Characterization of Long-Term Effector-Memory T-Cell Responses in Patients with Resected High-Risk Melanoma Receiving a Melanoma Peptide Vaccine

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Abstract: The authors determined whether long-term memory T cells could be detected in patients who received a multipeptide vaccine for high-risk resected melanoma. Five HLA-A*0201 patients received a vaccine that included the gp100209-217 (210M) peptide with Montanide ISA 51. Peripheral blood mononuclear cells were obtained before therapy, after 6 months of vaccinations, and from 18 months to 36 months later. The presence of gp100 antigen-specific cytolytic T cells was measured by ELISPOT, tetramer and chromium release assays. Tetramer-positive CD8+ cells were phenotyped by flow cytometry for markers including CD44, CD45RA, and CCR7. T-cell avidity and its evolution over time were examined in selected patients. Epitope spreading was analyzed by assessment of gp100280-288 (288V) T cells. All patients exhibited a significant increase in tetramer-positive gp100-specific CD8+ T cells that decayed at different rates over 18 to 36 months after vaccinations. Cells from all patients exhibited an effector-memory phenotype and were generally CD45 RA low/CCR7 negative and CD44 positive. Tetramer-positive cells declined over time in four of the five patients, but the proportion of tetramer-positive CD8+ cells that secreted gamma-interferon rose, suggesting enrichment for effector cells. Epitope spreading for the gp100280-288 (288V) epitope was detected. One patient maintained a population of 2.5% circulating gp100 tetramer-positive cells over 36 months. Avidity analysis showed no changes over time after induction of antigen-specific T cells. Vaccination with a heteroclitic melanoma antigen peptide with Montanide ISA 51 generated populations of circulating functional effector-memory T cells that were specific for gp100 and long-lived in the circulation for periods of 18 to 36 months after vaccination.

Key Words: antigen-specific, tetramers, ELISPOT, gp100, immunotherapy

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over time would improve our ability to generate long-term effector and memory responses and might induce protection against progressive tumor growth.

To characterize the long-term effector and memory T-cell responses in melanoma patients after vaccination, we analyzed the number, phenotype, cytokine expression, and function of resulting effector cells 6 to 42 months after initiating vaccination with multiple peptides on two different clinical trials for patients with resected high-risk melanoma. We chose patients alive and free of disease at least a year after finishing vaccination who agreed to have another pheresis and had a tetramer response to peptide gp100\textsubscript{209-217} (210M) after vaccination of at least 0.1% of the circulating CD8\textsuperscript{+} T cells.

**MATERIALS AND METHODS**

**Patient Selection**

Patients who had been on one of two Institutional Review Board-approved protocols in which they received the gp100\textsubscript{209-217} (210M) peptide with adjuvant Montanide ISA 51 with GM-CSF or IL-12 were chosen. They had to be alive at least 12 months after their last vaccination and free of disease, have a 6-month post-vaccination gp100 tetramer positive immune response of 0.1% or more, and be able to have a leukapheresis.

**Peptides**

Peptides used for in vitro studies were synthesized at the USC/Norris Microchemical Core Facility. They were the gp100\textsubscript{209-217} (210M) (IMDQVPFSV), gp100\textsubscript{209-217} WT (ITDQVPFSV), tyrosinase\textsubscript{368-376} (370D) (YMDGTM5QV), gp100\textsubscript{280-288} (YLEPGPVTV), HPV E7\textsubscript{86-93} (TLGIVZPI), and the HIV RT\textsubscript{476-484} (ILKEPVHGV) peptides.

**MHC-Peptide Tetramer Assay**

Tetramers containing the gp100\textsubscript{209-217} (210M) and tyrosinase\textsubscript{368-376} (370D) peptides were produced following the approach of Altman et al.\textsuperscript{15} The tetramer assay technique has been previously published by our group.\textsuperscript{16,17}

**Validation and Titration of Tetramers**

Each tetramer was validated by staining against a CTL line or clone specific for HLA-A2.1 in association with the peptide of interest. The limit of detection was 0.01% of CD8\textsuperscript{+} T cells, as previously described.\textsuperscript{16,17}

**Preparation of PBMC Specimens**

Pheresis samples were processed to purify peripheral blood mononuclear cells (PBMCs) and frozen at -168°C, as previously described.\textsuperscript{16}

**ELISPOT Assay**

PBMCs were thawed and cultured overnight, then tested in an ELISPOT assay with a 24-hour effector-stimulator incubation set-up, as previously published by our group.\textsuperscript{18} After processing, ELISPOT plates were read on a KS ELISPOT Reader (Carl Zeiss, Thornwood, NY). Values were normalized to spots per 100,000 cells.

**Growth of DC and T Cells**

Dendritic cells (DCs) were grown from thawed, frozen PBMCs. Immediately after thawing, cells were washed with cold Hanks buffered saline solution twice and cells were incubated at 37°C in 25 mL AIM V (with a target of 10\textsuperscript{8} cells in each flask) in T150 cell culture flasks for 1.5 to 3 hours. Non-adherent cells were washed away with 10 mL AIM V. GM-CSF and IL-4, mixed with AIM V to a concentration of each at 1,000 µM/mL, were then added to each T150 flask. The flasks were then incubated in a 37°C incubator. On day 6, GM-CSF and IL-4 were again added to each flask for a final concentration of 1,000 units/mL of each. On day 7, cells were removed from each flask and resuspended in 4 mL AIM V. Peptide was then mixed with 1 mL AIM V for each flask and added to a final concentration of 10 µg/mL. The flasks were then incubated at 37°C for 4 hours and then 1 mcg/mL LPS was added. On day 9 DCs were harvested and CTL growth was initiated. A total of 10 µL phosphate-buffered saline (PBS) was added, and each flask was then incubated for 30 minutes at 37°C. The flasks were hit with the heel of the palm three times each to loosen the DCs, and PBS was removed with a pipette and kept. DCs were then centrifuged to pellet them, then they were suspended in AIM V with 2% human AB serum at a concentration of 10\textsuperscript{7} cells/mL. PBMCs were then thawed in a 37°C water bath after removing them directly from a liquid nitrogen tank. The cells were washed twice with cold Hanks buffered saline solution, then centrifuged to pellet them and they were suspended at 2 × 10\textsuperscript{6} cells/mL in AIM V with 2% human AB serum. One milliliter of DCs and 1 mL of PBMCs were then mixed in each well of a 24-well plate. IL-7 was added to a concentration of 20 ng/well. On day 12, 20 units IL-2 was added to each well. On day 14 all cells were replated and concentrated or diluted as needed into more or fewer wells. Day 16 cells were again concentrated or diluted. On day 19 CTLs were harvested for assay.

**Chromium Release Assay Using Restimulated Cells**

For the chromium release assay, 10\textsuperscript{6} T2 cells (ATCC, Rockville, MD) pulsed with 10 µg/mL of applicable gp100 peptides and the irrelevant HPV-E7\textsubscript{86-93} peptide were then incubated with 100 µL Cr-51 for 2 hours at 37°C. In 96-well plates, 5,000 T2s were used as targets with 50,000 nonpulsed K562 cells and a serial dilution starting at a ratio of 100:1 CTL effectors to targets, diluted 3:1 for six total dilutions with 200 µL in each well. Maximal lysis was determined with the addition of 1N HCl and spontaneous lysis was determined by T2 cells in medium. The plates were then incubated together at 37°C for 4 hours. After incubation the plates were centrifuged...
at 500 rpm for 5 minutes without the brake, and the released radioactivity was quantitated in a gamma counter (Packard, Meriden, CT) using 100 µL culture medium harvested from the wells in triplicate.

**Chromium Release Assay With Fresh Cells**

A chromium release assay was also performed using thawed/unstimulated and selected CD8+ cells. Frozen PBMCs were thawed quickly in a 37°C water bath and diluted with 10 volumes of cold Hanks buffered saline solution. Cells were then centrifuged at 1,000 rpm for 5 minutes and treated with 4 units/mL DNase (Roche, Indianapolis, IN) for 30 minutes. Cells were centrifuged at 1,000 rpm for 5 minutes, then washed with 10 mL cold MAC buffer (PBS with 0.5% BSA and 2 mmol/L EDTA). Positive selection of CD8 cells was done using magnetic beads from Miltenyi Biotec (Miltenyi Biotec, Germany), following their protocol. Briefly, cells were suspended in 80 µL MAC buffer per 10^7 cells, and 20 µL of magnetic beads were added per 10^7 cells. The mixture was incubated at 4°C for 15 minutes, then washed with 10 mL cold MAC buffer and centrifuged at 1,000 rpm for 10 minutes. The cells were then suspended in 1 mL cold MAC buffer and loaded onto the columns. Columns were washed with 3 mL cold MAC buffer three times, and then cells were eluted from the column with 7 mL cold MAC buffer. Serially diluted CD8+ cells were mixed with 5,000 T2 target cells that had been pulsed for 18 hours with peptide (10 µg/mL) and labeled with Cr-51 for 2 hours, in a total volume of 200 µL. To eliminate natural killer cell reactivity, 50,000 unlabeled K562 cells were added to each well. A chromium release assay was carried out as above.

**Peptide Titration**

Peptide titration Cr-51 assays were carried out to determine cytotoxic avidity data over time. This was accomplished by setting up the Cr-51 assay without varying the effector to target ratio and keeping it at 33:1. Instead, targets were pulsed the night before with six different concentrations of peptide, starting at 10 µg/mL and going down at 10:1 for five more dilutions at 1 µg/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, and 100 pg/mL. Each condition was set up in triplicate and chromium release assays were carried out as above.

**RESULTS**

**Patients and Treatment**

Five patients treated on two different vaccine clinical trials for resected high-risk melanoma were chosen for this study. All five received the gp100209-217 (210M) and tyrosinase368-376 (370D) peptides at 1 mg per dose emulsified with Montanide ISA 51. Two patients received IL-12, two received GM-CSF, and one received no additions. The ages ranged from 44 to 57; three patients had resected stage III disease and two had stage IIB disease. All patients had tumors that stained with HMB-45, the antibody that recognized gp100. All patients received eight injections of vaccine given deeply subcutaneously at weeks 0, 2, 4, 6, 10, 14, and 18, then week 26. The vaccine in all cases consisted of the two peptides emulsified with ISA 51 by vortexing the mixture at high speed for 12 minutes. GM-CSF was given at 250 mcg/dose subcutaneously at the vaccine site for 5 days after each vaccine in the two patients with stage IIB disease. IL-12 was given at 30 ng/kg intradermally with each vaccine at the site to two of the patients with stage III disease. Leukaphereses were performed at time 0 and within 2 weeks of the final or eighth injection. All patients had an immune response to the vaccine as indicated by a gp100209-217 (210M) tetramer-specific population of at least 0.02% CD8+ cells detected in circulating PBMCs, with baselines in all vaccinated patients of less than 0.01%. The patients were again leukapheresed to collect PBMCs for immune assays at times ranging from 18 to 36 months after finishing the 6-month vaccine regimen (Table 1). They are all currently well and free of disease at a median of more than 4.5 years after finishing vaccinations. No patient developed vitiligo.

**Development of Long-Term Memory Cells**

Before vaccinations, after 6 months of vaccinations, and 18 to 36 months after vaccinations, PBMCs were analyzed by eight color flow cytometry for the presence of CD8+, gp100 tetramer-positive cells, and those cells were enumerated as a percentage of total CD8+ cells. The phenotype of those CD8+

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**TABLE 1. Patient Details**

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Stage of Disease</th>
<th>Age</th>
<th>Time Since 1st Diagnosis (to 2/1/04)</th>
<th>Time Since Vaccine Ended to Long-Term Specimen</th>
<th>Prior Treatment</th>
<th>Treatment Given</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IIB</td>
<td>57</td>
<td>87 months</td>
<td>24 months</td>
<td>Surgery, vaccine</td>
<td>Tyr/gp100/ISA 51 + GM-CSF</td>
</tr>
<tr>
<td>2</td>
<td>IIB</td>
<td>47</td>
<td>56 months</td>
<td>24 months</td>
<td>Surgery</td>
<td>Tyr/gp100/ISA 51 + GM-CSF</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>44</td>
<td>79 months</td>
<td>20 months</td>
<td>Surgery, vaccine</td>
<td>Tyr/gp100/ISA 51 + IL-12</td>
</tr>
<tr>
<td>4</td>
<td>III</td>
<td>55</td>
<td>191 months</td>
<td>18 months</td>
<td>Surgery, radiation</td>
<td>Tyr/gp100/ISA 51</td>
</tr>
<tr>
<td>5</td>
<td>III</td>
<td>48</td>
<td>279 months</td>
<td>36 months</td>
<td>Surgery</td>
<td>Tyr/gp100/ISA 51 + IL-12</td>
</tr>
</tbody>
</table>
tetramer-positive cells was also analyzed. The gp100 tetramer
response at month 6 after vaccination ranged from 0.016% to
6.1% of CD8\(^+\) cells (Table 2). No gp100-specific cells were
detected before vaccination, defined as less than 1 cell in
10,000, or less than 0.01%. The actual flow profiles for pa-
tients 2 and 5 are shown in Figure 1, indicating good separation
and clustering of positive cells gated for CD3, CD8, and tet-
ramer positivity. No detectable tetramer-positive cells were
seen before vaccination (left panels), but significant staining
was observed after vaccination (middle panels; 6.1% and
2.5%) and in the long term (right panels; 1.4% and 2.6%).

The ratios of gamma interferon (IFN-\(\gamma\))-secreting func-
tional gp100 specific cells detected by an ELISPOT assay to
tetramer-positive cells enumerated by flow cytometry were
calculated for four patients. As shown in the two right columns
of Table 2, there appeared to be an enrichment over time for
functional IFN-\(\gamma\)-secreting cells, with an increase in the
ELISPOT/tetramer ratio over time for the four patients tested.
This suggests that the nonfunctional but tetramer-positive cells
may apoptose or be eliminated over time, and the functional
cells may remain in the circulation.

Phenotyping of the cells showed that for all 6-month
(post-vaccine) and long-term post-vaccine (long-term)
samples, the cells were uniformly CD44\(^+\) activated T cells that
were CCR7 negative, suggesting that they were not naive cells,
nor were they central-memory cells, since both express CCR7,
but were effector-memory T cells (Table 3). Expression of
CD45RA varied, but cells were predominantly CD45RA nega-
tive, indicating that they were activated effector cells, with
some proportion of effector-memory cells. CD27 was variably
positive, as was CD28, and CD25 was undetectable on all cells
(not shown), indicating that they were not resting central
memory T cells.

**Post-Vaccine and Long-Term T Cells Are
Cytolytic-Effector Cells**

PBMCs were pulsed with gp100\(^{209-217}\) (210M) peptide
in the presence of low-dose (20 IU/mL) IL-2 for 10 days, then
harvested and tested in a chromium release assay at effec-
tor/target ratios ranging from 1:1 to 100:1. Peptide-pulsed T2
cells were used as targets. No significant gp100-specific lysis
was detected before vaccination, but after 6 months of vacci-
nation, and in all five patients tested 18 to 36 months later, a
significant level of lysis was detected (Fig. 2). In all five pa-
tients, no significant changes were seen between post-vaccine
and long-term post-vaccine samples, with equal levels of lysis
on a cell-for-cell basis, suggesting that lytic long-term
memory-effector cells could be amplified rapidly in a recall
response despite the decay shown by the tetramer analysis in
Table 2, and that those cells circulated up to 36 months after
finishing vaccine. The series of recall lysis assays was repeated
with gp100\(^{209-219}\) (210M) for all five patients showing an
identical pattern of and a similar level of lysis. For three of the five
patients, the wild-type gp100\(^{209-217}\) peptide was used in a simi-
lar restimulation chromium release assay with the same growth
conditions, and the results (Fig. 3) indicate that lytic cells that
recognized the wild-type gp100 peptide naturally processed
and expressed by tumor cells were generated 6 months after
starting vaccination with the heteroclitic epitope peptide and
were also present long term, albeit at a lower level of lysis than
observed with the gp100\(^{209-217}\) (210M) peptide. These recall
assays were also repeated in the three patients shown with
similar results.

**Evidence of Epitope Spreading by
Long-Term T Cells**

In the assays shown in Figure 2 described above, a gp100
epitope not contained in the vaccine but previously shown to
be immunogenic in HLA A*0201 melanoma patients,
gp100\(^{280-288}\), was pulsed onto autologous PBMCs as antigen-
presenting cells to determine whether the generation of a cy-
tolytic response to one HLA-A *0201 epitope, gp100\(^{209-217}\),
could cause epitope spreading to a different epitope from the
same antigen. The results (Fig. 4) indicated that significant up-
regulation of gp100\(^{280-288}\) specific lysis was observed after
vaccination with the gp100\(^{209-217}\) epitope in two patients
and was maintained over time long term, with lysis of peptide-
pulsed targets of 40% at effector/target ratios of 30:1 seen only
after vaccination. These results for gp100\(^{280-288}\) were observed
in repeated assays, and one set of representative profiles is
shown. This phenomenon was observed in restimulated cells

<table>
<thead>
<tr>
<th>Pt. No.</th>
<th>Pre-Vaccine (%)</th>
<th>Post-Vaccine (%)</th>
<th>Long-Term (%)</th>
<th>Post Ratio: ELISPOT/Tetramer</th>
<th>Long-Term Ratio: ELISPOT/Tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.9</td>
<td>0.058</td>
<td>0.098</td>
<td>0.712</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>6.15</td>
<td>1.4</td>
<td>0.41</td>
<td>1.14</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0.16</td>
<td>0.074</td>
<td>0.49</td>
<td>n/d</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0.16</td>
<td>0.043</td>
<td>1.021</td>
<td>2.807</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>2.5</td>
<td>2.6</td>
<td>1.60</td>
<td>1.62</td>
</tr>
</tbody>
</table>
ELISPOT Assays of Long-Term T Cells

In four patients, there were sufficient cells available for fresh unmanipulated CD8 T cells to be isolated before vaccination, after vaccination, and long term after vaccination using a Miltenyi affinity column. The resulting effectors were subjected to ELISPOT analysis using the gp100209-217 wild-type and heteroclitic gp200209-217 (210M) peptides. Reactivity to a human immunodeficiency virus HLA A*0201 restricted peptide constituted a negative control, and PHA or pokeweed mitogen were used as positive control. The number of spots per 100,000 input CD8+ cells is shown on the ordinate. The data are shown in Figure 5, in which the light bars indicate the negative control values using an irrelevant HPV peptide to pulse the APC, and the dark bars are specific for the gp100209-217 epitopes. No or minimal detectable reactivity was observed to either gp100 epitope before vaccination (the left portion of each set of profiles). All patients had a significant immune response after vaccination (the middle portion of each set of profiles), ranging from 75 to 1,235 spots per 10^5 effectors for wild-type gp100209-217 (left set of profiles) and from 88 to 4,244 spots per 10^5 effectors for heteroclitic peptide gp100209-217 (210M) (right set of profiles). The reactivity to the wild-type gp100209-217 peptide was less in all cases than the heteroclitic 210M variant, although it varied from 25% less to more than a factor of four decrease. No consistent response to tyrosinase368-376 (370D) or gp100280-288 peptides was observed by fresh ELISPOT, suggesting that the precursor frequency to those epitopes was low. Over the long term, the right part of each set of profiles in Figure 5 shows that a decay in ELISPOT gp100

TABLE 3. Phenotype of gp100 Specific T Cells

<table>
<thead>
<tr>
<th>Pr. no.</th>
<th>CD45RA</th>
<th>CCR7</th>
<th>CD27</th>
<th>CD44</th>
<th>CD28</th>
<th>CD38</th>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Post</td>
<td>neg</td>
<td>100% neg</td>
<td>50% pos</td>
<td>100% pos</td>
<td>32.6% pos</td>
</tr>
<tr>
<td></td>
<td>Long-term</td>
<td>neg</td>
<td>100% neg</td>
<td>50% pos</td>
<td>100% pos</td>
<td>54% pos</td>
</tr>
<tr>
<td>2</td>
<td>Post</td>
<td>50% pos</td>
<td>100% neg</td>
<td>50% pos</td>
<td>100% pos</td>
<td>8% pos</td>
</tr>
<tr>
<td></td>
<td>Long-term</td>
<td>pos</td>
<td>100% neg</td>
<td>50% pos</td>
<td>79% inter pos</td>
<td>21% hi pos</td>
</tr>
<tr>
<td>3</td>
<td>Post</td>
<td>neg</td>
<td>100% neg</td>
<td>50% pos</td>
<td>100% pos</td>
<td>89% pos</td>
</tr>
<tr>
<td></td>
<td>Long-term</td>
<td>neg</td>
<td>100% neg</td>
<td>50% pos</td>
<td>100% pos</td>
<td>92% pos</td>
</tr>
<tr>
<td>4</td>
<td>Post</td>
<td>neg</td>
<td>100% neg</td>
<td>50% pos</td>
<td>24.6% inter pos</td>
<td>75.4% hi pos</td>
</tr>
<tr>
<td></td>
<td>Long-term</td>
<td>neg</td>
<td>100% neg</td>
<td>50% pos</td>
<td>100% pos</td>
<td>16.4% intermed</td>
</tr>
<tr>
<td>5</td>
<td>Post</td>
<td>neg</td>
<td>100% neg</td>
<td>n.d.</td>
<td>100% inter pos</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Long-term</td>
<td>neg</td>
<td>100% neg</td>
<td>n.d.</td>
<td>100% pos</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
reactivity was seen, ranging from a 20% to a 50% diminution. This result is in contrast to the greater decay of all enumerated gp100-specific cells observed by tetramer analysis in Table 2, in which the decay varied from two- to five-fold. These data suggest that there was a relative sparing of the IFN-γ-secreting functional effector cells over time, which appeared to turn over more slowly than the remaining tetramer-positive CD8+ cells. These results were observed in repeated assays and demonstrated that functional IFN-γ-secreting effector CD8+ T cells were detected in fresh PBMCs and were maintained at high levels over 18 to 36 months after the final vaccination for all four patients tested.

Avidity Analysis of Long-Term T Cells

T-cell avidity can be defined as the sensitivity of T cells for recognition of MHC–peptide complex on the target surface, with high-avidity cells recognizing very low concentrations of peptide. The avidity of long-term T cells was analyzed by generation of antigen-specific T cells using a single peptide stimulation in vitro followed by a chromium release assay with varying concentrations of peptide pulsed onto T2 cells as the chromated targets. The concentration of peptide at which half-maximal lysis occurs reflects the avidity with which antigen-specific CTLs recognize the peptide target. In the two patients in whom there were adequate cells for the analysis (Fig. 6), the data suggested that there was no change over time in the avidity of the resulting gp100-specific CTLs after vaccination, consistent with the existence of a long-term memory-effector population without a true central memory component. The half-maximal value for both patients’ effector cells was 1 to 10 ng/mL, an intermediate value for avidity. The absolute level of lysis per effector cell was also similar for both patients at all concentrations of peptide, again indicating that surviving effector cells maintained their lytic capacity over the long term.

FIGURE 2. Chromium release assay after 10 days to gp100209-217 (210M) before and after vaccination and long term. The percentage lysis using 10-day effector cells as calculated in text is shown on the ordinate with effector/target (E:T) ratios on the abscissa, with pre-vaccine samples in the left panels, post-vaccine samples in the middle panels, and long-term samples in the right panels. Negative controls included the HPV-16 E786-93 peptide. Samples from five separate patients are shown, with similar results in a repeated experiment.

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We performed a flow assay that has been shown to correlate with avidity shown in a recent publication by Rubio et al\textsuperscript{18} for the two patients who had an analysis by a peptide titration technique in Figure 6, and found that the proportion of and intensity of staining of high-avidity, tetramer-positive/CD107a-positive cells was constant over time; we found that similar levels of avidity detected in the titration assay over time are supported by the consistency in the CD107a/tetramer assay at the different time points. For example, the ratio of CD107a/tetramer-positive cells was 1/6.4 = 0.156 after vaccination in patient 10450, compared with 0.3/1.7 = 0.176 in patient 10545 3 years later. Intensity of CD107a staining was virtually identical. The generation of low- to intermediate-avidity long-term memory T cells that are gp100 specific is consistent with the detection of a low proportion of high-avidity T-cell clones from post-vaccination blood samples.\textsuperscript{18}

**Effector Cells From Fresh PBMCs Are Lytically Active**

One hallmark of a potent functional immune response after vaccination would be the detection of lytically active, effector T cells in the fresh circulating peripheral blood that are long-lived. To test whether fresh PBMCs contained detectable CD8 effector cells that were peptide-specific after vaccination, CD8 cells were affinity purified from pre-vaccine, post-vaccine, and long-term samples from two patients who had sufficient cells available (patients 2 and 5). Fresh cells derived from thawed unstimulated PBMC samples were used directly in a 4-hour chromium release assay, with peptide-pulsed T2 cells as targets. The data from these two patients (Fig. 7) indicated that for both patients, significant specific lysis was absent before vaccination but was detected both after vaccination and long term. In patient 2, the lysis diminished from 50% to 20% at an effector/target ratio of 100:1. The lysis by effectors from patient 5 remained undiminished over 3 years after the post-vaccine sample, with lysis of nearly 50% at an effector/target ratio of 100:1 for both samples.

**DISCUSSION**

In this study, we analyzed the phenotype and function of tumor antigen-specific T cells in the peripheral blood of patients up to 3 years after finishing peptide vaccination. The antigen expressed by the majority of melanomas was recognized by cytolytic T-cell clones and by tumor infiltrating lymphocytes (TILs) from patients with regression of tumor after adoptive transfer of TILs.\textsuperscript{19–21} Although we had only a small sampling of five patients, an in-depth analysis of T-cell phenotype and effector function in these patients was performed.
FIGURE 5. ELISPOT assay using fresh unstimulated CD8+ T cells. ELISPOT assays are shown with the gp100 209-217 WT epitope in the left group of profiles and the gp100 209-217 (210M) epitope in the right group of profiles. The number of positive IFN-γ-secreting spots per 100,000 input CD8+ T cells with K562 cells as APC is shown on the ordinate, and the abscissa shows the type of cells used as effectors, before vaccination, 6 months after vaccination, and long term (18–36 months later). Light bars show the negative control values in which an irrelevant HPV 16 E7 86-93 peptide was used to pulse APC, and the dark bars show the values for gp100 specific spots. Similar results were seen with repeat experiments.

FIGURE 4. Chromium release assay after 10 days to gp100280-288 before and after vaccination and long term. The percentage lysis using 10-day effector cells as calculated in text is shown on the ordinate with effector/target (E:T) ratios on the abscissa, with pre-vaccine samples in the left panels, post-vaccine samples in the middle panels, and long-term samples in the right panels. Negative controls included the HPV-16 E7 86-93 peptide. Samples from two patients are shown, with nearly identical results in a repeated experiment.
Little is known about the kinetics of development and decay over time of antigen-specific immune responses to tumor self-antigens induced by vaccination. This is one of the first reports of functional long-term effector-memory T cells detected in the circulation of vaccinated cancer patients years after vaccination, and one of the few reports of lytic activity by fresh unstimulated long-term effector cells. Our findings suggest that vaccination with an immunogenic, heteroclitic peptide derived from a melanoma self-antigen and administered with adjuvant Montanide ISA 51 stimulates high levels of circulating functional effector-memory T cells. The T cells are phenotypically activated memory-effector and effector T cells that decayed at varying rates over several years after finishing a 6-month course of vaccinations. The relative decay of the functional IFN-γ-secreting CD8+ cells was slower than the turnover of the total tetramer-positive population. In one patient (patient 5), there was essentially no diminution in enumerated tetramer-positive effector-memory T cells over a 3-year period after finishing a vaccine regimen, which was confirmed by the stable lytic activity of fresh unstimulated CD8 effector cells over time. Epitope spreading, which has been detected for MART-126-35 in at least two prior vaccine trials including the gp100209-217 210M peptide, was demonstrated for epitope gp100280-288.

The antigen-specific T cells were highly functional, indicated by secretion of IFN-γ and other Tc1 cytokines and cytolysis demonstrated toward T2 cells pulsed with peptides as well as HLA-A2.1 tumor cell lines expressing the gp100 antigen. An avidity analysis using cytolysis against targets pulsed with varying titrations of relevant peptide showed that the avidity for the bulk population of T cells was low to intermediate but was maintained over time by long-term effector and effector-memory T cells. Virtually no antigen-specific T cells were detected by any assay in patients before vaccination. No central memory T cells were induced in any patient up to 3 years after vaccination. The generation of high-avidity T cells

![Figure 6](https://example.com/figure6.png)

**FIGURE 6.** Chromium release assay after 10 days with a gp100209-217 (210M) peptide titration after vaccination and long term. The percentage lysis using 10-day effector cells as calculated in text is shown on the ordinate with the peptide titrated downward in 10-fold increments at the concentrations shown on the abscissa. An effector/target ratio of 30:1 was maintained for all points on the abscissa, with post-vaccine samples in the left panels and long-term samples in the right panels for two patients. Negative controls included the HIV26-35 peptide. Data were derived from two patients (patients 2 and 4).
was found to correlate with functional cytolytic activity after vaccination\textsuperscript{18,27} (Lee P, et al, unpublished data), but for the bulk of CTLs tested in this work, only low- to intermediate-avidity cells were detected.

The absence of CCR7 expression was a consistent finding for the five patients in this study, indicating that the resulting effector cells would not be able to migrate to lymphoid tissue\textsuperscript{28,29} and are not central memory T cells. They would not be able to migrate in response to MIP-3\textbeta or secondary lymphoid chemokine (SLC), both of which are expressed in nodal tissue.\textsuperscript{29} The persistence of CCR7 effector cells up to 3 years after finishing vaccinations suggests that the schema in which effector cells differentiate to effector-memory cells and thence to central memory cells after clearance of pathogen antigen,\textsuperscript{30} as postulated by Ahmed et al,\textsuperscript{31,32} has not been followed in melanoma patients vaccinated with a self-antigen peptide. Although none of the five patients had evidence of disease at the time of vaccination and all have remained disease-free at a median of more than 4 years after vaccination, they still might harbor antigen in the form of circulating tumor cells, which can be detected in a surprisingly large proportion of patients with high-risk resected melanoma.\textsuperscript{33,34} The presence of chronic antigenic exposure may alter the development of memory and effector cells, leaving the balance in favor of sustained effector-memory T cells.

The generation of effector, effector-memory, and central memory T-cell responses has been studied in normals, in melanoma patients, and in several other cancer vaccine trials. The best-studied antigen is MART-1/Melan-A, to which normals have been shown to mount immune responses that are likely cross-reactive and show a naive CD45 RA\textsuperscript{+} phenotype.\textsuperscript{35,36} Patients who have not been vaccinated have detectable circulating MART-1-positive cells, but by ex vivo tetramer staining they have a naive phenotype. Upon in vitro restimulation, those cells can transform to CD45 RA effector cells. T cells with an effector or effector-memory phenotype have been detected using tetramer staining for gp100, tyrosinase, and MART-1 in melanoma patients.\textsuperscript{37} In patients who have received a MART-1\textsubscript{26-35} (27L) or gp100\textsubscript{209-217} (210M) peptide vaccine, effector and effector-memory T cells develop soon after vaccination, but central memory cells have not been demonstrated to develop, and the follow-up analyses have been over a brief duration.\textsuperscript{37-39} In contrast, the current work is the first detailed assessment of long-term T-cell responses in melanoma patients years after vaccination. We confirm that the majority of T cells generated after a peptide vaccine have a memory-effector phenotype and function, and although central memory cells were not detected, the decay of the effector and effector-memory cells occurs at a variable and often slow rate.

The therapeutic implications of these data are several. First, long-term memory responses can be generated in high-risk melanoma patients against a melanoma self-antigen, supporting the continued development of a peptide vaccine approach in which occasional antitumor responses have been observed that correlate with detection of antitumor immunity.\textsuperscript{40-43} In contrast to endogenous T cells that are tumor antigen-specific but anergic,\textsuperscript{44} long-term T cells in our vaccinated patients are highly lytic and secrete IFN-\gamma, the hallmarks of effector function in CD8\textsuperscript{+} cells. In two patients with greater than a 1% tetramer-positive gp100-specific response, fresh cir-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Chromium release assay with fresh unstimulated CD8 T cells to gp100\textsubscript{209-217} (210M). The percentage lysis using freshly thawed and washed CD8\textsuperscript{+} T effector cells derived from pre-vaccine, post-vaccine, and long-term PBMCs as indicated in text is shown on the ordinate with effector/target (E:T) ratios on the abscissa, with the profile from patient 2 in the left panel and that from patient 5 in the right panel. Negative controls included the HPV-16 E\textsubscript{6} peptide pulsed onto T2 cells as targets. Samples from two patients are shown, with nearly identical results in a repeated experiment.}
\end{figure}
culating CD8 T cells were lysically active. The variable decay over time of the effector/effector-memory population suggests that patient-related factors may affect the survival of T-cell responses after vaccination, such as relative levels of apoptosis factors such as Bcl-2 and Bcl-xL or endogenous cytokine levels. In future work we intend to sort the tetramer-positive cells detected in our patients after vaccination and analyze levels of signaling molecules that affect T-cell survival such as Bcl-X and Bcl-2. The presence of effector and effector-memory T cells after peptide vaccination with significant decay of the response without central memory development justifies the continued boosting of patients at intervals to maintain a high level of antitumor immunity. The absence of CCR7 also suggests that the effector cells may not migrate to lymphoid tissue and may not be useful for eradication of tumor in that location, a common occurrence in melanoma.

The patients in this study represent immune responders observed in two different clinical trials. A high variation in level of response has been observed in our studies and those of other groups. Significant heterogeneity in clonal T-cell receptor rearrangement has also been seen in patients on peptide vaccine trials. The factors that influence whether a patient will be a high or a low immune responder may be tumor-dependent but also may be host-specific, and the studies on effector, effector-memory, and central memory cells in this study and others may shed light on the optimal way to generate high sustained levels of tumor-specific immune cells for protection against progression of tumor. The data shown in Figures 1, 2, and 3 argue for the ability to boost peptide vaccine responses over the long term, indicating that long-term memory-effector responses can be rapidly amplified after exposure to antigen. A recent study in which antigen-specific T-cell clones were adoptively transferred and were associated with tumor regression yet remained detectable in the circulation and functional for up to 9 months suggests that further study of the factors that influence the survival and longevity of antitumor effector cells is important.

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