**Immunologic and Clinical Outcomes of a Randomized Phase II Trial of Two Multipeptide Vaccines for Melanoma in the Adjuvant Setting**

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**Abstract**

**Purpose:** Human melanoma cells express shared antigens recognized by CD8⁺ T lymphocytes, the most common of which are melanocytic differentiation proteins and cancer-testis antigens. However, peptide vaccines for melanoma usually target only one or two MHC class I–associated peptide antigens. Because melanomas commonly evade immune recognition by selective antigen loss, optimization of melanoma vaccines may require development of more complex multipeptide vaccines.

**Experimental Design:** In a prospective randomized clinical trial, we have evaluated the safety and immunogenicity of a vaccine containing a mixture of 12 peptides from melanocytic differentiation proteins and cancer-testis antigens, designed for human leukocyte antigen types that represent 80% of the melanoma patient population. This was compared with a four-peptide vaccine with only melanocytic differentiation peptides. Immune responses were assessed in peripheral blood and in vaccine-draining lymph nodes.

**Results:** These data show that (a) the 12-peptide mixture is immunogenic in all treated patients; (b) immunogenicity of individual peptides is maintained despite competition with additional peptides for binding to MHC molecules; (c) a broader and more robust immune response is induced by vaccination with the more complex 12-peptide mixture; and (d) clinical outcome in this peptide vaccine trial correlates with immune responses measured in the peripheral blood lymphocytes.

**Conclusions:** These data support continued investigation of complex multipeptide vaccines for melanoma.

**Tumor vaccines can induce protective T-cell responses in murine models and may induce regressions of established tumor (1, 2); however, vaccine-based immunotherapy against human cancers needs significant additional optimization. Tumor deposits contain a heterogeneous population of cells with respect to antigen expression (3, 4). Vaccines using only one or two melanoma-derived antigens may be inadequate in generating a complete immune response against the tumor. Thus, a vaccine incorporating larger numbers of antigenic epitopes would be more broadly applicable.**

The present study focused on evaluation of vaccine strategies using 12 defined, shared melanoma peptides from melanocytic differentiation proteins (tyrosinase and gp100) and cancer-testis antigens (MAGE-A1, MAGE-A3, MAGE-A10, and NY-ESO-1; ref. 5), including five peptides that had not, to our knowledge, previously been evaluated for immunogenicity in humans. We hypothesized that vaccines incorporating larger numbers of peptide antigens (a) would induce T-cell responses in a larger percentage of patients, (b) would lead to T-cell responses directed against multiple antigens, and (c) would not be compromised significantly by competition of multiple peptides for the same MHC molecules; we also hypothesized (d) that all 12 peptides incorporated in this vaccine would be immunogenic. These hypotheses were tested by randomizing patients to either a four-peptide vaccine, previously shown to...
be immunogenic (6, 7), or to a 12-peptide vaccine, and evaluating T-cell responses to the peptides in peripheral blood and in lymph nodes draining the vaccine sites [sentinel immunized nodules (SIN)]. Preliminary clinical outcome data have also been assessed as they relate to immune response data.

Materials and Methods

Patients. Patients with resected American Joint Committee on Cancer stage IIB to IV melanoma, who were human leukocyte antigen (HLA)-A1+, -A2+, or A3+, and whose melanoma cells expressed gp100 and/or tyrosinase by immunohistochemistry, were studied following informed consent and with institutional review board (HIC 8878) and Food and Drug Administration approval (BB-IND 9947). The patients were enrolled at the University of Virginia from October 2001 to October 2003 and have been followed continuously.

Inclusion criteria included age >18 years, Eastern Cooperative Oncology Group performance status 0 to 1, adequate liver and renal function, and completion of surgical therapy within the preceding 10 months. Exclusion criteria included ocular melanoma; extensive brain metastases; other invasive cancers within 5 years; pregnancy; cytotoxic chemotherapy, IFN, radiation, or steroids within 4 weeks; allergies to vaccine components; class III or IV heart disease; HIV or hepatitis C virus positivity; and systemic autoimmune disease with visceral involvement.

Patients who were candidates for high-dose IFN, but chose not to take it, initially were not eligible because of Food and Drug Administration instructions. After a 2002 meeting of the Oncologic Drugs Advisory Committee (8), the protocol was revised such that patients who refused IFN were eligible for this protocol if they showed tumor regression, an unexpected treatment-related adverse event, or unusual patient symptoms or physical findings. After 24 months, patients were offered a second composite vaccine, in a different extremity.

Toxicities were recorded by each patient using daily toxicity diaries, reviewed weekly by interview with a study clinician. Toxicities were graded by National Cancer Institute Common Toxicity Criteria v2.0. Dose-limiting toxicities were defined as treatment-related grade 2 allergic reactions, unexpected grade 3 non-hematologic toxicities, and unexpected grade 4 hematologic toxicities. Ulceration at the vaccine site was considered a grade 3 toxicity, but was not considered dose-limiting unless the ulcer was >2 cm diameter, or required antibiotic therapy, surgical debridement, or narcotic management. Those are the criteria for grade 3 injection site reactions defined in v3.0 of the National Cancer Institute Common Toxicity Criteria.

Patients with dose-limiting grade 3 toxicities had dose reductions of granulocyte macrophage colony-stimulating factor (reduced by 75 µg/dose) and had vaccine halted until toxicities were resolved to grade 1. Protocol treatment was discontinued for (a) patients with persistent or recurrent grade 3 toxicity not responding to dose reductions of granulocyte macrophage colony-stimulating factor, (b) disease progression requiring other therapy, or (c) noncompliance with study requirements. Six patients had granulocyte macrophage colony-stimulating factor dose reductions, generally followed by decreased toxicity. Two patients had delays in one vaccine >2 days (<1 week); two patients had protocol treatment discontinued after the fourth and fifth vaccinations, respectively. One other patient missed the sixth vaccination. Forty-three patients (84%) completed six vaccines without delay or dose reduction. All are considered evaluable.

Harvest of the SIN. On day 22, the lymph node draining the replicate immunization site (the SIN) was localized and harvested. Sixteen patients had at least annual communication with our staff. Two different vaccine preparations were used, with each preparation assessed independently. Each vaccine for group A contained 100 µg each of four peptides listed in Table 1 (DAEKSDICT-DEY, YMDGTM5QV, YL6CQPTA, and ALAVYATK). Plus 190 µg tetanus toxoid peptide AQYIKANSKFIGITEL (9). Each vaccine for group B contained 100 µg each of the 12 peptides listed in Table 1 (10–22), plus 190 µg tetanus toxoid peptide. The vaccines were administered in an emulsion of granulocyte macrophage colony-stimulating factor (110 µg) and Montanide ISA-51 adjuvant (1 mL), made using the two-syringe method. Emulsion stability was verified. Each vaccine was administered s.c. and intradermally at days 1, 8, 15, 29, 36, and 43. At days 1, 8, and 15, patients also received a second replicate vaccine, in a different extremity.

Peptides were synthesized and purified either by the University of Virginia Biomolecular Core Laboratory, or under GMP conditions by Multiple Peptide Systems (now NeoMPS). The 12–melanoma peptide mixture, four–melanoma peptide mixture, and tetanus peptide preparation were each made as separate sterile aqueous solutions. Quality testing included sterility, identity, purity, general safety, pyrogenicity, and stability.

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Toxicity assessment and stopping rules. Toxicities were recorded by each patient using daily toxicity diaries, reviewed weekly by interview with a study clinician. Toxicities were graded by National Cancer Institute Common Toxicity Criteria v2.0. Dose-limiting toxicities were defined as treatment-related grade 2 allergic reactions, unexpected grade 3 non-hematologic toxicities, and unexpected grade 4 hematologic toxicities. Ulceration at the vaccine site was considered a grade 3 toxicity, but was not considered dose-limiting unless the ulcer was >2 cm diameter, or required antibiotic therapy, surgical debridement, or narcotic management. Those are the criteria for grade 3 injection site reactions defined in v3.0 of the National Cancer Institute Common Toxicity Criteria.

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Harvest of the SIN. On day 22, the lymph node draining the replicate immunization site (the SIN) was localized and harvested. A central slice of the SIN was preserved in formalin, and the remainder was dissected mechanically into a single-cell suspension of lymphocytes and cryopreserved.

Clinical follow-up schedule. Patients received vaccines through the first 6 weeks of study, with the last vaccine on day 43. Scheduled follow-up visits were included in the study at 3, 6, 12, and 24 months. At each of these visits, history, physical examination, immunologic studies, and laboratory studies were done. Chest radiographs were done at each of these follow-up visits, and other imaging studies were done based on patient symptoms or physical findings. After 24 months, patients were followed by their referring oncologists or at the University of Virginia, with at least annual communication with our staff.

Cell lines and peptides used in vitro. C1R-A1, C1R-A2, and C1R-A3 are human EBV-transformed B-cell lines, provided by P. Cresswell.
(Department of Immunology, Yale University, New Haven CT), that lack class I MHC expression, except by transfection with genes for human HLA-A1, HLA-A2, and HLA-A3, respectively (24). T2 cells were also used as antigen-presenting cells in several assays (25), and a melanoma cell line VMM39 was used in one assay to minimize background reactivity associated with C1R cells. In addition to peptides in the vaccines, irrelevant peptides from HIV gag protein, SLYNTVATL (26), and from malarial circumsporozoite antigen (malaria CSP, 134-142), YLKIKKNSL (27), were used in laboratory analyses as negative controls.

ELISpot assays. Peripheral blood lymphocytes (PBL) were isolated by Ficoll gradient centrifugation and were cryopreserved. Samples from prevaccination and throughout the study were evaluated simultaneously, in parallel with SIN lymphocytes, using IFN-γ ELISpot assay. Blood samples were collected 1 week after vaccine administration, except the sample collected after vaccine 6 (collected at week 12). Lymphocytes were assayed 14 days after one in vitro sensitization (IVS) with the 12-peptide mixture (40 μg/mL each). ELISpot assay methods have been described (28). Each sample was tested in quadruplicate at each of two dilutions of lymphocytes. Two changes in assay methods occurred during the period of analysis: (a) Lymphocytes harvested from culture after 14 days were either tested immediately (fresh) or were cryopreserved and tested on a different day. (b) The latter half of assays were done with methanol prewetting of polyvinylidene difluoride filters in the assay plates. Analysis of immunologic data failed to detect a difference in assay results attributable to either the use of cryopreserved lymphocytes or prewetting of the membranes.

Assessment of immunologic response was based on a fold-increase measure as well as on the number of spots counted per 100,000 cells plated, using the following definitions: N_vax is the number of T cells responding to peptide in the vaccine; N_neg is the number of T cells responding to negative control (maximum of two negative controls: antigen-presenting cell alone or pulsed with irrelevant peptide); and R_vax is the ratio of N_vax/N_neg.

For evaluations of PBL, a patient is considered to have a T-cell response to vaccination only if all the following criteria are met: 1. N_vax exceeds N_neg by >30 cells per 100,000 (corresponds to >0.15% of CD8+ cells); 2. R_vax > 2; 3. (N_vax - 1 SD) > (N_neg + 1 SD); and 4. R_vax after vaccination > 2R_vax prevaccine. (In the rare event of an unevaluable prevaccine PBL sample, a negative result after vaccine was accepted as a surrogate for prevaccine response).

Observed fold increases <1 were converted to 1 to indicate no response. When the prevaccine PBL value was zero, this value was converted to 1 to avoid dividing by zero. Criterion 4 above was not applied to SIN. Differences in proportions (response rates) between the two groups were assessed by the χ² test. Differences in response magnitude were assessed using the two-sample t test. To evaluate relationships between clinical outcome and immune response, Kaplan-Meier survival functions were estimated, and the log-rank test was used to test statistical significance.

Results

Patient population. The patient populations were similar for group A (n = 26) and group B (n = 25), except that patients in group A were slightly younger (median age 49 versus 56 years) and had fewer males (54% versus 68%). Patients were stratified by stage: stage III/B/C, III, and IV patients represented 4, 19, and 3 patients, respectively, in group A and 4, 17, and 4 patients, respectively, in group B. HLA-A1 expression was more common in group A (10 versus 6 patients); HLA-A2 expression was similar (15 versus 13 patients) and HLA-A3 expression was more common in group B (7 versus 14 patients). Twenty-one percent expressed two of those alleles.

Summary of clinical toxicities. There were no grade 4 toxicities and no treatment-related deaths. The overall rate of grade 3 toxicities was 35% for group A and 40% for group B (P = 0.69). The most frequent toxicity was a local vaccine reaction (100%), which was twice as likely to be grade 3 in group B (32% versus 15%, P = 0.16). Other toxicities were similar between groups (data not shown). Overall, the highest-grade toxicity was three in 37%, two in 57%, and one in 6%. Systemic toxicities were usually limited to the first 24 h after vaccine administration (data not shown). The most common constitutional toxicities that occasionally were grade 3 were fatigue (78%, grade 3 in 14%), dyspnea (20%, grade 3 in 10%), headache (65%, grade 3 in 4%), and myalgia (45%, grade 3 in 2%). Other common systemic toxicities, limited to grades 1 to 2, were rigors/chills (57%), anorexia (35%),

Table 1. Melanoma-associated peptides in vaccines for groups A and B

<table>
<thead>
<tr>
<th>HLA</th>
<th>Four-peptide mixture, group A</th>
<th>Twelve-peptide mixture, group B</th>
<th>Epitope source protein (residues)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>DAEKSDICTDEY</td>
<td></td>
<td>Tyrosinase (240-251)*</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td>SSDYVIPGTY</td>
<td></td>
<td>Tyrosinase (146-156)</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>EADPGHSHY</td>
<td></td>
<td>MAGE-A1 (161-169)</td>
<td>(12)</td>
</tr>
<tr>
<td></td>
<td>EVDPIGHLY</td>
<td></td>
<td>MAGE-A3 (168-176)</td>
<td>(13, 14)</td>
</tr>
<tr>
<td></td>
<td>YLEPGPVTYA</td>
<td></td>
<td>Tyrosinase (369-377)</td>
<td>(15)</td>
</tr>
<tr>
<td>A2</td>
<td>YMDGTMQOY</td>
<td></td>
<td>gp100 (209-217)</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td>IMQVPFSV</td>
<td></td>
<td>gp100 (280-288)</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>YLEPGPVTYA</td>
<td></td>
<td>MAGE-A10 (254-262)</td>
<td>(18)</td>
</tr>
<tr>
<td>A3</td>
<td>ALLAVGATK</td>
<td></td>
<td>gp100 (17-25)</td>
<td>(19)</td>
</tr>
<tr>
<td></td>
<td>LIYRRRLMK</td>
<td></td>
<td>gp100 (614-622)</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>SLFRAVTK</td>
<td></td>
<td>MAGE-A1 (96-104)</td>
<td>(20)</td>
</tr>
<tr>
<td></td>
<td>ASGPGGGAPR</td>
<td></td>
<td>NY-ESO-1 (53-62)</td>
<td>(21, 22)</td>
</tr>
</tbody>
</table>

NOTE: Index peptides are in bold. These peptides, plus a fourth peptide (YLPVPVTYA), are included in the four-peptide mixture.

*Substitution of S for C at residue 244.
† Posttranslational change of N to D at residue 371.
‡ 209-2M, substitution of M for T at position 210.
§ We have found that the NY-ESO-1 peptide ASGPGGGAPR, reported to be immunogenic in association with HLA-A31, is naturally processed and presented also by HLA-A*0301 (A3) on human melanoma cells. This analysis was done by mass spectrometric analysis of peptides eluted from immunoaffinity purified HLA-A3 molecules from the HLA-A3+ melanoma VMM1B (22).
nausea (33%), sweating (29%), fever (25%), and flushing (24%). Laboratory abnormalities included hyperglycemia in 49% (in nonfasting samples, grade 3 in 2%) and decreases in hemoglobin (27%, all grade 1). Ocular/visual toxicities were all grade 1 and included dry eye in 1 patient (group B) and transient decreased night vision in two patients (both in group A). One patient with decreased night vision had noticed it first before the vaccine trial and was evaluated by an ophthalmologist who did not consider it significant. The other patient later acknowledged that decreased night vision was associated with fatigue and that it resolved with rest. None had persistent or progressive ocular/visual symptoms. New vitiligo was observed in 6% of patients.

Four patients discontinued vaccinations early due to toxicity, three on arm A and one on arm B. Of the 12 patients with grade 3 injection site reactions (ulceration), only two had dose-limiting toxicities: one of these two also had grade 3 dyspnea. Two additional patients discontinued vaccination early for other adverse events: grade 3 fatigue and headache in one patient, and a constellation of grade 1 and 2 constitutional toxicities in another.

![Fig. 2. CD8 T-cell responses to the four-melanoma peptide and 12-melanoma peptide vaccines. T-cell responses to vaccination were assessed by ELispot assay after one IVS with the 12-peptide mixture. Lymphocytes were tested before vaccination (Pre), and weekly through week 6, then at week 12 and/or beyond. Subsequent blood samples were many weeks after the last (sixth) vaccine. Data from the SIN are also given. Reactivity was evaluated against peptides pulsed on C1R cells. The peptides are abbreviated by their first three to four amino acids. GAG, irrelevant peptide from the HIV gag protein (SLYNTVATL). GAG and the C1R cells alone serve as negative controls. A, C, and E are from group A; B, D, and F are from group B. A and B, HLA-A1. C and D, HLA-A2. E and F, HLA-A3.](https://www.aacrjournals.org/clinicancrea.../6389/fig2)
Eleven patients were vaccinated with a 12-peptide mixture (Group B), and 10 of them had evaluable baseline blood samples. Ten of the 12 patients were evaluable in the SIN. One patient was not evaluable in both SIN and PBL, and another had no evaluable SIN sample. Ten of the 12 patients had no evaluable baseline blood sample and a nonevaluable PBL. Forty-nine patients (96%) were evaluable in the SIN. One patient was not evaluable in both SIN and PBL, and another had no evaluable SIN sample. Fifty patients (98%) were evaluable overall, and in the clinical trial was originally designed and powered for analysis of IVS ELIspot analyses (data not shown); however, the clinical trial was completed all six vaccines.

Ten immunogenic peptides identified. We evaluated T-cell responses by IVS ELIspot assay, as described in Materials and Methods. T-cell responses were also detected by direct analysis of cryopreserved lymphocytes using either ELIspot or tetramer analyses (data not shown); however, the clinical trial was originally designed and powered for analysis of IVS ELIspot assay data. Fifty patients (98%) were evaluable overall, and in PBL. Forty-nine patients (96%) were evaluable in the SIN. One had no evaluable baseline blood sample and a nonevaluable SIN; another had no evaluable SIN sample. Ten of the 12 peptides were immunogenic (Fig. 2; Table 2). Tyrosinase(146-156) and MAGE-A1(161-167) had no T-cell responses, but were evaluable in only six HLA-A1+ patients (upper limit on a one-sided 95% confidence interval for 0 of 6 is 39%). Most patients with positive responses had responses that were at least 10 times the background (Fig. 3). There was a strong correlation between fold increase and measured magnitude [$R^2 = 0.723$ (SIN) and 0.512 (PBL); both $P < 0.001$; Fig. 3].

Twelve-peptide vaccine induces greater immune response rates. In patients vaccinated with the 12-peptide mixture, T-cell responses were observed to multiple melanoma peptides restricted by each class I MHC molecule. Representative immune response data are shown in Fig. 2. The median numbers of peptides to which each patient responded was 1.0 (mean 1.0) in group A and 3.0 (mean 3.2) in group B (based on reactivity in PBL or SIN, $P < 0.001$, Wilcoxon rank-sum test, data not shown). Similar increases in peptide reactivity were observed in PBL alone and in SIN alone ($P < 0.001$). Overall, for patients vaccinated with the 12-peptide mixture, 100% had an immune response to at least one peptide, detectable in the blood in 83% or the SIN in 92% (Table 2). Immune response rates were lower in patients vaccinated with the four-peptide mixture, for the response overall (77%, $P = 0.012$, $\chi^2$ test of association).

Twelve-peptide vaccine induces greater cumulative T-cell response magnitude. The cumulative T-cell response is defined, for this study, as the sum of responses to all of the 12 peptides for which the patient expressed an appropriate HLA class I allele. Specifically, this was measured as a sum of maximum fold increases for each peptide. The cumulative T-cell response was greater for patients in group B than in group A (Fig. 4A). For groups A and B, respectively, the median cumulative T-cell response values were 4.6 and 12.6 in PBL (ratio 2.7, $P = 0.08$, Wilcoxon) and 6.7 and 28.6 in SIN (ratio 4.3, $P = 0.03$).

### Table 2. Mel39 peptide immunogenicity

<table>
<thead>
<tr>
<th>HLA Peptide Source protein</th>
<th>% Patients with immune responses in PBL SIN Overall Group A Group B</th>
<th>% Evaluable patients with positive T-cell response overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA A</td>
<td>DAEKSIDICTDEY Tyrosinase</td>
<td>69% 86% 81%</td>
</tr>
<tr>
<td></td>
<td>SSDVYVIPGY Tyrosinase</td>
<td>0% 0% 0%</td>
</tr>
<tr>
<td></td>
<td>EADPTGHSY MAGE-A1</td>
<td>0% 0% 0%</td>
</tr>
<tr>
<td></td>
<td>EVDPICHLY MAGE-A3</td>
<td>67% 33% 83%</td>
</tr>
<tr>
<td>HLA A2</td>
<td>YMDOQTMSQV Tyrosinase</td>
<td>44% 44% 56%</td>
</tr>
<tr>
<td></td>
<td>IMDQVPSFSV Gp100</td>
<td>75% 100% 100%</td>
</tr>
<tr>
<td></td>
<td>YLEPGPVTA Gp100</td>
<td>0% 8% 7%</td>
</tr>
<tr>
<td></td>
<td>GLYDGMELH MAG-A10</td>
<td>75% 67% 83%</td>
</tr>
<tr>
<td>HLA A3</td>
<td>ALLAVGATK Gp100</td>
<td>75% 84% 90%</td>
</tr>
<tr>
<td></td>
<td>LIYRRMLMK Gp100</td>
<td>62% 31% 62%</td>
</tr>
<tr>
<td></td>
<td>SLFRAVITK MAGE-A1</td>
<td>62% 75% 77%</td>
</tr>
<tr>
<td></td>
<td>ASGGPGGQR NYESO1</td>
<td>46% 38% 46%</td>
</tr>
</tbody>
</table>

Group A (four peptides) overall: 26 patients, 69% 72% 77% 0* 83%
Group B (12 peptides) overall: 24 patients, 83% 92% 100% 0* 100%

*In group A patients, T-cell responses were not detected to these eight peptides, except in two cases, one with reactivity in a single peripheral blood mononuclear cell sample to MAGE-A3168-176 and once in the SIN to gp100209-2M.

NOTE: Immunogenicity is reported in both groups A and B for the four peptides DAEKSIDICTDEY, YMDOQTMSQV, YLEPGPVTA, and ALLAVGATK, which are included in both peptide vaccines. However, for the other eight peptides, only patients in group B were vaccinated; immunogenicity is reported only for those patients. The numerator differs for the different peptides based on the number of evaluable patients with each HLA type. In the two columns to the right, patients with the appropriate HLA type in both groups are considered for overall reactivity to each peptide, even if they were not vaccinated with it.

**Fig. 3.** ELIspot assay characteristics. For each patient, the maximum T-cell response is represented by two different measures, with the ratio (Peptide-Control, $R_{\text{MC}}$) on the Y axis and the difference (Peptide-Control, $N_{\text{MC}}$ - $N_{\text{BG}}$) on the X axis (PBL SIN). For correlation between these two measures of T-cell response magnitude, $R^2$ values are 0.723 and 0.512 for SIN and PBL, respectively.

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**Cancer Therapy: Clinical**

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plots.Box, 25th to 75th percentile; horizontal line, median; +, mean; *, values at response. For SIN, this represents the complete dataset. Data are shown as box this was calculated as the sum of the fold increases in the sample with the highest response. For SIN, this represents the complete data set. Data are shown as box plots. Box, 25th to 75th percentile; horizontal line, median; +, mean; *, values at or beyond 5 and 95th percentile. Y axis, the natural log of the ratio. P values for differences between group A (12 Pep.) and group B (4 Pep.) for all 12 peptides were 0.08 for PBL and 0.03 for SIN (A), and for the four index peptides were 0.82 for PBL and 0.94 for SIN (B). P values were calculated by Wilcoxon rank test.

Fig. 4. Magnitude of T-cell responses, based on IVS ELISPOT assay, expressed as fold increase (ratio) over background. These are shown for the maximum cumulative T-cell response for PBL and for SIN, presented both as a cumulative value for all 12 peptides (A) and as a cumulative value for the four index peptides (B). For PBL, this was calculated as the sum of the fold increases in the sample with the highest response. For SIN, this represents the complete data set. Data are shown as box plots. Box, 25th to 75th percentile; horizontal line, median; +, mean; *, values at or beyond 5 and 95th percentile. Y axis, the natural log of the ratio. P values for differences between group A (12 Pep.) and group B (4 Pep.) for all 12 peptides were 0.08 for PBL and 0.03 for SIN (A), and for the four index peptides were 0.82 for PBL and 0.94 for SIN (B). P values were calculated by Wilcoxon rank test.

Wilcoxon). Thus, there is a significant increase in cumulative reactivity for 12 peptides versus 4 peptides, in the SIN by a factor of 4.3, and a 2.7 × increase in the PBL that approaches significance.

Peptide competition. To test for possible inhibition of immunogenicity due to competition of multiple peptides for the same MHC molecule, this trial was designed to detect a significant decrease in responses to index peptides [tyrosinase240-251 (DAEKSDICTDEY) for HLA-A1; tyrosinase369-377 (YMDGTMSQV) for HLA-A2; and gp10017-25 (ALLAVGATK) for HLA-A3] for group B compared with group A. The cumulative data (HLA-A1, HLA-A2, and HLA-A3) reveal no difference in the frequency of T-cell responses to the index peptides in PBL, SIN, or overall [n = 26 in A and 24 in B; P values (χ² test of association) in PBL = 0.85, SIN = 0.75, overall = 0.57; Table 2 and data not shown]. Furthermore, for index peptides, median (mean) cumulative responses did not differ between groups A and B (Fig. 4B). These values were 4.6 (12.6) and 4.7 (11.6) in the PBL (Wilcoxon P = 0.82) and 6.7 (16.3) and 8.1 (12.7) in the SIN (Wilcoxon P = 0.94) for groups A and B, respectively.

Persistence of T-cell responses. The focus of the immune evaluation was on the SIN and in PBL through week 12. Eighty-eight percent of patients were evaluable for T-cell responses at week 12. Among these patients, 20 (45%) had T-cell responses to one or more peptides evident at week 12 (mean fold-increase 8.7). Nine of these 20 patients were evaluated again at weeks 26 to 39. Seven (78%) of them had persistent responses at that later time point (mean fold-increase 3.9, data not shown).

Preexisting T-cell responses. ELISPOT assays were done simultaneously on all PBL samples for each patient. In four patients (8%), responses to at least one peptide were observed in the prevaccine samples. They included two HLA-A1+ patients with reactivity to tyrosinase240-251, one HLA-A2+/HLA-A3+ patient with reactivity to tyrosinase369-377 and to MAGE-A196-104, and one HLA-A3+ patient with reactivity to gp10017-25; all were in group B. Only one of these had a marked increase in reactivity with vaccination, meeting criteria for a positive response to vaccination in the PBL (data not shown). Responses in the SIN were considered nonevaluable in patients with preexisting responses in PBL. Considering all 50 evaluable patients, and four to eight (mean 5.04) HLA-relevant peptides per patient, there were 252 assessments for preexisting responses, for which these five cases represent a 2% incidence of preexisting immune responses. In comparison, there were 171 evaluable opportunities for vaccine-induced responses [50 patients × 1-8 (mean 3.42) HLA-relevant peptides in vaccine], of which there were 82 vaccine-induced responses to peptides (48%, data not shown).

Evaluation of the effect of IVS. For ELISPOT assays, all patient PBL and SIN were sensitized once in vitro with all 12 peptides used in the vaccine, before assaying responses to each peptide individually, even for patients vaccinated only with the four peptides. This served as a prospective randomized assessment of the effect of IVS for the eight peptides not used in vaccines for group A patients. Responses were observed to one of these eight peptides for only two patients in group A, each in just one lymphocyte sample per patient. Evaluable patients in groups A and B, respectively, had T-cell responses to each of these eight peptides at the percentages shown in Table 2. Thus, the mean rates of T-cell response to these eight peptides were 2% and 56% in groups A and B, respectively. Median values were 0% and 70%, respectively.

Clinical outcomes. At the time of this analysis, median follow-up was 2 to 2.5 years, but follow-up was <2 years for some patients, and there have been too few events to make a formal assessment for differences in outcome between patients in groups A and B. However, overall Kaplan-Meier survival data have been reviewed. Overall survival estimates for all 51 patients are 98%, 89%, 69%, and 61% at 1, 2, 3, and 4 years, respectively (data not shown), with 95% confidence interval at 2 years (76-95%) and at 4 years (36-79%). Survival analyses by stage show no mortality for stage II patients, 58% (95% confidence interval, 31-78%) 4-year survival for stage III patients, and 71% (26-92%) 2 year survival for stage IV patients (Fig. 5A).

Disease-free survival (DFS) data are estimated for the subset of stage IIB to III melanoma patients (Fig. 5B). For these patients, median DFS has been 35 months (2.9 years). The 95% confidence interval for this median DFS ranges from 15 months.
to an upper limit that cannot be defined yet. This corresponds to 76% 1 year DFS (95% confidence interval, 60-86), 59% 2-year DFS (95% confidence interval, 42-72), and 47% 3-year DFS (95% confidence interval, 27-64). These compare favorably to the outcome for the high-dose IFN arm on E1694 (29) and seem favorable to outcome with either study arm on E1684 and E1690 (30, 31), and similar to the DFS data from both arms of the Canvaxin trial in patients with resected stage III melanoma (32).

**Correlation between measured T-cell response and clinical outcome.** We have evaluated DFS of patients on this trial, depending on whether there was a T-cell response (by ELIspot assay) to one or more of the index peptides. Those data (Fig. 5C) represent a landmark analysis (33), where DFS is plotted from week 7, the time of completion of vaccination. Only three patients were excluded from this analysis: One patient was excluded from all immunologic analyses because there were no usable baseline laboratory data; one other patient on each study arm was excluded because follow-up data on disease status were missing after the last vaccine. Thus, this landmark analysis included 96% of the patients evaluable for immune response in each arm. No patients were excluded for progression before week 7. This landmark analysis indicates that patients who developed a T-cell response have a higher probability of longer DFS ($P = 0.041$).

**Discussion**

The data presented in this article address the following five aims of this clinical trial of multipeptide vaccination: (a) to evaluate the safety of vaccination with a 12-peptide mixture containing five peptides not previously tested in humans; (b) to evaluate the immunogenicity of 12 melanoma peptides restricted by HLA-A1, HLA-A2, or HLA-A3, derived from cancer-testis antigens and melanocytic differentiation proteins; (c) to determine whether vaccination with 12 class I MHC-restricted melanoma peptides induces immune responses in a greater proportion of patients than vaccination with four peptides; (d) to determine whether vaccination with 12 class I MHC-restricted melanoma peptides induces greater cumulative T-cell responses than vaccination with four peptides; and (e) to evaluate whether the addition of two or three peptides binding the same class I MHC allele will inhibit immunogenicity of index peptides restricted by HLA-A1, HLA-A2, or HLA-A3. The findings are discussed below.

**Safety of vaccination with a 12-peptide mixture containing five peptides not previously tested in humans**

The vast majority of systemic symptoms were mild, and usually did not interfere with activities of daily living, including work. Interestingly, the proportion of patients with ulcerated (grade 3 by Common Toxicity Criteria v2; grade 2 by Common Toxicity Criteria v3) injection site reactions may have been higher in group B patients than in group A patients (32 versus 15%, $P = 0.16$), suggesting that these reactions may have reflected a greater *in vivo* immune response associated with the larger number of antigens in group B patients.

**Immunogenicity of 12–melanoma peptide restricted by HLA-A1, HLA-A2, or HLA-A3, derived from cancer-testis antigens and melanocytic differentiation proteins**

We have previously shown the three index peptides tyrosinase269-277, gp10017-25, and tyrosinase240-251, and the gp100280-288 peptide to be immunogenic (6, 7, 9). The gp100209-216, MAGE-A1161-169, and MAGE-A3168-176 peptides

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**Fig. 5.** Survival data. Kaplan-Meier plots are shown for (A) overall survival of the 51 patients enrolled in the Mel39 trial, as subsets that are stage IIIB/IIIC (solid line), stage III (dashed line), and stage IV (dotted line), and for (B) DFS for the 44 patients with resected stage II to III melanoma. For both plots, time zero is the date of study entry. In C, DFS was plotted, using Kaplan-Meier curves, for patients with (dotted line) or without (solid line) a T-cell response in PBL (by ELIspot assay) to one or more of the index peptides. Data in C represent a landmark analysis, where DFS is plotted from the time of completion of vaccination at week 7.
have been shown to be immunogenic by others (3, 34, 35). In the present trial, we showed immunogenicity of four additional peptides (MAGE-A10254-262, gp100614-622, MAGE-A196-104, and NY-ESO-153-62). Overall, 10 of the 12 peptides were demonstrably immunogenic. The lack of detectable reactivity to the HLA-A1 peptides tyrosinase146-156 and MAGE-A196-104 may reflect low immunogenicity that is below the sensitivity of IVS ELIspot assays, or may reflect the low number of evaluable patients (n = 6). Regardless, this 12-peptide preparation will be useful for ongoing and future trials evaluating methods to increase immunogenicity. There are several related findings worth highlighting, summarized below.

**Nonmutated peptides can be reliably immunogenic.** The modification of the gp100209-2M peptide (IMDQVPFSV) from its natural sequence ITDQVPFSV has been widely highlighted as critical to the immunogenicity of that peptide, attributable to improvements in binding affinity to the HLA-A2 molecule (34). We have also found in prior studies that the modified peptide DAEKSDICTDEY is the most immunogenic of the four-peptide preparation we have used (6, 7). That peptide is modified from the natural sequence DAEKCDICTDEY because of the problematic bioreactivity of free sulfhydryl side chains on cysteine residues (10). However, the nonmodified gp10017-25, MAGE-A196-104, and MAGE-A10254-262 peptides were also very immunogenic in the present study, with frequencies and magnitudes rivaling those of the modified peptides.

**Peptides derived from cancer-testis antigens can be good immunogens.** Prior experience with the MAGE-A161-169 and MAGE-A3168-176 peptides is that they are weakly immunogenic (36–38); these findings are corroborated in the current report. This has led to a generalized view that cancer-testis antigen–derived peptides are not good immunogens. In response to that, an anecdotal report of immunogenicity of an NY-ESO–derived peptide in one patient argued that a cancer-testis antigen can, in fact, be a good immunogen (39). Here, we provide substantial data that the MAGE-A196-104 and MAGE-A10254-262 peptides restricted by HLA-A3 and HLA-A2, respectively, are very good immunogens. We have previously published data from this trial that these two peptides induce CTL with adequate avidity to lyse antigen-expressing melanoma cells in vitro (28). Overall, data from this clinical trial reveal that there is no clear hierarchy of immunogenicity based on whether a peptide is from a melanocytic differentiation protein or from a cancer-testis antigen. Instead, there are substantial variations in the immunogenicity of individual peptides that likely are attributable to combinations of MHC-binding affinity, tolerance, patient repertoire, and other factors.

**Temporal changes in T-cell response during and after vaccination may provide clues to regulation of T-cell responses to cancer antigens.** Most vaccine trials evaluate immunogenicity prevacination and at one time point postvaccination. In such studies, the tyrosinase169-177 peptide has been reported to be nonimmunogenic or very weakly immunogenic. Interestingly, in the present study (and in prior work; refs. 6, 7), we have found the peptide to be immunogenic in most patients vaccinated. Interestingly, however, the reactivity to this peptide is often transient, peaking after three or four vaccines, then returning to baseline, or nearly to baseline after the full sequence of vaccines (Fig. 2C-D). This could explain the reports of nonimmunogenicity when monitoring is done only before and after vaccination. We are currently investigating the mechanisms that explain transient response to vaccination with certain peptides.

**Characterization of assay methods.** ELIspot assays done after IVS can be criticized compared with assays done directly ex vivo. To evaluate the validity of the finding of immunogenicity in an IVS ELIspot assay, we assessed whether the IVS could be sufficient for a positive finding by IVS ELIspot. T-cell responses were rarely detected in PBL before vaccination (2%) but were common after vaccination (~48%). Also, responses to the eight peptides administered to group B patients, but not to group A patients, were rarely observed in group A patients (mean 2%, versus 56% in group B), although lymphocyte samples from both groups were stimulated in vitro with all 12 peptides. Thus, the finding of an immune response by IVS ELIspot assays reflects, with rare exception, an immune response to antigen exposure in vitro. The significant correlation between T-cell response and clinical outcome (Fig. 5C) also further support the biological relevance of the IVS ELIspot assay.

**Vaccination with 12 class I MHC–restricted melanoma peptides induces greater cumulative T-cell responses than vaccination with four peptides**

Overall, there is a 2.7-fold increase in T-cell reactivity to the vaccine peptides, as evaluated in the PBL (P = 0.08), and a 4.3-fold increase in reactivity as evaluated in the SIN (P = 0.03). This trial was powered to detect a 2.5-fold increase in reactivity, which was approximately the result achieved. These data support the stated hypothesis.

**Peptide competition: The addition of two or three peptides binding the same class I MHC allele does not significantly inhibit immunogenicity of an index peptide restricted by HLA-A1, HLA-A2, or HLA-A3**

If multiple peptides binding the same MHC molecule are coadministered, binding of lower-affinity peptides to the MHC may be competitively inhibited by higher-affinity peptides. Because of this concern, multipptide vaccines at other centers have most commonly been administered such that each peptide is administered at a different body site (40). This approach will become increasingly unwieldy if the number of peptides exceeds three or four. In preclinical studies from our group, we have found that competition among peptides for MHC binding does not significantly inhibit T-cell induction or T-cell effector function (1, 41, 42). Thus, for this trial, we hypothesized that addition of two to three peptides binding the same class I MHC allele would not inhibit immunogenicity of an index peptide when administered at equimolar concentrations. The findings of the present study support that hypothesis.

Interestingly, a recently published study raises concern about the effect of mixing peptides, specifically the peptides YMDGTMSQV (tyrosinase169-177) and IMDQVPFSV (gp100209-2M; ref. 43). However, that report was based on two sequential preparations we have used (6, 7). That peptide is modified from the natural sequence DAEKCDICTDEY because of the problematic bioreactivity of free sulfhydryl side chains on cysteine residues (10). However, the nonmodified gp10017-25, MAGE-A196-104, and MAGE-A10254-262 peptides were also very immunogenic in the present study, with frequencies and magnitudes rivaling those of the modified peptides.
peptides. In contrast to the study mentioned above, our data revealed that immune reactivity to the lower-affinity index peptide tyrosinase369-377 was maintained despite competition from the higher-affinity peptide gp100-209-234.

Clinical outcome

The present phase II study was designed for primary immunologic end points and was not designed for definitive comparison of clinical outcomes between the two treatment groups. However, a secondary aim was to estimate whether there is preliminary evidence that either melanoma vaccine comprising 12 or 4 peptides delays tumor recurrence in patients with metastatic melanoma. The study was designed also to compare overall DFS for stage IIB to III patients on this vaccine trial to historical control data for stage IIB to III patients on Eastern Cooperative Oncology Group trials E1680, E1684, and E1694. The data are not yet robust enough for definitive assessments of survival and DFS, as median follow-up is only ~2 years. However, the preliminary data suggest that median survival for the entire patient population on this trial approaches 3 years, which is a favorable finding compared with published data for this patient population (Fig. 4B). The overall stage-specific survival data meet or exceed expectations based on recent data (refs. 32, 44; Fig. 4A).

Correlation between immune response and clinical outcome

Importantly, the clinical outcome correlates significantly with the T-cell response to index peptides, suggesting that this T-cell response measure may have prognostic significance (Fig. 5C). In summary, the data from the current study support continued investigation of multipeptide vaccines targeting melanocytic differentiation antigens and cancer-testis antigens.

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