

Clinical and Immunologic Results of a Randomized Phase II Trial of Vaccination Using Four Melanoma Peptides Either Administered in Granulocyte-Macrophage Colony-Stimulating Factor in Adjuvant or Pulsed on Dendritic Cells

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Purpose: To determine clinical and immunologic responses to a multi-peptide melanoma vaccine regimen, a randomized phase II trial was performed.

Patients and Methods: Twenty-six patients with advanced melanoma were randomly assigned to vaccination with a mixture of four gp100 and tyrosinase peptides restricted by HLA-A1, HLA-A2, and HLA-A3, plus a tetanus helper peptide, either in an emulsion with granulocyte-macrophage colony-stimulating factor (GM-CSF) and Montanide ISA-51 adjuvant (Seppic Inc, Fairfield, NJ), or pulsed on monocyte-derived dendritic cells (DCs). Systemic low-dose interleukin-2 (Chiron, Emeryville, CA) was given to both groups. T-lymphocyte responses were assessed, by interferon gamma ELISPOT assay (Chiron, Emeryville, CA), in peripheral-blood lymphocytes (PBLs) and in a lymph node draining a vaccine site (sentinel immunized node [SIN]).

Results: In patients vaccinated with GM-CSF in adjuvant, T-cell responses to melanoma peptides were observed in

42% of PBLs and 80% of SINS, but in patients vaccinated with DCs, they were observed in only 11% and 13%, respectively. The overall immune response was greater in the GM-CSF arm ($P < .02$). Vitiligo developed in two of 13 patients in the GM-CSF arm but in no patients in the DC arm. Helper T-cell responses to the tetanus peptide were detected in PBLs after vaccination and correlated with T-cell reactivity to the melanoma peptides. Objective clinical responses were observed in two patients in the GM-CSF arm and one patient in the DC arm. Stable disease was observed in two patients in the GM-CSF arm and one patient in the DC arm.

Conclusion: The high frequency of cytotoxic T-lymphocyte responses and the occurrence of clinical tumor regressions support continued investigation of multi-peptide vaccines administered with GM-CSF in adjuvant.

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PEPTIDE VACCINES for cancer offer the prospect of inducing protective immune responses with a preparation that can be made synthetically and administered with minimal toxicity.¹ However, there is no consensus about how best to vaccinate with peptides. Effective vaccines require that peptides be presented on dendritic cells (DCs) or other antigen-presenting cells. One approach is to prepare DCs ex vivo and to pulse them

with peptides before injection into patients as a vaccine. An alternate approach is to present peptides to Langerhans cells (LCs); epidermal DCs) in vivo and to activate those cells in vivo using cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF).²⁻⁵ Peptide vaccination in a murine model was most effective when that peptide was administered in an emulsion with incomplete Freund's adjuvant and GM-CSF.⁶

We report clinical and immunologic results of a phase II randomized trial of peptide vaccination using the two different vaccine approaches. Patients were randomly assigned to vaccination with DCs pulsed with peptides (arm 1) or to vaccination with peptides in adjuvant plus GM-CSF (arm 2). All patients also were administered low-dose interleukin-2 (IL-2). A two-stage design was used to assess response for each arm individually. This report summarizes results from the first stage of patients accrued to each arm. Novel aspects of this study were evaluation of peptide vaccination in emulsions of GM-CSF in adjuvant; random assignment to vaccination with peptide pulsed on DCs; and immunologic monitoring in a lymph node draining a vaccine site, in addition to peripheral blood.

PATIENTS AND METHODS

Patients

Patients with advanced melanoma were studied with informed consent and with institutional review board and US Food and Drug Administration (FDA)

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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Table 1. Patient Demographics

	Arm 1, DC		Arm 2, GM-CSF		Total	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Age, years						
Range	32-74		29-63		29-74	
Mean	57		49		53	
Race or ethnicity, white	13	100	13	100	26	100
Sex, female	7	54	3	23	10	38
Extremity or unknown primary site	5	38	5	38	10	38
Trunk, head or neck primary site	5	38	5	38	10	38
Mucosal or ocular primary site	3	23	3	23	6	23
Stage						
III	0	0	1	8	1	4
IVA	3	23	2	15	5	19
IVB	4	31	5	38	9	35
IVC	6	46	5	38	11	42
Prior systemic chemotherapy, biochemotherapy, IFN, or IL-2	3	23	4	31	7	27

Abbreviations: DC, dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL-2, interleukin-2.

approval, under investigator-initiated investigational new drug 7593. Clinical characteristics of patients are summarized in Table 1. Inclusion criteria were diagnosis of advanced melanoma (unresectable stage III or measurable stage IV); Eastern Cooperative Oncology Group performance status 0 to 1; expression of HLA-A1, -A2 or -A3; and tumor expression of the melanoma-associated glycoproteins gp100 and/or tyrosinase. Other inclusion criteria included ability and willingness to give informed consent, absolute neutrophil count more than 1,000/ μL , platelets more than 100,000/ μL , hemoglobin more than 9 g/dL, AST and ALT $\leq 2.5 \times$ upper limit of normal (ULN), bilirubin and alkaline phosphatase $\leq 2.5 \times$ ULN, and creatinine $\leq 1.5 \times$ ULN. Patients were allowed to have as many as three brain metastases if the metastases were all less than 2 cm in diameter, were asymptomatic, and there was no mass effect, or they had been treated successfully by surgical excision or by gamma knife radiation therapy. Patients were excluded if they had received cytotoxic chemotherapy, cytokine therapy, corticosteroids, or other investigational agents within the preceding 3 months; had known or suspected allergies to any component of the vaccine; had unresectable tumor likely to cause symptoms and for which chemotherapy or radiation therapy was anticipated within 3 months of entering onto the study; were pregnant; or had New York Heart Association class II, III, or IV heart disease, or other medical contraindication.

Peptides

Class I major histocompatibility complex (MHC)-associated peptides used in these studies include: HLA-A1-associated DAEKSDICTDEY (tyrosinase_{240-251S})⁷; HLA-A*0201-associated YMDGTMSQV (tyrosinase_{368-376D})⁸; YLEPGPVTA (gp100₂₈₀₋₂₈₈)⁹; and YLKKIKNSL (malaria circumsporozoite protein₃₃₄₋₃₄₂)¹⁰; HLA-A3-associated ALLAVGATK (gp100₁₇₋₂₅) and LIYRRRLMK (gp100₆₁₄₋₆₂₂)^{11,12}; and the modified tetanus peptide AQYIKANSKFIGITEL.¹³⁻¹⁵ The peptides were synthesized and purified (> 90%) by the Biomolecular Core Facility at the University of Virginia (UVA; Charlottesville, VA).

The peptides for vaccination were placed in vials under investigational new drug 7593, in aqueous salt solutions. The four melanoma peptides were prepared as a single mixture, and the tetanus helper peptide was prepared as a separate sterile solution. These preparations were submitted for multiple quality-assurance studies including sterility, identity, potency, general safety, pyrogenicity, and stability.

Immunization Protocol

Peptides plus GM-CSF in adjuvant. Patients in arm 2 received a vaccine comprising four melanoma peptides (100 μg each of the HLA-A1-restricted peptide DAEKSDICTDEY, the HLA-A2-restricted peptides YMDGTMSQV and YLEPGPVTA, and the HLA-A3-restricted peptide ALLAVGATK); plus 190 μg of the modified HLA-DR-restricted tetanus helper

peptide AQYIKANSKFIGITEL. The vaccines were prepared as a stable emulsion containing 225 μg GM-CSF (Schering-Plough Research Institute [SPRI, Kenilworth, NJ] for patients 1 to 25 and Immunex [Seattle, WA] for patient 26) and 1 mL of Montanide ISA-51 adjuvant (Seppic Inc, Fairfield, NJ). Each patient was immunized six times: days 0, 7, 14, 28, 35, and 42.

The first three vaccinations were divided between two injection sites (primary and replicate), and the last three vaccinations were delivered to the primary injection site only. At each injection site, half was administered subcutaneously and half was administered intradermally. Patients also received outpatient IL-2 (Chiron Corp, Emeryville, CA) daily for 6 weeks at a dose of 3×10^6 U/m²/d ideal body weight subcutaneously, days 7 to 49. The trial is presented schematically in Figure 1.

Vaccine regimen: DCs pulsed with peptides. Patients in arm 1 were vaccinated with monocyte-derived DCs pulsed with the same peptides described previously and were vaccinated on the same schedule, with the same regimen of low-dose IL-2.

Preparation of monocyte-derived DCs. Peripheral-blood mononuclear cells were obtained by leukapheresis performed on a COBE Spectra (COBE, Denver, CO) apheresis system in the Bone Marrow Transplant Unit at UVA. Leukapheresis consisted of a 3- to 5-hour collection of 8 to 12 L, containing approximately 1 to 2×10^8 nucleated cells per patient kilogram (average, 9×10^9 cells total). Mononuclear cells were plated in serum-free Aim-V medium (Life Technologies, Grand Island, NY) into each of 20 to 30 T225 flasks (Corning, Acton, MA), yielding 2×10^8 cells/50 mL. After the contents were cultured for 2 hours (37°C, 5% CO₂), the flasks were gently washed two or three times with prewarmed buffered saline, and nonadherent cells were removed. Adherent cells were cultured in Aim-V medium containing 50 mmol/L of 2-mercaptoethanol (Gibco, Carlsbad, CA), 1,000 U/mL human GM-CSF (SPRI), and 1,000 U/mL human IL-4 (SPRI). After 7 days, nonadherent cells and adherent cells were washed gently two times with buffered saline.

Seven-day DCs were harvested, counted, and suspended into fresh medium (2×10^6 cells/mL), then incubated 4 hours (37°C) with the mix of four melanoma peptides (40 $\mu\text{g}/\text{mL}$ each) and tetanus peptide (76 $\mu\text{g}/\text{mL}$), then washed three times with Dulbecco phosphate-buffered saline (Life Technology), counted, and cryopreserved.

On the day of vaccination, peptide-loaded DCs were thawed rapidly in a 37°C water bath and evaluated for viability, recounted, and injected within 1 hour. The cells were divided into three equal volumes for the first three vaccine dates, with one aliquot administered intravenously in 50 mL of normal saline, and the other two administered subcutaneously at the primary and replicate immunization sites, respectively. At the fourth, fifth, and sixth vaccine dates, the DCs were divided into two equal volumes, with one administered intravenously in 50 mL of normal saline, and the other administered subcutaneously at the primary immunization site. The average

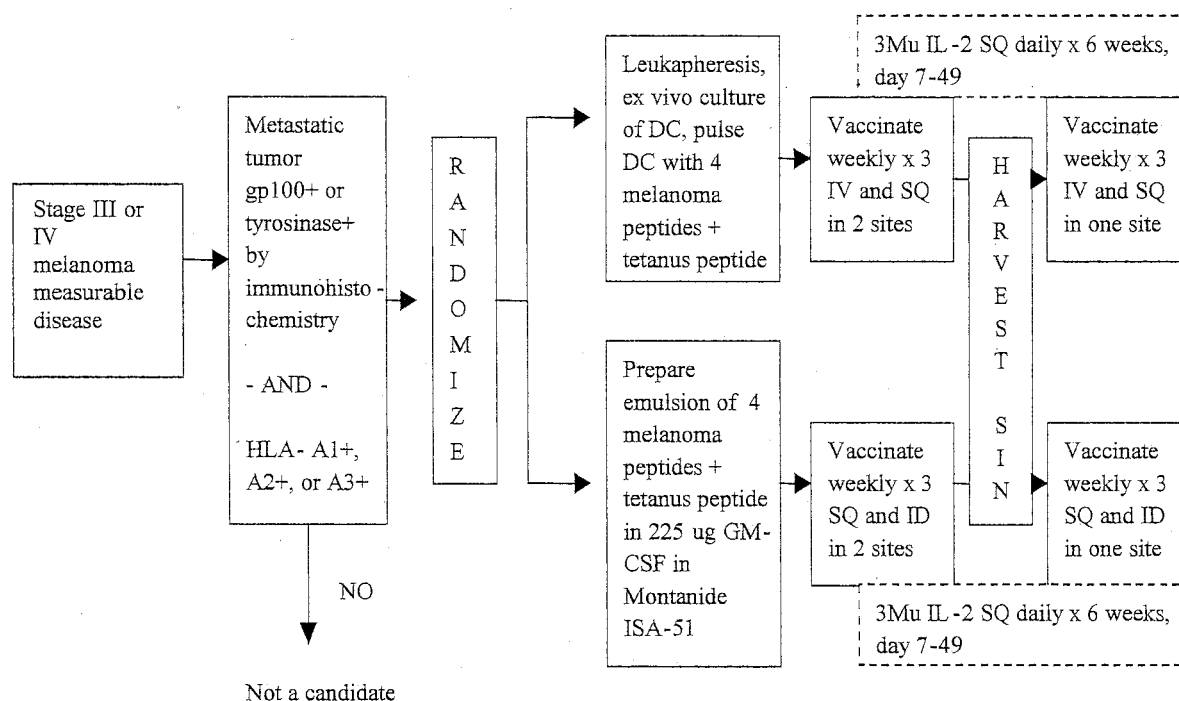


Fig 1. Schematic flow diagram for clinical trial design, University of Virginia melanoma vaccine trial Mel 31. gp100, melanoma-associated glycoprotein; DCs, dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; IV, intravenously; SQ, subcutaneously; IL-2, interleukin-2; SIN, sentinel immunized node; ID, intradermally

number of DCs administered per vaccine site was 13.4×10^6 (range, 6.5 to 23.6×10^6). The DCs were evaluated for multiple DC markers and met lot release criteria. They were CD86⁺, CD80⁺, and class II MHC⁺ (data not shown). Expression of CD83 was tested on several preparations and was borderline positive. DC samples were capable of stimulating lymphocyte proliferation in mixed lymphocyte reactions in vitro and were recognized by cytotoxic T-lymphocyte (CTL) with specificity for the loaded peptides (data not shown).

Sentinel immunized node (SIN). To provide a replicate immunization site at which a node draining that site could be harvested, patients were vaccinated at two cutaneous sites for the first three injections. The primary vaccination site was the patient's arm. The replicate immunization site was on the thigh, except in one patient (patient 2) with prior bilateral inguinal node dissections, in whom the replicate immunization was done in the opposite arm. The replicate vaccine site was placed in an extremity distant from known melanoma deposits so that the immune response detected in the SIN represented a response to the vaccine rather than a pre-existing response to tumor.

A lymph node draining the replicate vaccine site (the SIN) was identified by lymphoscintigraphy 1 week (6 to 9 days) after the third vaccine, as described.¹⁶ Selective biopsy of the SIN was performed (by C.L.S.) under local anesthesia with the intraoperative aid of a sterile hand-held gamma probe (Care Wise, Morgan Hill, CA). The patients consented to this procedure as part of the initial informed consent process for the trial. One patient (patient 21) refused SIN collection. The incision was routinely 2 to 3 cm long, and the node was removed in the outpatient clinic under local anesthesia, without any major infections or complications at the surgical sites. A central section of the node was submitted for histologic assessment. The remainder of the SIN was dissociated mechanically into a single-cell suspension of lymphocytes and cryopreserved.

Cell lines used. C1RA1 and C1RA3 are human Epstein-Barr virus-transformed B-cell lines that lack expression of class I MHC molecules, and that have been transfected with the genes for HLA-A1 and HLA-A3, respectively. T2 is a mutant human T/B-cell hybrid that lacks the transporter associated with antigen processing but expresses *HLA-*

*A*0201*.¹⁷ C1RA1, C1RA3, and T2 were provided by P. Creswell (Yale University, New Haven, CT).

HLA typing. HLA typing was performed by clinical laboratories in some samples. In other samples, it was determined either by microcytotoxicity assay on autologous lymphocytes or by DNA typing using polymerase chain reaction methods (One Lambda, Canoga Park, CA).

Evaluation of clinical outcome. The primary end point for this trial was clinical response. This was assessed by measurement of assessable metastatic deposits by computed tomography scan, magnetic resonance imaging scan, or direct measure of cutaneous deposits. Baseline tumor measurements used for assessment of clinical response were those obtained most immediately before the first vaccine administration and within 6 weeks of protocol entry. Measurements were made and reviewed by a multidisciplinary team. The original protocol defined tumor response on the basis of changes in cross-sectional area calculated as the product of two perpendicular measures. However, since the initiation of this study, the Response Evaluation Criteria in Solid Tumors Group (RECIST) system was employed as the current standard for clinical trials, in which response is based on changes in maximum cross-sectional dimensions.^{18,19} Computed tomography scans of clinical responders were reviewed again by a senior faculty radiologist not otherwise involved in the study.

Statistical considerations. The trial was designed as a phase II evaluation of two vaccine regimens, with each assessed separately for response. For either group of patients entered onto this study, interest in the treatment regimen would increase if the data indicated a response rate (complete response [CR] plus partial response [PR]) of at least 20%, and would diminish if the data indicated a response rate of 5% or less. A minimax two-stage design was used for each group to allow for early closure of either treatment regimen if the preliminary data indicated that the treatment was not sufficiently active.²⁰ It was estimated that 27 eligible patients were required for the analysis of each treatment regimen. For each treatment group, the following two-stage rule was used to test the null hypothesis that the response rate is $\leq 5\%$ versus the alternative that the response rate is $\geq 20\%$. For stage I, 13 eligible patients were accrued to each arm. If no responses were observed, then the study would be closed to this group of patients, with

the conclusion that the data supported the null hypothesis that the response rate was $\leq 5\%$. If one or more responses were observed in the first stage, an additional 14 eligible patients would be accrued, for a total of 27. If accrual proceeded to completion of stage 2 and, at the final analysis, three or fewer responses (11%) were observed, then we would conclude that the data supported the null hypothesis. If four or more responses (15%) were observed, then we would conclude that the data supported the alternative hypothesis. This design had approximate size $\alpha = .05$ and power = 0.8 to test the previously stated hypothesis. At study completion, 95% CIs were provided to aid in estimating true response rates. Kaplan-Meier curves were used to estimate time to progression.

Patients were prospectively randomly assigned to arms 1 and 2 during the first stage of accrual; the findings from this study that allow comparisons in results between the two arms thus are based on random assignment to the two arms. Therefore, results for the two arms are comparable. Comparisons between arms that would include both stages could be challenged because those in stage 2 are not based on prospective randomized assignment. The findings in terms of immune response are most reliable when evaluated at this point, after stage 1. The encouraging immunologic and clinical results from stage 1 have led to an amendment to the protocol such that patients with evidence of clinical tumor regression or stable disease were offered a series of booster vaccines during the course of 18 months. We plan to report the summary clinical and immunologic data for arm 2 of this study, with all 27 patients (plus the booster vaccines in seven patients), when those data are complete.

Toxicity assessment. Toxicity during the vaccine regimen was recorded by each patient by means of a daily diary of toxicities, which were reviewed by a member of the immune therapy team each week and supplemented by direct questioning regarding a series of specific toxicity categories. Toxicities were graded in accordance with the National Cancer Institute common toxicity criteria. In patients with grade 3 toxicities or greater, IL-2 dosing was withheld until toxicities resolved completely or were reduced to grade 1, when IL-2 dosing was resumed with a 25% dose reduction. Up to two such dose reductions were permitted. Patients with evidence of autoimmunity (eg, hyperthyroidism suggestive of autoimmune thyroiditis), whether symptomatic or not, continued to receive vaccines, but IL-2 was discontinued. Patients with unequivocal disease progression or severe symptoms of disease progression were taken off protocol.

ELISpot assays. Peripheral-blood lymphocytes (PBLs) were isolated by Ficoll gradient centrifugation and were cryopreserved. After completion of the vaccine regimen, samples from prevaccination and representative samples after one or more vaccinations were evaluated simultaneously, in parallel with SIN lymphocytes, by interferon gamma ELISpot assay. Lymphocytes were assayed 2 weeks after a single sensitization in vitro with peptide. This approach, plus methods for the ELISpot assays, has been reported.¹⁶ In one patient (patient 18), the ELISpot data from the SIN were not assessable because the negative control sample was not assessable; that SIN was evaluated by a separate ELISpot assay performed in parallel with samples from other patients on this study, and repeat data confirmed the immunogenicity results.

Evaluation of T-cell responses was based on the following definitions:

N_{vax} = number of T cells responding to peptide in the vaccine

N_{neg} = number of T cells responding to negative control (maximum of two negative controls: C1R-A1, T2, or C1R-A3 alone; or C1R-A1, T2, or C1R-A3 pulsed with an irrelevant peptide)

R_{vax} = ratio of $N_{\text{vax}}/N_{\text{neg}}$

For evaluations of PBLs, a patient was considered to have a T-cell response to vaccination only if all of the following criteria had been met: N_{vax} exceeded N_{neg} by at least 30 cells per 100,000 (corresponds to $\sim 0.15\%$ of CD8⁺ cells); $R_{\text{vax}} > 2$; ($N_{\text{vax}} - 1$ standard deviation) \geq ($N_{\text{neg}} + 1$ standard deviation); and R_{vax} after vaccination $\geq 2 \times R_{\text{vax}}$ prevaccine.

The peak CTL response to any peptide after the first vaccine is reported as a fold-increase over the negative control, and the increase resulting from vaccination is reported as a ratio of the postvaccine measure to the prevaccine measure. For evaluations of SIN, the first three listed criteria are required for a T-cell response to vaccination. Because prevaccination lymph node samples were not routinely evaluated in this study, the last criterion was

not applied to SIN. Differences in proportions (ie, response rates in the SIN) between the two arms were assessed using the χ^2 test, and differences in the magnitude of response were assessed using the Wilcoxon rank sum test.

RESULTS

Clinical Responses

Using RECIST criteria, two PRs were experienced by patients in the GM-CSF arm and one PR was experienced by a patient in the DC arm. One of the PRs, in patient 3, was transient and met criteria for a PR by a narrow margin, with a 34% decrease in the sum of assessable tumor diameters. However, the other two PRs were dramatic and persisted for several months or more. Additional stable disease was observed in two patients in the GM-CSF arm and in one patient in the DC arm. Thus, favorable outcomes were observed in four of 13 patients (31%) in the GM-CSF arm but in only two of 13 patients (15%) in the DC arm. Details are listed in Table 2. The objective clinical response rates in the GM-CSF arm and DC arm were 15% (95% CI, 2% to 45%) and 8% (95% CI, < 1% to 36%), respectively.

The trial had a two-stage design, in which 13 patients were accrued to each arm, and if there was at least one clinical response (PR or CR) in those 13 patients, then 14 additional patients would be accrued. After completion of accrual in arm 1, there were no clinical responses. Thus, that arm was closed. However, one patient (patient 23) subsequently had a gradual decrease in tumor burden and met criteria for a PR. Arm 1 was not reopened for three reasons: literature became available suggesting that monocyte-derived DCs may induce tolerance,²¹ the immunologic studies showed minimal immunogenicity in arm 1, and the GM-CSF and IL-4 required for DC preparation that was being provided by the SPRI was no longer available from that source. Thus, for patient safety and logistic concerns, this arm was not reopened. There were clinical responses in arm 2 at the time of completion of stage 1; therefore, the trial was kept open for accrual of 14 additional patients to that arm. This article reports the immunologic and clinical outcome of the two vaccine regimens at the completion of stage I because within the first stage, the two groups are comparable.

Patients With Early Discontinuation of Vaccines or IL-2

Among the 13 patients randomly assigned to receive vaccination with peptide-pulsed DCs, three patients progressed rapidly during the DC preparation period and required other interventions that caused their removal from this study before the first vaccine. To address the potential question of whether there may have been unequal delays in starting therapy between the two arms, the time from consent to initiation of therapy was calculated. The preparation of DC vaccines required a 7-day culture period for DC plus 2 to 3 weeks for quality assurance studies, including an FDA-mandated 14-day sterility test. Thus, we would expect DC vaccines to start 21 to 28 days later than peptide vaccines. The median time to first vaccine (or to the decision to come off study) after the patients signed consent was 37 days for those in the DC arm and 11 days for those in the peptide arm. This difference of 26 days is within the range expected because of the time required for preparation and quality assurance studies for the DC.

Table 2. Clinical Responses and Summary of T-Cell Responses per Arm

Patient No.	Dendritic Cells + Peptide (arm 1)				Peptide + GM-CSF in Adjuvant (arm 2)					
	Immune Response		Clinical Response	No. of Patients	%	Patient No.	Immune Response		Clinical Response	No. of Patients
	SIN	PBL				SIN	PBL			
4	NA	NA	PD		1	+	+	PD		
5	–	–	PD		2	+	–	PD		
6	+	–	PD		3	+	+	PR, vitiligo		
7	NA	NA	PD		8	+	–	PD		
9	NA	NA	PD		10	NA	NA	PD		
12	NA	NA	PD		11	+	–	PD		
14	–	–	SD 5 months		13	+	+	SD 3 months until surgery, vitiligo		
15	–	–	PD		17	–	–	PD		
16	–	–	PD		18	+	+	PR		
20	–	+	PD		19	–	–	PD		
22	–	–	PD		21	NA	–	PD		
23	–	–	PR		24	NA	+	SD 3 months → PD		
25	NA	–	PD		26	+	–	PD		
PR				1	8				2	15
SD				1	8				2	15
Alive with disease				1	8				1	8
Vitiligo				0	0				2	15

NOTE. Plus (+) and minus (–) symbols indicate the presence or absence, respectively, of a response.

Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; SIN, sentinel immunized node; PBL, peripheral-blood lymphocytes; NA, not assessable; PD, progressive disease; PR, partial response; SD, stable disease.

Five additional patients developed symptomatic tumor progression requiring discontinuation of treatment before the completion of all six vaccines. This included two patients in the DC arm (arm 1) and three patients in the GM-CSF arm (arm 2).

Toxicity and Tolerability

Twenty-three patients received at least one vaccine and were, therefore, assessable for toxicity. Transient grade 1 and 2 toxicities were experienced by the majority of patients and were attributable to IL-2 in most patients (Table 3). Grade 3 and 4 toxicities were observed in 11 patients (48% of enrolled patients; Table 3). Excluding three patients whose adverse events are considered unrelated to therapy (attributable to tumor progression and/or not related to vaccination), there were grade 3 and 4 adverse events in five patients in the GM-CSF arm (38%) and four patients in the DC arm (40%). IL-2 was stopped early in two patients who experienced hyperthyroidism, although it was generally asymptomatic. In five patients, IL-2 delays and dose reductions of 25% were needed to manage grade 3 toxicities. In one patient, a second dose reduction of 25% (total, 50%) was required. The only serious adverse event was the single episode of severe hyperglycemia (one of 23 patients; 4%) after which IL-2 and vaccines were stopped at week 3. No treatment-related mortality occurred.

Peptide-Specific Immune Responses

Among 13 patients randomly assigned to arm 1, five required withdrawal from study because of tumor progression before collection of the SINS. Among 13 patients randomly assigned to arm 2, two progressed before completion of vaccines and did not have the SIN collected. One additional patient in arm 2 refused SIN harvest. Thus, SINS were harvested and assessable from eight patients in

arm 1 and 10 patients in arm 2. Both exceeded the protocol requirement that at least six patients on each arm have assessable SINS.

Immunologic data are presented in Figure 2 and Tables 4, 5, and 6. CTL responses were detectable in the SINS of eight of 10 patients (80%) vaccinated with peptides in GM-CSF in adjuvant, but in only one of eight patients (13%) vaccinated with peptide-loaded DCs (Table 4). In addition, the magnitude of the responses was substantially higher in the GM-CSF arm than in the DC arm (Fig 2). The strongest observed CTL responses were to the two tyrosinase peptides (Fig 2, Table 5), but responses were also observed to each of the gp100 peptides (Table 5).

The CTL response rate in the SIN was higher for the GM-CSF arm than for the DC arm (80% v 13%; $P = .004$; Table 4). The response rate in the PBLs was numerically higher in the GM-CSF arm, but was not statistically significant (42% v 11%; $P = .13$; Table 4). The overall CTL response rate was higher in the GM-CSF arm (75% v 22%; $P = .017$; Table 4).

For patients vaccinated with GM-CSF, the median ratio of SIN response to the PBL response after three vaccines was 10:1 (Table 6). The median ratio of SIN responses to the maximal PBL response was 6.3:1 (Table 6). Concordance between CTL response in the PBL and in the SIN was observed for three of the four peptides. In the GM-CSF arm, four of five patients with a response in the PBL also had a response in the SINS. CTL responses in the SIN in the remaining patient (patient 24) could not be evaluated. In contrast, the one patient in the DC arm who had a CTL response in the PBL did not have a detectable CTL response in the SIN (Table 2).

Helper T-Cell Responses to Tetanus Peptide

The circulating T-cell response to the tetanus peptide was evaluated by proliferation assay as described previously¹⁴ (Fig

Table 3. Clinical Toxicities in Patients 1 Through 26

Toxicity	Total		Grades I and II		Grade III		Grade IV	
	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
Malaise or fatigue	21	91	20	87	1	4		
Anorexia or weight loss	17	74	17	74				
Injection site reaction	16	70	16	70				
Nausea	15	65	15	65				
Headache	14	61	14	61				
Pruritus	13	57	13	57				
Arthralgia or myalgia	13	57	13	57				
Chills	12	52	12	52				
Fever	11	48	11	48				
Rash	10	43	9	39	1	4		
Sweats	10	43	10	43				
Diarrhea, without colostomy	9	39	8	35	1	4		
Dyspnea	9	39	7	30	2	9		
Vomiting	8	35	8	35				
Pain, nonlocal	8	35	7	30	1	4		
Other	7	30	6	26	1	4		
Metabolic or laboratory	7	30	5	22			2	9
Mood changes	6	26	6	26				
Stomatitis	5	22	5	22				
Edema	5	22	5	22				
Constipation	4	17	4	17				
Cough	3	13	3	13				
Dizziness or lightheadedness	3	13	3	13				
Erythema	3	13	3	13				
Gastric ulcer	2	9	2	9				
Insomnia	2	9	2	9				
Facial flushing	2	9	2	9				
Endocrine	2	9	1	4	1	4		
Liver function abnormality	2	9	2	9				
Acute vascular leak syndrome	1	4					1	4
Paresthesia	1	4	1	4				
Conjunctivitis or keratitis	1	4	1	4				
Ocular or visual	1	4	1	4				
Hemoptysis	1	4	1	4				

NOTE. A total of 23 patients was assessable.

3). A stimulation index of 4 or greater is considered positive (closed circles; Fig 3B). There was a significant correlation (Spearman coefficient, 0.631; 95% CI, 0.17 to 0.92; $P = .016$) between proliferative response to the tetanus peptide and CTL response in the SINs to the class I restricted melanoma peptides (Fig 3C). The sample size evaluated was too small for meaningful statistical comparison between groups; however, in arm 1, one of five tested patients responded (20%); in arm 2, four of nine patients responded (44%; Fig 3B).

Vitiligo

We observed vitiligo in two patients in this protocol. Both were in arm 2 and represent 17% of assessable patients in this arm. One patient had intervening therapy (biochemotherapy) before the vitiligo was evident (patient 3), which may have contributed to the vitiligo. Vitiligo was noted over most of the skin of the chest and neck and, also, over a subcutaneous tumor nodule in the right arm that regressed completely after appear-

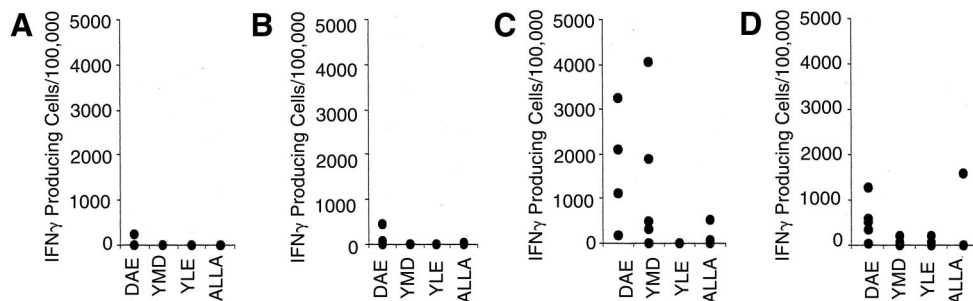


Fig 2. Magnitude of the maximal sentinel immunized node and peripheral blood lymphocyte levels for arm 1 (A and B, respectively) and arm 2 (C and D, respectively), by ELISpot assay, for each of the four peptides. IFN γ , interferon gamma; DAE, tyrosinase_{240-251D}; YMD, tyrosinase_{368-376D}; YLE, gp100₂₈₀₋₂₈₈; ALLA, gp100₁₇₋₂₅.

Table 4. Number and Percentage of Patients With T-Cell Responses in PBL, SIN, or Either, by Study Group

Arm	Sample Source		
	PBL	SIN	Either
Arm 1, dendritic cells			
Responses, No.	1	1	2
Total No. of patients	9	8	9
Responses, %	11	13	22
Arm 2, GM-CSF + peptides			
Responses, No.	5	8	9
Total No. of patients	12	10	12
Responses, %	42	80	75
<i>P</i> , χ^2 , arm 1 v arm 2	.13	.004	.017

Abbreviations: PBL, peripheral-blood lymphocytes; SIN, sentinel immunized node; GM-CSF, granulocyte-macrophage colony-stimulating factor.

ance of the vitiligo. The other patient developed vitiligo within 2 months after completion of the vaccine regimen, and without intervening therapy (patient 13). In this patient, the vitiligo was dramatic and involved the large majority of his skin. These vitiligo events were not associated with visual changes or other adverse symptoms, and were not considered toxicity. Both patients developed cellular immune responses to vaccination. Patient 3 had a partial clinical response, and patient 13 had stable disease (Table 2).

Correspondence of Clinical Responses and T-Cell Responses in Patients in the GM-CSF Arm

Patient 3 had a PR during vaccination, and the T-cell response in that patient has been reported.¹⁶ There were CTLs responding to the tyrosinase_{240–251S} peptide DAEKSDICTDEY in the SINS, in the PBLs, and in metastatic tumor deposits, as long as 8 weeks after completion of vaccination.¹⁶ This PR was transient and its assessment was complicated by the removal of one metastasis that grew during 7 days and was regressing at week 3. However, we consider that the observed changes meet RECIST criteria for a PR. That tumor deposit, and another

removed after completion of vaccination, both contained CTLs reactive to the tyrosinase peptide.¹⁶

Patient 18 had two intrathoracic tumor masses, which had decreased in size by 70% (cross-sectional area; 44% by diameter, according to RECIST) within 4 months after vaccination. The larger of those two masses is shown in Figure 4. Both masses continued to shrink after this time to a maximal response of 91% (area) or 76% (diameter). The patient subsequently developed recurrence of an isolated brain metastasis, which was re-treated with gamma knife surgery. Otherwise, there has been no progression of systemic disease, and the two intrathoracic lesions remain in regression. He is asymptomatic. Evaluation of his T-cell responses revealed a transient response to YLEPGPVTA in PBLs, plus responses to YMDGTMSQV and ALLAVGATK in the SINS.

Survival Analysis

Median survival time for patients in the GM-CSF arm was 14.8 months (95% CI, 6 to 17 months) and 6.2 months (95% CI, 4 to 11 months) for patients in the DC arm (log-rank *P* = .26; data not shown). Estimated survival for patients in the GM-CSF arm is 62% at 1 year (95% CI, 35% to 88%) and 23% at 2 years (95% CI, < 1% to 46%).

DISCUSSION

In this study, we report clinical and immunologic results of a randomized phase II trial of vaccination with four melanoma peptides, plus a tetanus helper peptide, using two vaccination approaches. Despite the theoretical advantage of vaccinating with DCs pulsed with peptides, immune responses were greater in frequency and in magnitude among patients vaccinated with peptides in an emulsion of adjuvant plus GM-CSF. This difference was evident in both node and blood. T-cell responses were identified in these patients by ELISpot assay after a single in vitro sensitization. In a prior article, we reported that responding T cells can also be detected directly ex vivo, and that these T cells

Table 5. Number and Percentage of Patients With T-Cell Responses to Each Peptide

Peptide	Arm 1, Dendritic Cells			Arm 2, GM-CSF + Peptides		
	PBL	SIN	Either	PBL	SIN	Either
DAEKSDICTDEY						
Responses, No.	1	1	2	4	4	5
Total No. of patients	6	4	6	5	4	5
Responses, %	17	25	33	80	100	100
YMDGTMSQV						
Responses, No.	0	0	0	1	4	4
Total No. of patients	3	3	3	6	6	6
Responses, %	0	0	0	17	67	67
YLEPGPVTA						
Responses, No.	0	0	0	1	0	1
Total No. of patients	3	3	3	6	6	6
Responses, %	0	0	0	17	0	17
ALLAVGATK						
Responses, No.	0	0	0	1	2	2
Total No. of patients	4	3	4	4	3	4
Responses, %	0	0	0	25	67	50

Abbreviations: GM-CSF, granulocyte macrophage colony-stimulating factor; PBL, peripheral-blood lymphocytes; SIN, sentinel immunized node.

Table 6. CTL Responses in PBL and SIN, in Patients in Arm 2 With Evidence of Immunogenicity in PBL or SIN

Patient No.	Peptide	CTL Response (cells per 10 ⁵)			Ratio (SIN:PBL maximum*)	Ratio (SIN:PBL3*)
		SIN	PBL Maximum	PBL After Three Vaccines (time of SIN)		
1	ALLA	512.0	1,601.0	272.5	0.32	1.88
1	DAE	2,092.0	1,271.0	431.5	1.65	4.85
2	YMD	1,902.3	< 1	ND	1,902.30	ND
3	DAE	3,237.0	529.0	529.0	6.12	6.12
8	YMD	476.7	72.0	ND	6.62	ND
11	YMD	4,040.3	195.0	2.3	20.72	1,756.65
13	DAE	1,114.5	601.0	< 1	1.85	1,114.50
18	ALLA	75.3	4.2	< 1	17.93	75.30
18	YLE	< 1	195.7	24.0	0.01	0.04
18	YMD	315.3	33.2	5.5	9.50	57.33
26	DAE	177.0	28.0	17.7	6.32	10.00
Mean		1,267.6	411.9	142.7	179.4	334.5
Median		512.0	195.0	17.7	6.3	10.0
Mean calculated from ln of ratios and back transforming					4.3	18.0

Abbreviations: CTL, cytotoxic T-lymphocyte; PBL, peripheral-blood lymphocytes; PBL3, PBL after three vaccines; SIN, sentinel immunized node; ND, not determined.

*For calculation of ratios, values < 1 are considered to be 1.

have cytotoxic function directed against the peptides used for vaccination and against tumor cells naturally expressing gp100 and/or tyrosinase.¹⁶ Thus, these data support continued investigation of vaccination with peptides in adjuvant plus GM-CSF.

The minimal T-cell responses with DCs, in most patients, could have several possible explanations. It is increasingly apparent that generating DCs ex vivo for vaccines is complicated by tight regulatory control of critical DC functional states, which remain incompletely understood. At the time this study was initiated, there was evidence of efficacy of vaccines with monocyte-derived DCs cultured in GM-CSF and IL-4.²²⁻²⁴

More recent work suggests that immature DCs generated from monocytes in IL-4 and GM-CSF may be tolerogenic.²¹ However, in our study, we did detect peptide-specific immune responses in two patients vaccinated with DCs, suggesting that this vaccine approach with DCs was not tolerogenic but was minimally immunogenic.

It is presumed that intradermal vaccination with peptides in adjuvant plus GM-CSF leads to peptide-MHC complexes on epidermal LCs that have been activated by the GM-CSF and adjuvant. It is believed that these activated LCs mature and migrate to the draining nodes where they present antigen to T

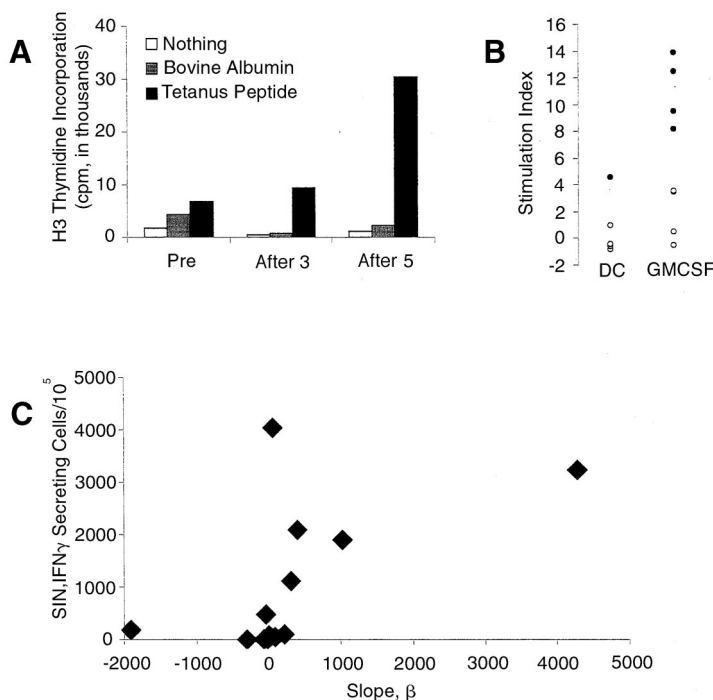


Fig 3. Proliferation of peripheral blood lymphocytes after 5 days in culture with tetanus peptide or bovine albumin. Sample data for responder (A); stimulation index, as ratio of proliferative responses after and before vaccination (B); slopes for each patient's measurements of tetanus minus background, in relation to number of immunizations, using simple linear regression (C). SIN, sentinel immunized node; IFN γ , interferon gamma; DC, dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor.

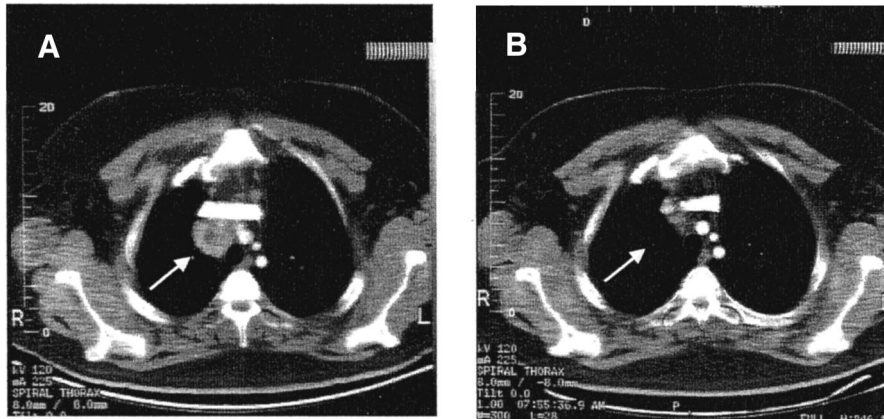
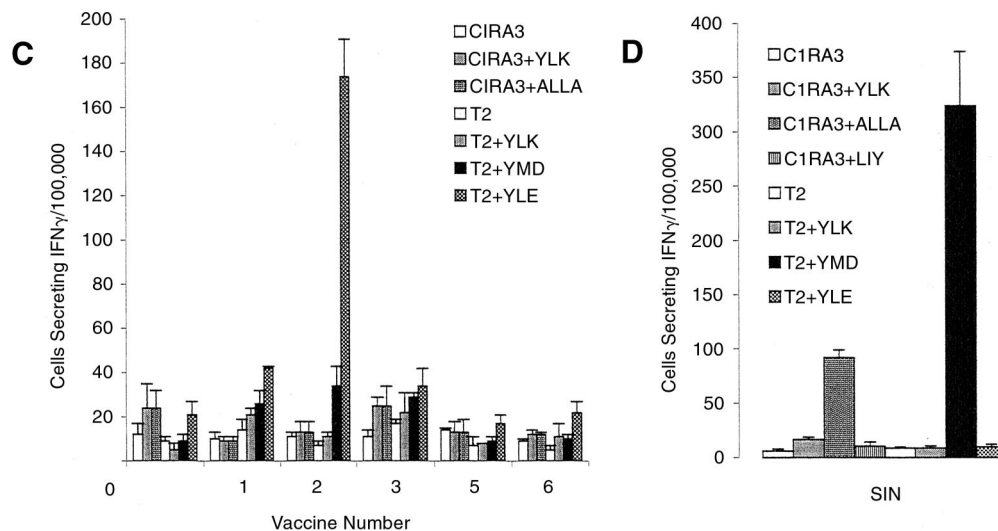


Fig 4. Computed tomography images showing size of the larger of two intrathoracic metastases (arrow) prior to vaccination (A) and 4 months after vaccination, when a major objective response had occurred (B). Ellsplot results from this patient's (C) peripheral-blood lymphocytes and (D) sentinel immunized node (SIN). IFN γ , interferon gamma; YLK, malaria CSP₃₃₄₋₃₄₂; ALLA, gp100₁₇₋₂₅; LIY, gp100₆₁₄₋₆₂₂; YMD, tyrosinase_{368-376D}; YLE, gp100₂₈₀₋₂₈₈.



cells. This sequence of events has not been studied directly with the current vaccine regimen, but is consistent with existing data on LC maturation and migration, and interactions between naïve T cells and DCs in lymph nodes.^{26,27} Studies are planned to address more directly the cellular events associated with vaccination using peptides in cytokine emulsions.

At the time this trial was conceived, few melanoma antigens recognized by class II MHC molecules had been defined, and they were restricted by a minority of class II MHC molecules. Thus, for this study, we included a nonspecific tetanus helper peptide, which we have reported to be immunogenic in humans when administered in adjuvant.¹⁴ Data from this study also support the immunogenicity of this helper peptide when administered in adjuvant. The positive correlation between CTL responses and tetanus responses suggests that the adjuvant plus GM-CSF approach can stimulate both helper and cytotoxic T cells. The ability of this helper peptide to augment responses to CTL epitopes will be tested as part of a planned clinical trial (Eastern Cooperative Oncology Group trial 1602).

Three patients in this trial experienced objective clinical tumor regressions, all of which were PRs. In arm 2, the two clinical

tumor regressions occurred in patients with immune responses to one or more peptides used in the vaccines. The rate of clinical tumor regressions (15%) was less than the observed CTL response rate (80%), which may be explained by heterogeneity of tumor antigen expression and other immune escape mechanisms manifested by tumor cells. However, one patient in arm 1 experienced an objective tumor regression, and that patient has no detectable immune response to the peptides used for vaccination. PBLs from that patient were found to recognize the melanoma antigen recognized by T-cells (MART-1/MdanA)₂₇₋₃₅ peptide before and after vaccination (data not shown). It is likely that the clinical regression in this patient was unrelated to the vaccines and either was a spontaneous regression or may have been contributed to by the low-dose IL-2 or the GM-CSF.

It is important to note that all patients in this study received low-dose IL-2, and its contribution to the observed clinical tumor regressions is unknown. The dose regimen was selected on the basis of prior published experience that 3 U/m²/d increases T-cell responses but is unlikely to contribute to clinical tumor regressions.²⁷⁻²⁹ However, there is evidence of toxic systemic effects of IL-2 at this dose, including evidence of autoimmune

thyroiditis and marked eosinophilia (E.M.H. Woodson et al, manuscript in preparation). Thus, there also may have been some therapeutic value at this dose. However, the daily dose of IL-2 used was only approximately 2% of the FDA-approved daily therapeutic dose.

Because of the uncertainty of the role of IL-2 in both the T-cell responses and the clinical regressions in patients receiving this protocol, and because of the noted toxicities of this IL-2 regimen, we have initiated two additional vaccine trials with this peptide vaccine regimen. One of these is evaluating the impact of this low-dose IL-2 on T-cell responses (UVA-Mel 36), and that trial has completed accrual of 40 patients. Results of that study are being reported separately. The other is a trial evaluating the clinical response rate with this vaccine regimen in the absence of low-dose IL-2 (UVA-Mel 42), which currently is accruing patients.

Results of this study support the hypothesis that immune responses to vaccination are detected more readily in the SInS than in the PBLs. As shown in Table 4, there were two patients in arm 1 with T-cell responses and nine patients in arm 2 with T-cell responses. Only six of these 11 immune responders were detectable by evaluation of the PBLs alone (one in arm 1, five in arm 2). Thus, evaluation of the SInS increased the detectable immunogenicity by 83%. Furthermore, the magnitude of CTL response detected in the SInS was at least four-fold higher than the maximal response in the PBLs (Table 6). Though there was no prevaccine lymph node to compare with the SInS results, the largely negative responses in the SInS of DC patients serve as a

valid negative control for the strong positive responses in the SInS of the GM-CSF patients.

It is not suggested that all vaccine trials must include evaluation of an SInS, but the cost and logistic challenges are manageable and permit greater power in comparing immunogenicity of two different vaccine regimens with a small sample size. The technology also will likely be valuable in characterizing whether failures to achieve therapeutic antitumor immunity are due to lack of immunogenicity or to inadequate dissemination and persistence of the immune response.

The principal findings of this study are that vaccination with the mixture of four melanoma peptides (restricted by HLA-A1, A2, and A3) leads to expansion of peptide-specific immune responses in 75% to 80% of patients and is associated with clinical tumor regressions in a proportion of patients.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices in a study if they are not being evaluated as part of the investigation. Received more than \$2,000 a year from a company for either of the last 2 years: Craig L. Slingluff Jr, Chiron Corp, Immunex/Berlex, Schering-Plough Research Institute.

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