days, cytotoxic activity and cytokine production were assessed, as described above. T cell lines were established from the initial cultures and maintained by weekly restimulations, as described above, except that 5 × 10⁶ T cells were incubated with 5 × 10⁶ peptide-pulsed stimulators in 12-well plates (Costar) after week 2, and cultures were supplemented with 10 μg/ml IL-2 on the day of restimulation and then 3 days later.

Intracellular cytokine staining

Cytokine expression was examined either ex vivo in freshly isolated spleen cells or in long-term T cell lines maintained in vitro. For ex vivo analysis, CD8⁺ T cells were isolated from spleens of immunized mice after incubation with a mixture of Abs to enrich for CD8 cells, followed by passage over StemSep column (StemCell Technologies, Vancouver, BC, Canada). Preparations were consistently 85–95% CD₈⁺, as assessed by flow cytometry. Enriched CD₈⁺ T cells were then directly assessed for cytokine production after 5-h incubation with C1R-AAD stimulator cells that had been pulsed overnight with the indicated concentration of peptide.

To measure the production of intracellular cytokines, peptide-pulsed stimulator cells were incubated with T cells for 5 h at a ratio of 1:1 in medium supplemented with 50 U/ml IL-2 and 10 μg/ml brefeldin A. Stimulated cells were counterstained with a 1/1000 dilution of either PE- or APC-conjugated anti-CD8 (BD PharMingen, San Diego, CA), washed, then fixed and permeabilized in PermWashFix (BD PharMingen), followed by staining with a 1/50 dilution of FITC-conjugated anti-IFN-γ (BD PharMingen). Flow cytometry was conducted on a FACScan using CellQuest software (BD Biosciences, San Jose, CA). Normalized staining values were calculated by using the formula (experimental value − background value for un pulsed stimulators)/(maximal value (using 100 μg/ml peptide-pulsed stimulators) − background value) × 100.

Cytotoxicity assay

Lytic activity of T cell populations was assessed using standard chromium release assays. Briefly, target cells were labeled overnight with ¹¹⁰⁵ (American, Boston, MA) at a concentration of 100 μCi/10⁵ cells. The target cells were washed and counted, and 2000 cells were aliquoted into wells containing the indicated concentration of peptide that had been diluted in medium containing 5 μg/ml β₂-microglobulin. After 2 h at 37°C, T cells were added at a ratio that had been predetermined to produce 65% lysis of target cells pulsed with 10 μg/ml peptide.

Tetramer staining

HLA-A*0201 tetramers that had been folded around ITDQVPFSV (gp100259) or IMDQVPFSV (gp100202,M) were produced by the National Institutes of Health Tetramer Facility at Emory University (Atlanta, GA). T cells were cocultured for 45 min at room temperature with the indicated concentration of tetramer and a 1/1000 dilution of anti-CD8, washed twice, and fixed in 2% paraformaldehyde. Staining was quantitated on a FACScan using CellQuest software. Tetramer-staining values on a CD₈⁺ T cell with irrelevant specificity were subtracted.

Adoptive transfer of CD8⁺ T cells

Subcutaneous tumors were established in AAD mice by injection of 4 × 10⁵ B16-F1-AAD or B16-F10-AAD in 200 μl of saline. Tumor cells were 100% viable by trypan blue exclusion and >98% AAD⁺ by flow cytometric analysis on the day of injection. All animals developed palpable tumors by day 10, at which point 1 × 10⁷ cells of the indicated CD8⁺ T cell line were injected directly into the tumor. A total of 500 Cetus units of IL-2 was coadministered with the T cells, and additional injections of 500 Cetus units of IL-2 were delivered i.p. on the subsequent 3 days. Tumor growth was measured every 48–72 h using a Vernier caliper, and was recorded as the product of two orthogonal diameters (a × b). The first diameter was identified as the longest surface length (a), with the second diameter being the subsequent orthogonal width (b). Repeated observations with the Vernier caliper resulted in <5% SD.

Results

The requirement for variant peptides to activate MDP-reactive CD8⁺ T cell populations in AAD mice is not overcome by using CD40-activated DC

The generation of CD8⁺ T cell populations that can be used for adoptive transfer requires the in vitro expansion of Ag-experienced T cells. We previously showed that after immunization with recombinant vaccinia viruses or peptide-pulsed mature DC, responses to the self-Ags Tyr₃₆₉ and gp100₂₀⁹ were extremely weak (5, 6). Since it has been reported that CD40L is a potent inducer of DC immunogenicity, we first asked whether populations of MDP-reactive memory cells that would be appropriate for further in vitro manipulation could be generated in AAD mice immunized with DC that had been activated in this manner. AAD mice were injected i.v. with CD40L-activated DC that had been incubated with either Tyr₃₆₉ or gp100₂₀⁹ peptides, and CD8⁺ T cells were isolated 7 days later at the peak of the primary response. These cells were incubated with peptide-pulsed stimulator cells for 5 h and then analyzed by flow cytometry for the accumulation of IFN-γ. No measurable number of gp100₂₀⁹-specific T cells was detected in
any of seven mice immunized with gp100<sub>209</sub>, and only two of six mice showed a discernible response to Tyr<sub>369</sub> (Table I). In contrast, consistently strong responses were seen in AAD mice immunized using DC pulsed with single amino acid-substituted variant peptides Tyr<sub>369</sub>(Y) or gp100<sub>209</sub>(M) (Table I). Tyr<sub>369</sub>(Y) is a variant of Tyr<sub>369</sub> carrying a conservative F to Y substitution at the P1 position, while gp100<sub>209</sub>(M) is a variant of gp100<sub>209</sub> with enhanced binding to HLA-A*0201 due to the substitution of M for T at P2 (22). Importantly, ~40% of the CD8<sup>+</sup> T cells activated by immunization with Tyr<sub>369</sub>(Y) or gp100<sub>209</sub>(M) also produced IFN-γ when stimulated with Tyr<sub>369</sub> and gp100<sub>209</sub>, respectively (Fig. 1 and Table I). These data confirm earlier work showing that T cells in AAD mice are largely unresponsive to immunization with gp100<sub>209</sub> and Tyr<sub>369</sub> self peptides, and demonstrate that CD40L-activated DC are not effective in altering this. However, immunization with closely related peptide variants enables the expansion of T cell populations that show appreciable cross-reactivity on the parental self peptides at the effector level, and provides a basis for the further development of MDP-reactive CD8<sup>+</sup> T cell populations for adoptive transfer.

Selection of CD8<sup>+</sup> T cells with high functional avidity

Although the preceding results were encouraging, it was of concern that over several independent immunizations, on average over half of the CD8<sup>+</sup> T cells activated with epitope variants, were not cross-reactive on the epitope itself (Table I and data not shown). Thus, further in vitro culture in the presence of the epitope variants might lead to loss of cross-reactive T cells altogether (6). On the other hand, the inability to demonstrate significant primary responses upon immunization with Tyr<sub>369</sub> or gp100<sub>209</sub> suggested that these peptides might be unable to drive in vitro proliferation of T cells. To evaluate these concerns directly, mice were immunized using DC pulsed with either 0.1 or 10 μg/ml gp100<sub>209</sub>(M). T cell lines were established from mice primed with the lower concentration of peptide by in vitro culture with 0.1 μg/ml of either gp100<sub>209</sub>(M) or gp100<sub>209</sub>, and corresponding lines were established from mice primed with the higher concentration by culture with 10 μg/ml of either peptide. CD8<sup>+</sup> T cell lines were successfully generated in each case, demonstrating that gp100<sub>209</sub> can stimulate proliferation of primed cells in vitro, despite its inability to stimulate naive T cells in vivo (Fig. 2 and data not shown).

The functional avidity of each line was determined as the concentration of gp100<sub>209</sub>(M) peptide that, when pulsed onto stimulator cells, induced half-maximal intracellular accumulation of IFN-γ in a 5-h assay (SC<sub>50</sub>). gp100<sub>209</sub>(M) was chosen to measure functional avidity for all T cell lines because it was the peptide used to prime all T cells in vivo. The T cell lines maintained with 0.1 μg/ml of either gp100<sub>209</sub> or gp100<sub>209</sub>(M) showed similar and relatively high functional avidities (SC<sub>50</sub> of 3.7 × 10<sup>-9</sup> and 2.9 × 10<sup>-9</sup> g/ml, respectively; Fig. 2, A and B, and Table II) compared with those of the T cell lines maintained with 10 μg/ml of either peptide (SC<sub>50</sub> of 4.9 × 10<sup>-7</sup> and 9.9 × 10<sup>-7</sup> g/ml, respectively; Fig. 2, C and D, and Table II). These relative differences in functional avidity for gp100<sub>209</sub>(M) among the lines were observed consistently in three independent experiments, and were in concordance with previously work demonstrating that the functional avidity of a T cell population inversely correlates with the concentration of Ag used to generate the T cells (18).

We next determined the cross-reactivity of each line by measuring its functional avidity for gp100<sub>209</sub>, and determining the ratio of the SC<sub>50</sub> values of the two peptides. The T cell line maintained with 0.1 μg/ml gp100<sub>209</sub> recognized this peptide ~40-fold better than did the line maintained with 0.1 μg/ml gp100<sub>209</sub>(M), despite their similar avidities for gp100<sub>209</sub>(M) (Fig. 2, compare A and B, and see Table II). Similarly, the T cell line maintained with 10 μg/ml gp100<sub>209</sub> recognized this peptide ~6-fold better than did the line maintained with 10 μg/ml gp100<sub>209</sub>(M) (Fig. 2, compare C and D, and see Table II). Similar results were observed by comparison of several other T cell lines developed from gp100<sub>209</sub>-immunized mice (data not shown). Two important conclusions can be reached from these data. First, they demonstrate that there are conformational differences between gp100<sub>209</sub> and gp100<sub>209</sub>(M) that are detectable by T cells, despite the fact that the residue altered between these two peptides is in a binding pocket and not directly accessible at the cell surface. Second, in vitro culture with

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Table I. Summary of AAD mouse CD8<sup>+</sup> T cell responses to immunization with MDP-derived peptides

<table>
<thead>
<tr>
<th>Immunizing DC Pulsed with</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (ng/ml)</th>
<th>Frequency of Primary Ex Vivo CD8&lt;sup&gt;+&lt;/sup&gt; T Cell Responses Detected by In Vitro ICS Using Stimulators Pulsed with</th>
<th>Proportion of CD8&lt;sup&gt;+&lt;/sup&gt; T Cell Response That Recognizes Parent Peptide* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr&lt;sub&gt;369&lt;/sub&gt; parent</td>
<td>65</td>
<td>2/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Tyr&lt;sub&gt;369&lt;/sub&gt;(Y) variant</td>
<td>74</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>gp100&lt;sub&gt;209&lt;/sub&gt; parent</td>
<td>70</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>gp100&lt;sub&gt;209&lt;/sub&gt;(M) variant</td>
<td>9</td>
<td>6/7</td>
<td>6/7</td>
</tr>
</tbody>
</table>

* Average of the size of the primary ex vivo CD8<sup>+</sup> T cell populations detected by intracellular cytokine staining (ICS) using stimulator cells pulsed with 100 μg/ml of the parent peptide, divided by the average size of the CD8<sup>+</sup> T cell populations detected by ICS using stimulator cells pulsed with 100 μg/ml of the variant peptide. Data obtained from three independent experiments.

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FIGURE 1. CD40L-activated DC pulsed with variant peptides can expand CD8<sup>+</sup> T cell populations that cross-react with the parent peptide. AAD-transgenic mice were primed i.v. with 10<sup>5</sup> activated DC that had been pulsed with 1 μg/ml of the indicated peptide. Spleens were removed 7 days after immunization. Splenocytes were enriched for CD8<sup>+</sup>CD40L-activated DC, and corresponding lines were established from mice immunized using DC pulsed with either 0.1 or 10<sup>3</sup> g/ml of either peptide. Data obtained from three independent experiments. Independent one experiment of three is presented.
Functional avidity and cross-reactive recognition of Tyr 369 could be generated by manipulating the concentration of Tyr 369 (Y) used to establish T cell lines, as measured by either IFN-γ accumulation or cytotoxic activity (Fig. 3, A and B, and Table II). Importantly, we also found that as functional avidity for Tyr 369 (Y) increased, there was a comparable increase in both the fraction of cells that recognized Tyr 369 at the highest peptide dose, as well as the cross-reactivity measured as the ratio of SC₅₀ values for Tyr 369 and Tyr 369 (Y) (Fig. 3C and Table II). Indeed, the T cell line maintained with the lowest concentration of Tyr 369 (Y) did not substantially discriminate between this peptide and Tyr 369 (Fig. 3, B and C).

Table II. Cross-reactivity increases with functional avidity of Tyr 369-specific T cells, but decreases for gp100 209-specific T cells

<table>
<thead>
<tr>
<th>Peptide Used for T Cell Line Maintenance</th>
<th>Peptide for T Cell Maintenance (µg/ml)</th>
<th>SC₅₀ Variant Peptide (g/ml)ᵃ</th>
<th>SC₅₀ Parent Peptide (g/ml)ᵇ</th>
<th>Cross-Reactivityᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp100 209 (M)</td>
<td>10</td>
<td>9.9 × 10⁻⁷</td>
<td>1.9 × 10⁻⁵</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.9 × 10⁻⁹</td>
<td>3.7 × 10⁻⁶</td>
<td>7.8 × 10⁻⁴</td>
</tr>
<tr>
<td>gp100 209</td>
<td>10</td>
<td>4.8 × 10⁻⁷</td>
<td>2.9 × 10⁻⁶</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3.7 × 10⁻⁹</td>
<td>8.6 × 10⁻⁸</td>
<td>0.04</td>
</tr>
<tr>
<td>Tyr 369 (Y)</td>
<td>100</td>
<td>3.3 × 10⁻⁶</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.4 × 10⁻⁶</td>
<td>1 × 10⁻⁵</td>
<td>7.3 × 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>9.1 × 10⁻⁷</td>
<td>6.3 × 10⁻⁶</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3.2 × 10⁻⁷</td>
<td>1.45 × 10⁻⁶</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1.3 × 10⁻⁷</td>
<td>2.15 × 10⁻⁷</td>
<td>0.59</td>
</tr>
</tbody>
</table>

ᵃ SC₅₀ determined by calculating the concentration of peptide producing half-maximal stimulation of each T cell line. Data determined from the assays presented in Figs. 2 and 3.

ᵇ Cross-reactivity determined by the formula: 1/SC₅₀ parent/1/SC₅₀ variant. Cross-reactivity increases as values approach 1.

The results obtained with the Tyr 369 (Y)-stimulated T cells were in keeping with our expectations, based on studies with Abs, that receptors with a higher affinity for Ag would also show greater cross-reactivity on closely related structures (23, 24). In contrast, the T cell lines maintained on 0.1 µg/ml of either gp100 209 or gp100 209 (M) showed less cross-reactivity than T cell lines maintained on 10 µg/ml of either peptide (Table II). To gain further insight into the reasons for this, we analyzed the interactions of TCR expressed on the four gp100 209-reactive T cell lines with tetramers composed of HLA-A*0201 folded around either gp100 209 or gp100 209 (M). Because of the multivalent nature of these interactions, we refer to this parameter as structural avidity. Unfortunately, <2% of the T cell lines maintained in the presence of 10 µg/ml of either gp100 209 or gp100 209 (M) bound to either tetramer (data not shown). Thus, it was not possible to evaluate whether the higher cross-reactivity of these lines was reflected in the structural avidity of their TCR.

This analysis did reveal unexpected complexity in the tetramer staining of T cell lines maintained with 0.1 µg/ml of either peptide. More than 75% of both lines expressed TCRs that bound to tetramers at the highest concentrations tested (Fig. 4, A and B). Interestingly, however, the T cell line maintained with 0.1 µg/ml gp100 209 bound to tetramers made with gp100 209 (M) only 5-fold.
are representative of multiple experiments. Most surprisingly, the structural avidities of the T cell line maintained with 0.1 μg/ml gp100_{209} were always much lower than those of the T cell line maintained with 0.1 μg/ml gp100_{209}(M), despite the fact that its functional avidities for both peptides were equivalent or higher (cf Fig. 4, A and B, with Fig. 2, A and B). Taken together, these results indicate that the functional avidities of these T cell lines do not correlate with their structural avidities, and that significant differences in effector activity can occur upon interaction with Ags for which the TCR has similar nominal structural avidity.

**Functional avidity for parent peptide, not variant, correlates with in vitro recognition of tumor and in vivo therapy of tumor by adoptive transfer**

Our ultimate goal was to develop CD8<sup>+</sup> T cells that could be used for adoptive immunotherapy of tumors. We therefore screened the T cell lines described above for in vitro and in vivo reactivity with B16-F1 or B16-F10 melanoma cells that had been transfected to express AAD. Although ~50% of cells in the line maintained with 0.01 μg/ml Tyr_{369}(Y) accumulated IFN-γ when stimulated with B16-F1-AAD, only 2% of the line maintained with 0.1 μg/ml Tyr_{369}(Y) did so, and no stimulation by B16-F1-AAD was detected in the lines maintained with 1 or 10 μg/ml of this peptide (Fig. 5A). Interestingly, none of these lines recognized B16-F10-AAD (data not shown), suggesting that it expresses a lower amount of this epitope than B16-F1-AAD. Among the T cell lines initiated with gp100_{209}(M), the line maintained with 0.1 μg/ml gp100_{209} was stimulated to the greatest extent by B16-F10-AAD, while the lines cultured with either 10 μg/ml gp100_{209} or 0.1 μg/ml gp100_{209}(M) were stimulated to a lower and similar extent, and the line cultured with 10 μg/ml gp100_{209}(M) was not stimulated at all (Fig. 5B). In the case of the gp100_{209}-reactive lines, recognition of the B16-F10-AAD tumor does not correlate with the functional avidity for the gp100_{209}(M) peptide nor with structural avidity as measured with MHC-tetramers based on either peptide. Thus, the ability of these T cell populations to produce IFN-γ upon

**FIGURE 3.** Functional avidity and cross-reactivity of T cell lines from mice immunized with Tyr_{369}(Y). A, C1R-AAD cells were labeled with <sup>51</sup>Cr and then incubated with increasing concentrations of Tyr_{369}(Y) for 2 h. CD8<sup>+</sup> T cells that had been generated by culture with graded concentrations of Tyr_{369}(Y) were added at a predetermined E:T ratio, and lytic activity was determined 4 h after T cell addition. B and C, C1R-AAD were pulsed with increasing concentrations of peptide overnight, washed, and then incubated at a 1:1 ratio with CD8<sup>+</sup> T cells for 5 h before staining for CD8 and accumulated IFN-γ, as described in Materials and Methods. Data are representative of multiple experiments.

**FIGURE 4.** Structural avidity of T cell lines from mice immunized with gp100_{209}(M). T cell lines established and maintained with 0.1 μg/ml gp100_{209}(M) (A) or gp100_{209} (B) were stained with serial dilutions of gp100_{209}(M) (■) or gp100_{209} (□) tetramer in the presence of anti-CD8 Abs. Data represent one of two similar assays.
recognized of this tumor in vitro correlates most closely with their functional avidities for Tyr369 or gp100209.

We next examined the ability of these T cell lines to control the outgrowth of established melanoma after adoptive transfer in vivo. Mice were injected s.c. with 4 × 10^6 tumor cells, rested for 10 days, and then 10 × 10^6 T cells were injected at the tumor site in conjunction with IL-2. For T cell lines maintained with Tyr369(Y), gp100209(M), or gp100209, there was a direct correlation between functional avidity and the ability to delay outgrowth of B16-F1-AAD or B16-F10-AAD (Fig. 6, in), keeping with their ability to recognize these tumors in vitro. Surprisingly, T cell lines maintained with 1 μg/ml Tyr369(Y) or 10 μg/ml gp100209(M), which showed no recognition of tumor in vitro, slowed tumor outgrowth to a limited degree. Neither CD8− T cells specific for an irrelevant epitope, which did not recognize B16-AAAD cells in vitro (data not shown), nor administration of IL-2 alone had an effect on tumor outgrowth. Thus, the ability of the T cell lines maintained with 1 μg/ml Tyr369(Y) or 10 μg/ml gp100209(M) to control tumor in vivo was Ag specific, and demonstrated that the in vitro measurements of tumor reactivity underestimated their therapeutic potential. Nevertheless, the ability of these Tyr369- and gp100209-reactive T cell populations to control tumor outgrowth in vivo correlated most closely with their functional avidity, and not with their structural avidity, for these two Ags.

Discussion

In this study, we used single amino acid substitution peptide variants derived from tyrosinase and gp100 in conjunction with activated DC to generate populations of MDP-specific CD8+ T cells. We further developed T cell lines in vitro from these populations using techniques to enhance either functional avidity, or cross-reactivity, or both. We found an inverse correlation between concentrations of peptide used to maintain T cell lines and their functional avidities measured by stimulation of IFN-γ accumulation. As expected, T cell lines with higher functional avidities for Tyr369(Y) also showed greater cross-reactivity on the parental Tyr369 peptide. However, this correlation was inverted for T cell lines from mice primed with gp100209(M). Surprisingly, we found that T cell lines with low cross-reactivity on gp100209, as determined using functional assays, exhibited similar structural avidities, as defined with MHC tetramers, for both peptides. However, the structural avidity of T cells maintained with gp100209 was substantially lower than the structural avidity of T cells maintained with gp100209(M). These results demonstrate that functional avidity, as measured by effector function, need not be directly correlated with structural avidity. Finally, we found that the ability of these T cells to control the outgrowth of a murine melanoma correlated with their functional avidity for the original parent peptides.

Effective adoptive immunotherapies against tumors are dependent upon the generation of large numbers of highly tumor-reactive CD8+ T cells. The data presented in this work and in previous studies (5, 6) demonstrate that the HLA-A*0201-restricted responses to Tyr369 and to several gp100-derived epitopes are usually undetectable in AAD mice, as is the response to gp10025–33 restricted by H-2Db (25). In contrast, responses to TRP-2281–288 restricted by H-2Kb were observed after single in vivo immunization (19, 26, 27). Collectively, these studies indicate that the immunogenicity of different class I MHC-associated MDP epitopes can vary significantly. Although intermediate to high MHC-binding affinity of foreign peptides has generally correlated with their ability to induce immune responses (28), many poorly immunogenic MDP-derived peptides have affinities in this range (5, 6). This suggests that self-tolerance also plays an important role in determining whether different MDP-derived peptides are effective immunogens. Successful expansion of highly tumor-reactive T cells will therefore depend on level of peptide presentation and the mechanism by which self-tolerance prevents responses to MDPs.

It is apparent from this study and previous work (5, 6) that peptide variants of the poorly immunogenic Tyr369 and gp100209 can provide an effective means to activate MDP-specific responses in AAD mice, and indicates that self-tolerance does not delete or inactivate all T cells reactive with these epitopes. The mechanism of action of these variant peptides remains to be elucidated. The relative weak HLA-A*0201-binding affinity exhibited by some MDP-derived peptides provided the rationale for the initial design of variant peptides (22), and enhanced responses were attributed to more stable MHC-peptide complexes, allowing more productive TCR engagement (6, 7, 22, 29, 30). However, the immunogenicity of Tyr369(Y) cannot be explained in this manner, as it binds to HLA-A*0201 with nearly identical affinity as Tyr369, and both peptides are equally immunogenic in AAD+ mice that do not express tyrosinase (5). An alternative explanation is that the Tyr369-specific T cells that persist in tyrosinase+ mice perceive this epitope as a partial or weak agonist (31, 32) that is capable of activating effector functions such as perforin and cytokine release, but not proliferation. However, these T cells would perceive Tyr369(Y) as a full agonist, which elicits the full range of effector activities and proliferation.

The substitution of Met for Thr at the P2 anchor position of gp100209 to produce gp100209(M) was originally shown to stabilize its binding to HLA-A*0201 while preserving its ability to be recognized by gp100209-reactive T cells (22). Indeed, some of the data reported in this work and elsewhere (6, 7) are consistent with this explanation of its improved immunogenicity. However, while the binding affinity of gp100209 for HLA-A*0201 is ∼10-fold lower than that of gp100209(M) (Table I), the cross-reactivity of different T cell lines was up to 1000-fold lower. These results demonstrate that these two peptides are antigenically distinguishable by at least some T cells, and that for many of those cells gp100209 is perceived as a weak agonist. Interestingly, while some studies have demonstrated that stronger agonist epitopes, as measured by functional assays, exhibited relatively higher affinities for TCR (33, 34), others have shown that this correlation does not
always hold (35–37). In the present work, the observation that a T cell line responded functionally to gp100\textsubscript{209} (M) up to 1000-fold better than gp100\textsubscript{209}, yet showed very similar structural avidities for these two peptides, is consistent with these latter observations. Furthermore, the T cell line maintained on gp100\textsubscript{209} responded functionally to gp100\textsubscript{209p} and gp100\textsubscript{209q} equivalently and 1000-fold better, respectively, than the line maintained on gp100\textsubscript{209q}(M). However, the structural avidities of the former line were up to 1000-fold lower than those of the latter. These data demonstrate a significant disparity between functional and structural avidities. This suggests that differences in the efficiency with which signals are transduced by these receptors in response to each of these epitopes play an important role in shaping T cell functional avidity, and that this may also be shaped by in vitro culture conditions.

It remains to be determined how functional avidity can be regulated independently of structural avidity, and particularly how a T cell line with low structural avidity can show high functional avidity. The expression of CD8 was similar between the two cell lines (data not shown), and CD8 involvement in tetramer binding was prevented by Abs to CD8 (38, 39). Thus, differences in structural avidities between these cell lines cannot be accounted for by the differential involvement of CD8. In addition, when CD8 involvement was minimized in the functional assay, there was no change in the relative avidities for gp100\textsubscript{209} and gp100\textsubscript{209q} (M) (data not shown). Together, these data suggest that the differences in structural and functional avidities are independent of CD8 engagement and/or p56\textsuperscript{6κ} recruitment (40). It is interesting that both structural avidity and T cell functional activity have been shown to be augmented by the organization of TCR into membrane lipid rafts (41–44). Thus, it is possible that the differences in these parameters for the lines maintained in gp100\textsubscript{209} and gp100\textsubscript{209q} (M) reflect differences in both the true affinity of their TCRs and the extent to which these TCR are distributed into lipid rafts.

Our data demonstrate that T cells with a high functional avidity may express TCR with a low structural avidity. Such dissociation of structural and functional avidity has also been noted in two other recent studies, suggesting that this phenomenon may be more prevalent than previously appreciated (45, 46). The implication of these observations is that the selection of T cell populations based solely on high structural avidity may exclude T cells that could have high functional avidity and be therapeutically useful. Thus, while tetramers are an important tool for monitoring the expansion of T cells in an immune response, functional studies are required to assess therapeutic potential of the activated populations.

The principal goal of this study was to use the AAD-transgenic mouse to examine strategies for the generation of CD8\textsuperscript{+} T cells specific for peptide Ags that are relevant to human melanoma. We have demonstrated a direct correlation between impact on tumor outgrowth and the T cell’s functional avidity for the exact peptide expressed on the tumor. Although these results are similar to those observed by others using either viral peptides or strongly immunogenic tumor-associated peptides (18, 19), we have extended this paradigm to include situations in which the tumor-associated peptides are weakly immunogenic at best. The challenge in this situation was to enrich for T cells that are capable of recognizing the tumor-associated Ag. We found that in one case, T cells with augmented cross-reactive recognition of the tumor-associated Ag could be selected simply by using very low concentrations of a closely related peptide variant. In the second case, however, selection of T cells with this specificity was augmented by sequential use of a relatively immunogenic peptide variant and the tumor-associated peptide, both in low concentration. It will be interesting to examine the utility of these alternative strategies to derive similar tyrosinase- or gp100-reactive CD8\textsuperscript{+} T cells for use in melanoma patients.

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