Immunization Using Autologous Dendritic Cells Pulsed with the Melanoma-Associated Antigen gp100-Derived G280-9V Peptide Elicits CD8$^+$ Immunity

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Abstract

Purpose: To determine the toxicity, maximal tolerated dose, and clinical and immunologic response to autologous dendritic cells pulsed with melanoma-associated antigen gp100-derived G280-9V peptide.

Patients and Methods: Twelve HLA-A*0201$^+$ patients with advanced melanoma were administered dendritic cells pulsed with G280-9V peptide. Cohorts of three patients were administered 5×10^6, 15×10^6, and 50×10^6 cells i.v. every 3 weeks for six doses according to a dose escalation scheme. Three additional patients were treated at the highest dose. No additional cytokines or therapies were coadministered. The immunogenicity of G280-9V-pulsed dendritic cells was measured by IFN-γ ELISPOT assay, tetramer assay, and ^{51}Cr release assay comparing prevaccination to postvaccination blood samples. Response to treatment was assessed by Response Evaluation Criteria in Solid Tumors.

Results: CD8$^+$ immunity to the native G280 was observed in 8 (67%) patients as measured by ELISPOT and in 12 (100%) patients as measured by tetramer assay. Of the 9 patients tested, 9 (100%) had measurable high-avidity CTL activity as defined by lysis of allogeneic melanoma lines, which coexpress HLA-A*0201 and gp100. The median follow-up of the entire cohort is 43.8 months. Two (17%) partial responses were observed and 3 (25%) patients had stable disease. The median survival of the treated population was 37.6 months. At this time, three patients are alive, including one patient who continues to respond without additional treatment.

Conclusion: The high rate of immunization as measured by three independent assays and the occurrence of clinical regression support continued investigation of G280-9V peptide as a candidate epitope in melanoma vaccine formulations.

Melanoma incites an immune response by the host, and a variety of strategies have been pursued in an attempt to take therapeutic advantage of this observation (1). Some of these strategies involve nonspecific attempts to augment antitumor immunity, such as the cytokines interleukin (IL)-2 and IFN-α2b (2, 3). These are now accepted conventional therapies. However, most recent investigational work has been focused on attempts to induce specific antitumor immunity by engendering responses to defined single or multiple antigens expressed by tumor cells. The central line of this research has been the identification and isolation of the antigens present on melanomas recognized by T cells (4).

The isolation of melanoma-associated antigens has allowed investigators to design peptide vaccines that can be coadministered with a variety of adjuvants (4, 5). Dendritic cells serve a critical role in capturing and presenting antigen in the initiation of immunity against infectious pathogens and neoplasia (6). A variety of technical advances in isolating human dendritic cells as well as an improved understanding of dendritic cell biology have led investigators to propose using autologous dendritic cells as adjuvant for peptide vaccination in cancer (7, 8).

The initial clinical reports conclude that dendritic cell immunization in patients with advanced melanoma is safe and well tolerated; however, response rates are <10% in most studies (9–11). Laboratory correlative studies show that some patients develop T-cell immunity after dendritic cell vaccination with a variety of schedules and routes of administration

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(12–14). Most *in vitro* studies conclude that immature dendritic cells are optimal at antigen capture and processing, whereas mature dendritic cells are best at presenting antigen to elicit immunity (15). Limited studies in human dendritic cell vaccine trials tend to support this conclusion, but additional controlled trials are needed to more carefully address this issue (16, 17).

We report the results of a phase I study of peptide-pulsed dendritic cells for patients with advanced melanoma. In the current study, monocyte-derived dendritic cells are loaded with soluble peptide antigen *ex vivo* and irradiated before reinfusion through an i.v. route every 21 days for six doses. We find immunization using G280-9V-pulsed dendritic cells elicited melanoma-reactive $CD8^+$ T cells specific for the native (G280) epitope in all 12 patients as judged by ELISPOT assays, tetramer analysis, and cytolytic assays using peripheral blood obtained from longitudinal samples from each patient.

Patients and Methods

Study design

Adult patients with histologically proven regional (unresectable stage III) or metastatic (unresectable stage IV) cutaneous melanoma were eligible. All subjects were HLA-A*0201$^+$, had Eastern Cooperative Oncology Group performance status of 0 or 1, had gp100$^+$ biopsy-proven melanoma metastases confirmed by immunohistochemistry using the monoclonal antibody HMB45, had no evidence of autoimmune disorder, and were >4 weeks from any therapy with corticosteroids, chemotherapy, radiotherapy, immunotherapy, or surgery. Measurable disease was required for enrollment. Other inclusion criteria included age ≥18 years, adequate hematologic, renal, and hepatic function as defined by WBC >3,000/mm^3, platelet count >75,000/mm^3, serum bilirubin <2.0 mg/dL, and serum creatinine <2.0 mg/dL. Patients were expected to have estimated life expectancy of >3 months. Table 1 summarizes the patients enrolled in this study. Patients were excluded if immunized previously with a gp100-containing formulation. Subjects were not eligible if they had active infection requiring treatment, had evidence of HIV/AIDS, had positive HBV serology, or were pregnant or nursing mothers. A negative pregnancy test was required from females of childbearing age.

All patients signed a valid informed consent for treatment on a phase I protocol reviewed and approved by the Dana-Farber Cancer Institute Human Protection Committee in accord with an assurance filed with the Department of Health and Human Services numbered as WA0001121. The Food and Drug Administration approved procedures for dendritic cell preparation and administration according to investigational new drug application IND 7847.

Preparation of dendritic cell vaccine

Patients underwent leukapheresis according to standard procedures. For the first immunization, fresh peripheral blood mononuclear cells (PBMC) were initiated into culture for generation of dendritic cells. For subsequent use, the remaining cells were aliquoted for single use according to dose level and cryopreserved in RPMI 1640 with 15% autologous plasma and 10% DMSO. Cryopreserved cells were thawed in sterile PBS containing 1.25% human serum albumin (Bayer 684-20, Research Triangle Park, NC), 40 units/mL heparin (Elkins-Sinn, Saint Davids, PA), and 10 μg/mL DNase I grade II (Boehringer Mannheim, Germany). After washing, cell viability was determined by trypan blue exclusion.

Dendritic cells were prepared as described (18). PBMC suspended in RPMI 1640 with 5% autologous plasma at 5×10^6 cells/mL were dispersed into T75 culture flasks. After 1-hour culture at 37°C in 5% CO_2, nonadherent cells were removed by three washes with PBS. Adherent cells were then cultured in RPMI 1640 with 1% autologous xplasma/25 mmol/L HEPES/L-glutamine/gentamicin supplemented with granulocyte macrophage colony-stimulating factor at 100 ng/mL (Immunex, Seattle, WA) and IL-4 at 20 ng/mL (Schering-Plough, Kenilworth, NJ). Fresh medium containing granulocyte macrophage colony-stimulating

Table 1. Details of subject characteristics, dose, and clinical responses

ID	Age*	Sex† (M/F, 5/7)	Stage (III, 2; IV, 10)	Disease sites	Prior therapy	Dose level‡	Total dendritic cell dose	Outcome
101	72	F	IV	Soft tissues, mesentery	Surgery	1	30×10^6	SD
102	49	F	IV	Distant lymph nodes	Surgery, IFN	1	30×10^6	SD Now NED
103	55	M	IV	Soft tissue, lung, mesentery	Surgery, XRT, chemotherapy	1	30×10^6	Twice§ PR; then PD
201	69	M	IV	Lymph node, lung, liver	Surgery, XRT	2	90×10^6	PD
202	51	F	IV	Skin, soft tissue, adrenal	Surgery, IFN	2	90×10^6	PD
203	29	F	III	Skin	Surgery, IFN	2	90×10^6	PD now NED
301	67	M	IV	Lung	Surgery	3	250×10^6	PD
302	75	M	III	Skin	Surgery, IFN	3	300×10^6	PR
303	59	M	IV	Skin, lung, liver, brain	Surgery, XRT	3	300×10^6	PD
304	71	M	IV	Liver	None	3	300×10^6	SD; then PR‖ AWD
305	60	M	IV	Abdominal mass	Surgery, IFN	3	300×10^6	PD
306	50	F	IV	Lung	Surgery, chemotherapy	3	200×10^6	PD

Abbreviations: PD, progressive disease; SD, stable disease; PR, partial response; NED, no evidence of disease; AWD, alive with disease; XRT, radiation therapy.
*Median age, 59.5; range, 29-75.
†Five men, seven women.
‡Dose level 1: 10×10^6: 2, 15×10^6: 3, 50×10^6.
§Subject 103 experienced a PR, underwent a second course of therapy, and then progressed.
‖Subject 304 showed stable disease after six treatments but went on to experience a partial response 4.5 months after the last treatment without additional therapy.

Table 2. Dendritic cell phenotype

Mean percentage of positive cells by two-color fluorescence-activated cell sorting (range)	
HLA-DR	0.67 (0-9.7)
CD11c	1.24 (0-15)
HLA-DR/CD11c	96.72 (79.2-99.8)
CD80	0.12 (0-2.15)
CD86	87.29 (54.6-98.6)
CD80/CD86	6.52 (0-35.4)
CD14	0.04 (0-1.2)
CD11c	75.87 (50.7-97.4)
CD14/CD11c	21.87 (0.2-49.2)
CD3	3.44 (0.02-29.2)
CD19	2.28 (0.02-27.2)
CD3/CD19	0.46 (0-5.84)

NOTE: Release criteria: HLA-DR, >75%; CD11c, >75%; CD86, >75%; CD14, <50%.

factor and IL-4 was added every 2 to 3 days. On day 5 of culture, an aliquot (2×10^6) of cells were removed for phenotypic analysis and microbiological studies. On day 7, dendritic cells were harvested, washed twice in serum free medium, and resuspended to 2×10^6 cells/mL. Generally, an average yield of 4% to 8% dendritic cells was achieved from PBMC. The dendritic cells were evaluated for multiple dendritic cell markers and met lot release criteria as shown in Table 2. Phenotyping was done on aliquots of cultured dendritic cells before pulsing. They were $CD86^+$, $CD80^{low}$, $CD83^{low}$, and HLA-DR/CD11c double positive (see Results).

Dendritic cells were resuspended in X Vivo 15 medium (BioWhittaker, Walkersville, MD) supplemented with recombinant human granulocyte macrophage colony-stimulating factor (100 units/mL) and IL-4 (20 ng/mL) at 2×10^6 cells/mL in a 50 mL conical tube. G280-9V peptide (100 μg/mL) was added in solution with the dendritic cells in 37°C water for 2 hours followed by centrifugation at 1,200 rpm for 10 minutes at 20°C. In the initial dose only, tetanus toxoid (10 μg/mL; Pasteur-Merieux Connaught, Swiftwater, PA) was added to the cell suspension. The dendritic cell/peptide preparation was submitted for multiple quality assurance studies, including sterility and endotoxin before infusion. After washing twice at room temperature with PBS, the dendritic cell number for the patient's dose level was resuspended in 100 mL normal saline containing 5% human serum albumin. Cells were then irradiated (25 Gy) for subsequent infusion given within 60 minutes after final preparation.

The purpose of this trial was to determine the toxicities, maximal tolerated dose, and clinical and immunologic effects of the peptide-pulsed dendritic cells. A dose escalation design was used. Patients were consecutively assigned to three patient cohorts. Dose levels were 5×10^6, 15×10^6, and 50×10^6 cells per dose i.v. every 3 weeks for a total of six doses. Three patients were added at the maximal tolerated dose, which was the highest dose level.

Patients underwent clinical evaluation every 3 weeks. Immunologic analysis to evaluate peptide-specific immunity was done using PBMCs collected before initiation and each vaccination, and most patients underwent apheresis 4 to 8 weeks after study completion for PBMC collection. Toxicities and adverse effects were graded according to the National Cancer Institute Common Toxicity Scale (SR). Clinical response was assessed by measurement of assessable metastatic deposits by computerized tomography scan, magnetic resonance imaging scan, or direct measure of cutaneous deposits. The Response Evaluation Criteria in Solid Tumors Group system was used (19).

Peptides. HLA-A*0201-associated gp100: 280-288 (G280) peptide (YLEPGPVTA) with modification at the terminal anchor residue (position 9) called G280-9V (YLEPGPVT*V*) was used in this study. GMP-grade lyophilized peptide with >99% purity by high-performance liquid chromatography was purchased from Bachem AG (Bubendrof, Switzerland). The peptide for vaccination was dissolved in 5% DMSO in sterile water at 2 mg/mL followed by 0.2 μm filtration. The peptide was divided into aliquots, vialed for single use, and frozen at −80°C. The peptide was tested for sterility, biological activity by T2 target cell assay, and stability before pulsing dendritic cells (data not shown).

Immunologic monitoring

ELISPOT assay. ELISPOT was done against the immunogen G280-9V (the anchor modified), native G280 (the relevant target expressed on melanoma), and HIV-gag (SLYNTVATL) peptide (background control) in batch to minimize interassay variation. Briefly, 96-well plates (MAHAS4510, Millipore, Bedford, MA) were coated with 100 μL anti-IFN-γ antibody (10 μg/mL, 1-D1k, Mabtech, Mariemont, OH) overnight at 4°C. After six washings with PBS, the plates were blocked with 150 μL/well RPMI 1640 with 5% human AB serum (Valley Biomedical, Inc., Winchester, VA) at 37°C for 2 hours. After removal of the blocking medium, thawed PBMCs were incubated at 2×10^5 PBMCs per well in triplicate in a total of 100 μL RPMI 1640 with 5% human AB serum with each of the peptides G280 and G280-9V at 20 μg/mL for 24 hours. Plates were washed thrice with 0.01% Tween 20 in PBS. Next, biotinylated anti-IFN-γ antibody (100 μL/well; 1 μg/mL, 7-B6-1, Mabtech) in PBS with 0.5% bovine serum albumin (Fisher Biotech, Fair Lawn, NJ) was added. After incubation at room temperature for 2 hours, plates were washed thrice with each of 0.01% Tween 20 in PBS and PBS alone. Next, streptavidin-APL (100 μL/well; 1:1,000 dilution in PBS) was added and incubated at room temperature for 1 hour. After four washings with PBS, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate solution (Bio-Rad, Hercules, CA) was added. The plates were incubated at room temperature in the dark to develop. The colorimetric reaction was then stopped by washing with running water. Plates were allowed to dry, and the number of spots was read on an ELISPOT reader (Autoimmune Diagnostika GmbH, Strassbery, Germany). Values are corrected by subtracting background spots caused by PBMC incubation with control peptide HIV-gag. Phytohemagglutinin-positive controls were employed and in all cases yielded >300 spots per well.

To define an ELISPOT response, we determined the mean number of spots at baseline for patients and the between-patient SD. A positive result was designated as an elevation of 2 SDs above baseline. Data from our laboratory (data not shown) have shown that in patients, using the average of duplicate values, the mean and SD were 1.9 ± 1.6 for the G209 peptide in 17 patients and 2.6 ± 2.7 for the G209-2M peptide in 15 patients in an assay using the same conditions as reported here. For this study (see Results), the mean and SD were 6 ± 6.8 spots for G280 and 4.4 ± 3.4 spots for G280-9V. Thus, a positive response to G280 was an increment of 14 spots over baseline. For G280-9V, a positive result was an increment of 7 spots over baseline.

Tetramer assay. PBMCs (1×10^6) were washed with 1% fetal bovine serum/PBS and followed by incubation with antigen-presenting cell–labeled G280:HLA-A*0201 tetramers and phycoerythrin-labeled anti-human CD8 antibody (Pharmingen, San Diego, CA) on ice for 30 minutes. In some experiments, G280-9V:HLA-A*0201 tetramers were used. After washing twice with 1% fetal bovine serum/PBS, cells were fixed by 2% paraformaldehyde in PBS and analyzed by a FACSCalibur with CellQuest (BD Biosciences, San Jose, CA). HIV-gag peptide:HLA-A*0201 tetramer (Beckman Coulter Immunomics, San Diego, CA) was used as background control. Data are presented as the percentage of $CD8^+$ cells positive for tetramer binding based on gating variables set with appropriate isotype control nonbinding antibodies. The optimal concentrations of CD8-phycoerythrin and antigen-presenting cell–labeled G280:HLA-A*0201 tetramers with temperature and time of reaction were determined in preliminary experiments.

The mean ± SD staining at baseline was 0.1 ± 0.04%. As for ELISPOT, a positive result was designated at 2 SDs above baseline or results greater than 0.18%.

Cytolytic assay. Purified $CD8^+$ T cells were obtained by negative selection (20). Autologous dendritic cells pulsed with 10 μg/mL peptide were added to purified $CD8^+$ cells at a 1:20 ratio and cultured in 48-well trays (Costar 3548, Cambridge, MA) in RPMI 1640 containing 10% autologous plasma. IL-2 (50 units/mL, Chiron, Emeryville, CA) was added to each well beginning on day 1 and every 2 to 3 days thereafter. T cells were harvested on day 7 and assayed for lytic activity in triplicate using a standard 4-hour ^{51}Cr release assay. T2 (TAP1-deficient, $A*0201^+$) cells, DM6 ($A*0201^+$ and $gp100^+$), DM13 ($A*0201^+$ and $gp100^+$), and A375 ($A*0201^+$ and $gp100^-$) cells were used as target cells in lysis assays as described.

Statistical methods. The Kaplan-Meier plot was used to present the survival curve of the 12 patients. The Kruskal-Wallis test was used to test differences in the three dose groups with respect to the three immunologic end points. The immunologic end points were defined as the difference between prevaccine and postvaccine readings. In addition, univariate analysis of the signed rank test on the three immunologic end points was done.

Results

Patient characteristics. From October 1998 to April 2001, we enrolled 12 patients evaluable for clinical and immunologic responses. Fourteen additional patients were deemed ineligible as a consequence of being typed $HLA\text{-}A2^-$. Two patients enrolled had unresectable recurrent stage III melanoma and 10 patients had stage IV disease (see Table 1). Five patients had prior adjuvant IFN, whereas two patients had received prior chemotherapy for metastatic melanoma. Two patients had prior brain metastases controlled with radiation therapy and were deemed stable before study entry.

Feasibility. The production of dendritic cells for therapeutic administration was feasible and reproducible. The mean yield of dendritic cells from initial PBMC was 6.77% (range, 2.62-17.75%). No dendritic cell lot failed release criteria. Dendritic cell phenotypes as determined by two-color flow cytometry are listed in Table 2.

Safety. The overall toxicities of the G280-9V-pulsed dendritic cell vaccine were mild. Five patients reported rashes during the study. Two of these were grade 1 eruptions possibly related to treatment. Two of them were grade 1 and related to contrast administration. One was grade 2 contact dermatitis and unrelated to treatment. Four patients reported upper respiratory or flu-like symptoms; all were grade 1. There were three reports of constitutional symptoms, nausea or vomiting. All were grade 1 in intensity. Generally, the vaccine was well tolerated, and no serious adverse events related to therapy were reported.

Efficacy. Using Response Evaluation Criteria in Solid Tumors, patients were evaluated by computed tomography imaging after three vaccinations and again after six vaccinations. Two patients (103 and 302) experienced a partial response after six vaccination cycles. One of these patients (103) had tumor in multiple soft-tissue sites and had already experienced a prior complete response to whole-brain radiation therapy as well as a partial response to prior chemotherapy, whereas the other patient (302) had disease confined to the skin of the scalp that responded after treatment. Three patients (101, 102, and 304) had stable disease after completion of the study. Patient 304 experienced stable disease of a solitary (biopsy-proven) liver metastasis after study but went on to show a near-complete tumor response with additional follow-up in the absence of further treatment and remains alive with disease. Patient 101 had stable disease but ultimately experienced disease progression of pulmonary nodules 11 months after completion of therapy. Patient 102 had stage IV disease with extensive i.p. adenopathy but is currently alive with disease after additional treatment with systemic biochemotherapy and i.p. chemotherapy for mesenteric disease.

The remaining seven patients had documented disease progression. Interestingly, patient 203 had extensive in-transit stage III disease at entry and had a vigorous CTL response as measured by ELISPOT, tetramer, and lysis assays and progressed to stage IV disease involving distant skin. After further investigational therapy with a farnesyl transferase inhibitor (no response) followed by isolated limb perfusion (complete response), the patient is disease free. Remarkably, the latter procedure prompted regression of the regional metastases as well as a distant metastatic lesion on the contralateral (nonperfused) limb.

The median follow-up of the entire cohort is 43.8 months and the median survival of the entire population is 37.6 months. The median survival of the 10 stage IV patients is 21.1 months. Three patients are still alive; two of them having undergone additional treatments as described above. Figure 1 illustrates the overall survival in a Kaplan-Meier plot.

Immunologic response. The ELISPOT (IFN-γ) assay was used to measure the frequency of cells reactive to the native G280 peptide as well as the anchor-modified peptide G280-9V used for immunization. Baseline (preimmunization) values were calculated, and the mean and SD for the population were determined. Positive values were those >2 SDs above the pretreatment mean. As shown in Fig. 2A, 8 (67%) patients responded to G280 immunization with a significant increase in ELISPOT number. Figure 2B illustrates the response to the immunizing peptide, G280-9V; 8 (67%) patients responded to the modified peptide. Two patients (202 and 305) show no detectable immunity to either peptide. Two additional patients (103 and 302) mounted weak yet significant responses to G280-9V without significant responses to the native peptide.

To evaluate the frequency of cells specific for the G280:HLA-A*0201 complex, tetramer analysis was done on PBMC from

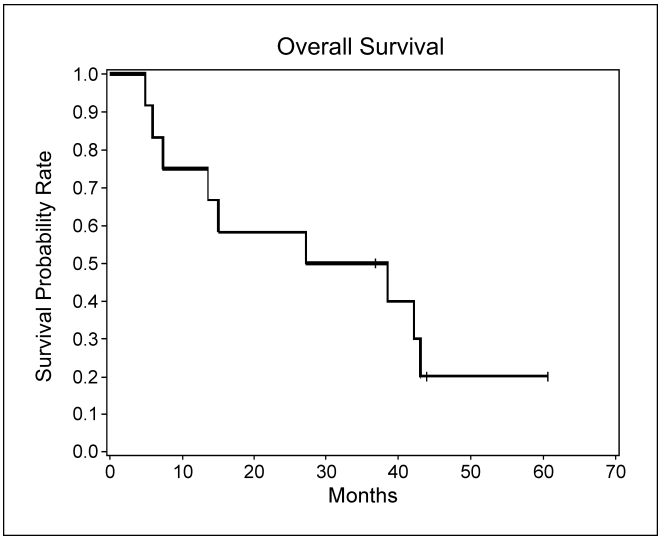

Fig. 1. Kaplan-Meier plot of survival of study population.

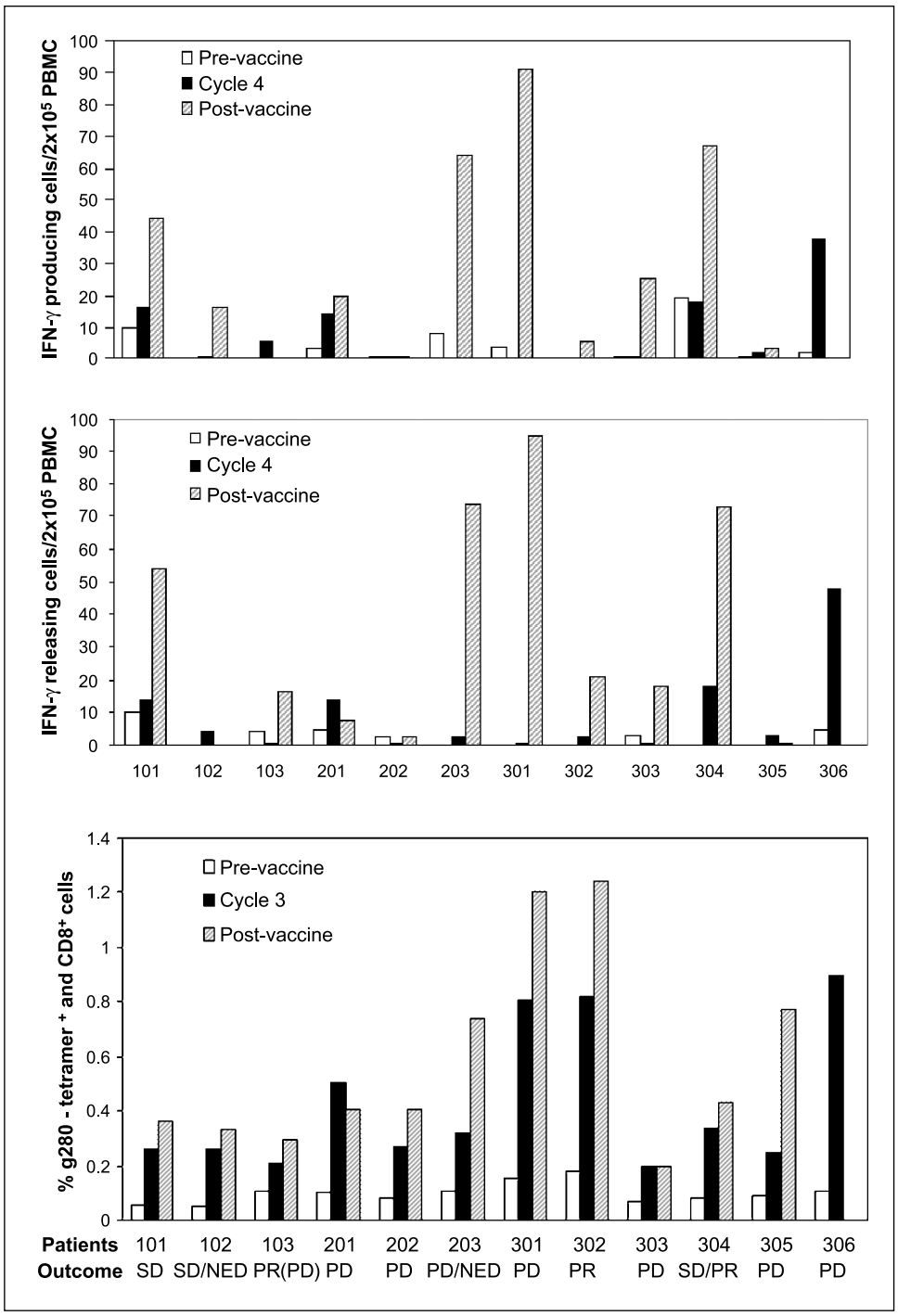

Fig. 2. Immunologic monitoring by ELISPOT analysis. Mean number of spots per 2×10^5 PBMCs (triplicate determinations) in an IFN-γ assay at baseline (*Pre-vaccine*), after the fourth vaccination (*Cycle 4*), or after the sixth vaccination (*Post-vaccine*). Background spots (after incubation with HIV-gag peptide) have been subtracted from the number of spots. *A*, ELISPOT to native peptide G280. *B*, ELISPOT to immunogen peptide G280-9V. The postvaccination sample for 306 was not obtained. *C*, immunologic monitoring by tetramer analysis. Percentage of G280:HLA-A*0201 tetramer$^+$/CD8$^+$ cells at baseline (*Pre-vaccine*), after the third vaccination (*Cycle 3*), and after the sixth vaccination (*Post-vaccine*). The postvaccination sample for 306 was not obtained. The clinical outcome is presented at the bottom and described as in Table 1.

12 patients after three vaccinations and after the final (sixth) vaccination. Baseline values were determined as above. As shown in Fig. 2C, all 12 immunized patients showed significant increases in CD8$^+$ cells specific for G280:HLA-A*0201 3 weeks after the third and sixth immunizations. Peripheral blood samples from patient 306 were tested at baseline and after the third immunization only.

Standard ^{51}Cr release assays were done and repeated at least once to measure the lytic activity of CD8$^+$ effector T cells from blood samples obtained at baseline and after study completion. Purified CD3$^+$CD8$^+$ cells from peripheral blood were cultured for 7 days with G280-9V-pulsed dendritic cells and tested using T2 target cells or allogeneic melanoma cell lines. All nine patients studied (304, 305, and 306 were not evaluated due to insufficient PBMC) had measurable immunity induced by dendritic cell immunization. In Fig. 3A, CD8$^+$ T cells from each donor were tested from prevaccination and postvaccination blood samples using T2 cells pulsed with the G280-9V peptide and all nine patients had detectable response. In parallel cultures, all nine patients

had detectable immunity against G280 peptide-pulsed T2 cells elicited by dendritic cell immunization (Fig. 3B). Moreover, dendritic cell vaccination elicited CTL that lysed DM6 melanoma coexpressing gp100 and HLA*0201 (Fig. 3C). As a control, $CD8^+$ T cells were cocultured with influenza A matrix (M1) peptide and tested against T2 cells pulsed with the M1 peptide. Both prevaccination and postvaccination blood samples contained influenza A–specific CTL, suggesting that all patients were immunocompetent at study enrollment and study conclusion based on reactivity to M1 peptide (Fig. 3D). Cultured $CD8^+$ T cells from all donors were tested for natural killer cell activity against K562 target cells and were nonreactive (data not shown).

Patients 103 and 203 were studied in greater detail to confirm the above findings and prove antigen specificity. In separate experiments, $CD8^+$ T cells from both donors were stimulated *in vitro* for 7 days with G280-9V-pulsed dendritic cells and tested at various E:Ts using either T2 target cells or additional melanoma cell lines. Postvaccination CTL from each donor lysed T2 target cells pulsed with the G280 native peptide as well as the G280-9V-pulsed targets (data not shown). In a second test of specificity, both donor CTL efficiently lysed DM6 and DM13 ($gp100^+$, $A*0201^+$) melanomas; however, the A375 ($gp100^-$, $A*0201^+$) melanoma target is not recognized supporting the requirement for naturally processed gp100 antigen. Postvaccination CTL from both donors can effectively kill G280-pulsed A375 melanoma cells showing reconstitution with exogenous antigen. As a control, prevaccination and postvaccination $CD8^+$ T cells specific for influenza A M1 peptide are detected from each patient.

As a final test of antigen specificity, $CD8^+$ T cells from 12-day *in vitro* cultures from postvaccination blood samples are evaluated by tetramer analysis with flow cytometry. At study completion, patient 103 had 0.65% tetramer/CD8 double-positive PBMC, whereas 203 had 0.82% tetramer/CD8 double-positive PBMC (data not shown). After 12 days of *in vitro* culture with G280-9V-pulsed dendritic cells, the expansion of peptide-specific $CD8^+$ T cells was significant with detection of 64% $tetramer^+CD8^+$ cells seen in donor 103 (Fig. 4B) and 88% $tetramer^+CD8^+$ cells seen in donor 203 (Fig. 4D). CTL from these cultures efficiently killed DM6, DM13, and G280-pulsed A375 melanomas as confirmation of specificity as shown for both donors (Fig. 4A and C).

In an effort to evaluate antigen receptor avidity, T2 target cells were pulsed with 10-fold dilutions (range, 10 fmol/L-100 nmol/L) of G280, G280-9V, or M1 peptide before addition of effector $CD8^+$ cells from postvaccination samples. We calculated the peptide concentration sufficient to render the target cells susceptible to lysis and express this value as 50% half-maximal lysis. A representative experiment is shown with donor 203 using effector T cells obtained by 12-day *in vitro* culture (Fig. 4E). The 50% half-maximal lysis was calculated as 12.53 pmol/L for G280-9V and 14.32 pmol/L for G280. The flu M1 peptide was not recognized at any concentration tested. A similar analysis was conducted using postvaccination blood from donor 103 and similar results were obtained which support the finding of high-avidity CTL after dendritic cell immunization. We conclude that dendritic cell vaccination of melanoma patients with G280-9V elicits CTL that fully cross-react and retain specificity for the native peptide sequence naturally presented by $A*0201^+$ melanomas.

The Kruskal-Willis test did not show significant differences between the vaccine dose groups with respect to the three immunologic end points. Because the dose groups did not differ significantly, the effect of the vaccine dose on the end points was assumed to be homogeneous. Next, the signed rank test (one sided *P*s) was used to evaluate the presence of an effect of the vaccine on the induction of immunity as measured by ELISPOT ($P = 0.003$), tetramer ($P = 0.005$), and lysis ($P = 0.0005$) assays. These data suggest that an increase in the magnitude of the response seen in each assay is due to the vaccine; however, the responses are not affected by the vaccine dose administered.

Discussion

We postulated that immunization with dendritic cells is effectively required to elicit antigen-reactive T cells capable of eradicating melanoma. A phase I dose escalation clinical trial was designed to test the hypothesis that immunization with G280-9V-pulsed dendritic cells can effectively elicit tumor-reactive CTL in patients with advanced melanoma. Results from this study allow us to conclude that peptide-specific $CD8^+$ T cells can be elicited through repeated dendritic cell immunization with a modified peptide designed for improved immunogenicity. Single amino acid substitution of neutral

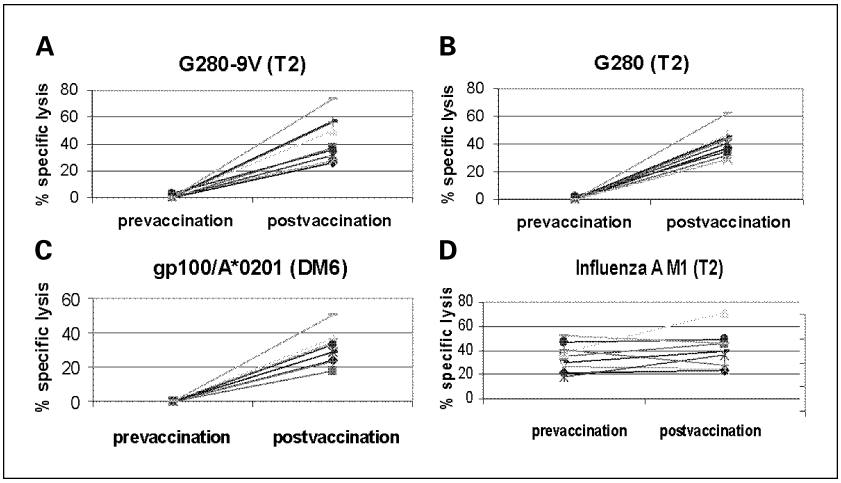

Fig. 3. Immunologic monitoring by ^{51}Cr lysis assay. $CD8^+$ T cells from either prevaccination (baseline) or postvaccination (sixth vaccination) blood samples were activated *in vitro* for 7 days with G280-9V-pulsed dendritic cells and tested in 4-hour ^{51}Cr release assays using T2 cells pulsed with either G280-9V (*A*), G280 (*B*), or DM6 melanoma (*C*). Influenza A M1 peptide-stimulated $CD8^+$ T cells were tested against T2 cells loaded with the M1 peptide as a positive control (*D*). E:T was 10:1 for all patients. All nine patients tested were deemed immunocompetent at baseline and at study conclusion based on reactivity to M1 peptide. All nine patients (101-303) responded to dendritic cell vaccination as judged by significant increases in lytic activity against G280-9V, G280, and DM6 ($gp100^+/A*0201^+$) melanoma. A375 ($gp100^-/A*0201^+$) melanoma was not killed by $CD8^+$ T cells from any patient (data not shown). This experiment was repeated at least once for all nine patients using prevaccination and postvaccination blood samples.

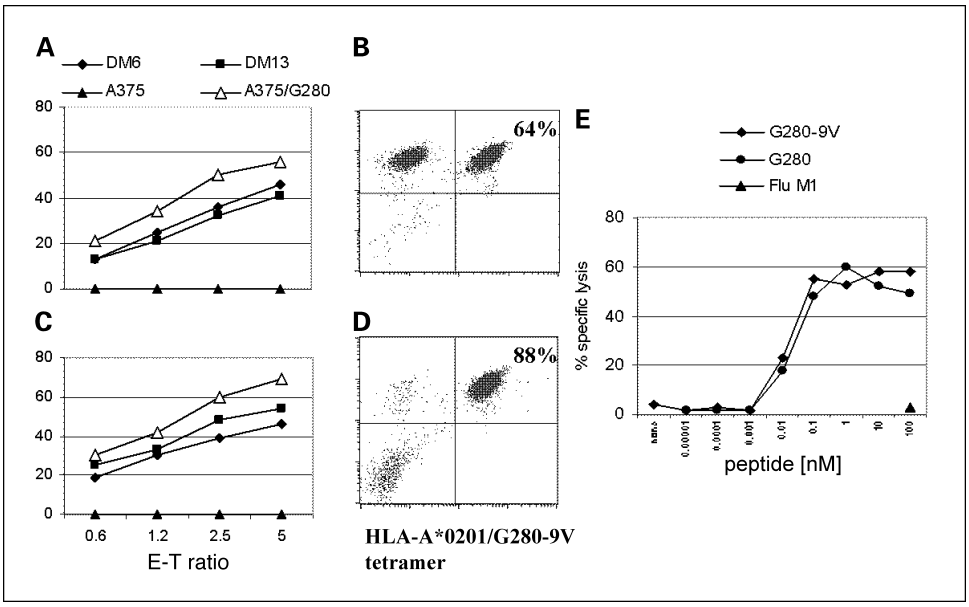

Fig. 4. Vaccine-induced peptide-specific $CD8^+$ T cells bind G280-9V:HLA-A*0201 tetramers and are high-avidity CTL. Postvaccination $CD8^+$ T cells from patients 103 and 203 were stimulated *in vitro* with G280-9V-pulsed dendritic cells for 12 days and analyzed by tetramer analysis. The percent positive $CD8^+$ cells that bind tetramer is indicated in the top right quadrant for 103 (*B*) and 203 (*D*). Effector CTL were tested in ^{51}Cr release assays using allogeneic melanoma cell lines: DM6 and DM13 ($gp100^+/A*0201^+$) and A375 ($gp100^-/A*0201^+$; *A*, 103; *C*, 203). Postvaccination CTL fail to recognize allogeneic CTL that are $A*0201^-$, such as DM14 (data not shown). *E*, 203 CTL were tested against T2 cells pulsed with 10-fold dilutions of G280-9V and G280 peptide in a standard 4-hour assay at E:T 2:1. The calculated 50% half-maximal lysis are 12.53 pmol/L for G280-9V/T2 and 14.32 pmol/L for G280/T2. M1/T2 target cells were not lysed at any peptide concentration tested. This experiment was repeated thrice with 203 postvaccination CTL with similar results.

(or detrimental) residues that influence binding to class I molecules was proposed as one approach to modify peptides that would result in improved immunogenicity (21). We choose to focus on the G280 epitope because this was initially described as a naturally processed 9–amino acid peptide eluted from HLA-A*0201 molecules from the DM6 melanoma and encoded by the gp100/pMel17 antigen (22). Tumor-infiltrating lymphocytes harvested from various melanoma patients clearly recognized the G280 epitope. Binding affinity experiments later suggested that G280 has relatively low affinity for HLA-A*0201 (20, 23). Clinical vaccination trials using the native G280 peptide confirm the poor immunogenicity of the native epitope in patients with melanoma (24). Parkhurst et al. (25) provided evidence that modification of gp100 peptides results in improved immunogenicity. Subsequent vaccine trials evaluating the G209-2M epitope supports the notion that anchor-modified peptides can serve as potent immunogens when coadministered with strong adjuvants (26, 27).

Dendritic cells are widely regarded as essential antigen-presenting cells that serve to initiate immunity; however, the optimal design for dendritic cell–based vaccine strategies remains yet defined (28). Emerging evidence supports the concept that maturation of dendritic cells serves as an important control point for immunity and ultimately dictates the outcome after encountering antigen (9). For example, current evidence from model systems supports the notion that immature dendritic cells are tolerogenic, whereas mature dendritic cells are immunogenic (29). In our study, dendritic cells best characterized as immature based on the phenotype ($CD80^{low}$, $CD83^{low}$, $CD86^+$, HLA class I^+, HLA class II intermediate) were used for the vaccination protocol. We provide evidence that melanoma-reactive $CD8^+$ T cells are easily measured in all patients after repeated immunizations with dendritic cells grown under the described conditions. Primate studies provide some evidence for maturation of immature dendritic cells when readministered after 7 days of *ex vivo* culture with granulocyte macrophage colony-stimulating factor and IL-4 (30). Moreover, successful immunization using immature dendritic cells with detection of SIV-specific CTL has shown that not all immunization strategies using immature dendritic cells leads to tolerance (31). By the methods and schedule described, we show that immunization with immature dendritic cells charged with a modified peptide can elicit tumor-specific CTL, but controlled studies are necessary to quantitatively assess potency relative to mature dendritic cells. It is important to note that fresh dendritic cells were generated *in vitro* from cryopreserved adherent PBMC for each dose and that strict quality-control measures were employed to ensure immunization with pure dendritic cell populations for each dose. We elected to irradiate all dendritic cell products before reinfusion based on a preclinical study showing improved dendritic cell vaccine efficacy in a murine model (32); at this time, the effects of irradiation on human dendritic cell maturation and possible effects on vaccine potency remain unclear. Merrick et al. (28) found that (*a*) irradiation did not change the surface phenotype (MHC class I and II,CD1a,CD80,CD83, CD40,and CCR7) of human PBMC-derived immature dendritic cells, although increased CD86 was seen at high dose (30 Gy); (*b*) irradiated immature dendritic cells mostly maintain their endocytic, phagocytic, and migratory functions; and (*c*) irradiation did not increase expression of RelB in immature dendritic cells, a marker of dendritic cell maturation. The significance of this finding is unclear.

Recent studies suggest that CTL avidity is an important factor influencing recognition of malignant and virally infected cells. Alexander-Miller et al. proposed that avidity is defined as the sensitivity to low peptide-MHC complex determinant density on target cells and is useful in characterizing the quality of CTL from immunized patients (33, 34). We evaluated avidity as lysis of allogeneic melanoma targets and carefully determined the 50% half-maximal lysis. In repeated experiments with postvaccination blood samples from this patient cohort, we consistently obtain 50% half-maximal lysis values of <50 pmol/L for G280 peptide.

We show here that G280-9V, when delivered on autologous dendritic cells, is uniformly immunogenic. When immunogenicity is measured by ELISPOT, 67% of patients at each dose level develop significantly increased levels of circulating IFN-γ

secreting $CD8^+$ T cells. Up to 25-fold levels of increase were observed. Fluorescence-activated cell sorting analysis, with tetramers of circulating levels of peptide-specific $CD8^+$ T cells, showed significant responses to vaccination in all patients. Two patients at the highest dose level exhibited >1% circulating $CD8^+$ T cells specific for the native G280. Finally, all nine patients tested had measurable high-avidity CTL that lysed allogeneic melanoma cell lines that coexpressed A*0201 and gp100.

Recently, Cerundolo et al. (35) reviewed progress in the dendritic cell therapy field. They chose as the standard for dendritic cell trials those using characterized peptides and ELISPOT or tetramer assays for measurements of T-cell frequencies in the blood. They compared these with trials done using peptide alone or recombinant viruses alone. There is a very wide variation in T-cell responses. Response to gp100 (209-217M) was 28% in the single trial they analyzed; other melanoma antigen dendritic cell peptide trials report responses ranging from 22% to 73%. The results reported here compare favorably with other studies.

We conclude that dendritic cell–based vaccination with modified class I restricted G280-9V peptide uniformly elicits antitumor immunity and offers the prospect of clinical benefit for some patients (35).

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