

A Phase I Trial of Tumor Lysate-pulsed Dendritic Cells in the Treatment of Advanced Cancer¹

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ABSTRACT

Purpose: The objectives of this study were to assess the toxicity and immunological response induced by the intradermal (i.d.) administration of tumor lysate-pulsed dendritic cells (DCs).

Experimental Design: Patients with stage IV solid malignancies were treated in cohorts that received 10⁶, 10⁷, and 10⁸ DCs i.d. every 2 weeks for three vaccines. Each vaccine was composed of a mixture of half DCs pulsed with autologous tumor lysate and the other half with keyhole limpet hemocyanin (KLH). Peripheral blood mononuclear cells (PBMCs) harvested 1 month after the last immunization was compared with pretreatment PBMCs for immunological response. Delayed-type hypersensitivity reactivity to tumor antigen and KLH was also assessed.

Results: Fourteen patients received all three vaccines and were evaluable for toxicity and/or immunological monitoring. There were no grade 3 or 4 toxicities associated with the vaccines or major evidence of autoimmunity. Local accumulation of CD4⁺ and CD8⁺ T cells were found at the vaccination sites. There was a significant proliferative response of PBMCs to KLH induced by the vaccine. In 5 of 6 patients, the vaccine resulted in increased IFN- γ production by PBMCs to KLH in an ELISPOT assay. Using the same assay, 3 of 7 patients' PBMCs displayed increased IFN- γ production in response to autologous tumor lysate. One patient with melanoma also was observed to have an increased frequency of MART-1- and gp100-reactive CD8⁺ T

cells after vaccination. By delayed-type hypersensitivity testing, 8 of 9 and 4 of 10 patients demonstrated reactivity to KLH and autologous tumor, respectively. Two patients with melanoma experienced a partial and a minor response, respectively.

Conclusion: The administration of tumor lysate-pulsed DCs is nontoxic and capable of inducing immunological response to tumor antigen. Additional studies are necessary to improve tumor rejection responses.

INTRODUCTION

DCs³ are highly potent antigen-presenting cells of bone marrow origin that are integral in the stimulation of primary and secondary T- and B-cell responses. Preclinical studies have demonstrated that DCs are preferentially responsible for the sensitization of naive T cells in their first exposure to antigen (1, 2). The ability to generate DCs *ex vivo* along with pulsing with antigen has enabled investigators to generate reagents that can be used for immunization purposes. An important advantage of using DCs as a mode of tumor vaccination is its ability to induce immunity to poorly immunogenic tumors (*i.e.*, human malignancies), thus potentially bypassing inherent deficiencies in antigen presentation within the host (3).

Our preclinical models demonstrated that tumor lysate-pulsed DCs were capable of mediating the regression of micro-metastatic disease upon s.c. administration (4). The antitumor response by tumor lysate-pulsed DCs comprised both CD4⁺ and CD8⁺ T cells. One advantage of using tumor lysates to pulse DCs is the multiple antigens that are included in such a preparation. Use of a tumor lysate may allow for the sensitization of T cells to a variety of antigens that may be heterogeneously expressed on growing tumors and is particularly relevant to those human cancers which to date do not have molecularly defined tumor-associated antigens.

On the basis of our preclinical animal studies, we have now completed a Phase I clinical trial of the intradermal administration of autologous tumor lysate-pulsed DCs in patients with advanced solid malignancies. Cohorts of patients received 10⁶, 10⁷, and 10⁸ DCs pulsed with tumor lysate and KLH every 2 weeks for a total of three injections. Besides toxicity, secondary end points included immune monitoring of PBMCs for reactivity to autologous tumor and KLH.

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³ The abbreviations used are: DC, dendritic cell; KLH, keyhole limpet hemocyanin; PBMC, peripheral blood mononuclear cell; DTH, delayed-type hypersensitivity; i.d., intradermally; GM-CSF, granulocyte/macrophage-colony stimulating factor; IL, interleukin; PE, phycoerythrin; FACS, fluorescence-activated cell sorter; mAb, monoclonal antibody; ELISPOT, enzyme-linked immunospot; TSH, thyroid-stimulating hormone; ANA, anti-nuclear antibody; SI, stimulation index.

PATIENTS AND METHODS

Patients and Study Design

Patient Selection. Patients with stage IV solid malignancies were eligible if they had a performance status of Eastern Cooperative Oncology Group 0 or 1 and had measurable or evaluable disease. Additional eligibility requirements consisted of absence of brain metastases by computed tomography or magnetic resonance imaging and absence of anergy to common recall antigens. Anergy was initially assessed with the Multitest CMI kit (Merieux Institute, Inc., Miami, FL), which measures DTH reactivity to seven common recall antigens. However, midway during the trial, these kits were no longer manufactured, and an alternate method of DTH testing to 4 antigens (*Candida albicans*, mumps, PPD tuberculin, and tetanus toxoid) was adopted. Reactivity to any one of these recall antigens (induration ≥ 5 mm) was considered evidence for lack of anergy.

Study Design. The primary end points for the Phase I study was to assess feasibility and toxicity of the DC vaccination regimen. Secondary end points were to assess immune responses induced by the vaccination procedure to the marker antigen, KLH, and to autologous tumor. There were three cohorts of patients who received 10^6 , 10^7 , and 10^8 DCs pulsed with KLH and autologous tumor lysate. Each cohort of patients received the DCs i.d. near an inguinal or axillary nodal region deemed clinically free of disease. A total of three vaccinations were administered at intervals of 2 weeks in the outpatient clinic of the General Clinical Research Center. One month after the third immunization, patients underwent leukapheresis for immune monitoring as well as radiological assessment of tumor status. If the tumor status was deemed to be stable or responding to therapy, an additional three vaccines were given as before. Conventional criteria for tumor response was used. A partial response was $\geq 50\%$ decrease in the perpendicular diameter of all measurable lesions without progression of evaluable disease. Progressive disease was $\geq 25\%$ increase in the perpendicular diameter of any measurable disease, appearance of a new lesion, or worsening of evaluable disease. This protocol was approved by the University of Michigan Institutional Review Board (IRB 97-064; UMCC 9702) and by the Food and Drug Administration under IND BB6958. Before protocol entry, each subject provided written informed consent.

Vaccine Toxicity Grading System

For systemic toxicities, the National Cancer Institute Common Toxicity scale was used. For local vaccine toxicities, the following scale was used: grade I, erythema and induration < 20 mm; grade II, erythema and induration ≥ 20 mm without ulceration; grade III, ulceration or painful adenopathy; and grade IV, permanent dysfunction related to local toxicity.

Tumor Cell Harvest and Cryopreservation

Tumors were harvested surgically for palliative or curative intent and cryopreserved. Tumors were kept sterile on ice and transported from the operating room to the laboratory. A single cell suspension was made by a combination of mechanical and enzyme dispersion techniques as described previously (5). The tumor cells were cryopreserved in 90% human AB serum plus

10% DMSO at -178°C in a liquid nitrogen freezer. Each freezing vial contained $2-4 \times 10^7$ tumor cells.

Leukapheresis and Cryopreservation of PBMCs

In accordance with the University of Michigan Blood Bank guidelines, protocol patients underwent a 4-h leukapheresis on a COBE spectrum apheresis system to ensure adequate numbers of PBMCs for DC culture and for immune monitoring. PBMCs were obtained by taking the apheresis product, diluting it 4-fold in HBSS (Life Technologies, Inc., Grand Island, NY), and overlaying it on Ficoll-Hypaque gradients. The cells were then centrifuged at $900 \times g$ for 30 min at room temperature. The interface representing the PBMCs were then collected and washed in HBSS twice to reduce platelets. Aliquots of PBMCs were then cryopreserved in 70% human AB serum 20% X-VIVO 15 and 10% DMSO for future use in cryopreservation bags (Baxter Corp., Deerfield, IL) or cryovials.

Vaccination Preparation

DC cultures and antigen pulsing were performed in the Human Applications Laboratory of the General Clinical Research Center, which is a facility that operates under good manufacturing procedures. The vaccine for each patient was prepared from the fresh leukapheresis sample. Subsequent vaccines were prepared from cryopreserved PBMCs obtained from the pretreatment leukapheresis. PBMCs were resuspended in serum-free X-VIVO 15 medium (BioWhittaker, Walkerville, MD) at 1×10^7 cells/ml for a total volume of 30 ml in 225-cm² flasks. The cells were allowed to adhere for 2 h at 37°C in 5% CO₂, and the nonadherent cells were removed after gentle rocking of the flasks and aspiration of the medium. Immediate replacement of 30 ml of X-VIVO 15 medium containing GM-CSF (100 $\mu\text{g/ml}$; Schering-Plough, Kenilworth, NJ) and IL-4 (50 $\mu\text{g/ml}$; Schering-Plough) was completed, and the cells were incubated for 6 days at 37°C , 5% CO₂ before pulsing with tumor lysate or KLH.

The adherent DCs were harvested from the flasks using 10 ml of EDTA (3 mM) for each flask and allowed to incubate for 10 min. The detached DCs were harvested, washed, and resuspended at 1×10^6 cells/ml in fresh X-VIVO 15 medium containing GM-CSF and IL-4. Ten ml of the cell suspension were placed in 75-cm² flasks (10^7 DCs/flask) for pulsating with tumor lysate or KLH. Single cell suspensions of tumor freshly removed or cultured short-term (< 2 weeks to ensure removal of contaminating enzymes used for disaggregation) were snap freeze-thawed three times in rapid succession, irradiated at 10,000 cGy, and stored in liquid nitrogen for later use. Tumor lysate suspension was added to DCs at 1:1 cell equivalent ratio. Specifically, a volume of tumor lysate equal to 10^7 tumor cells was added to each flask and incubated for 18 h at 37°C , 5% CO₂. KLH pulsation was performed in separate flasks of DCs. A volume of 300 μl of KLH stock solution diluted in PBS (50 $\mu\text{g/ml}$; Calbiochem, San Diego, CA) was added to each flask and incubated for 18 h.

After incubation, the tumor lysate-pulsed and KLH-pulsed DCs were harvested and counted. For each vaccine dose, 50% was composed of tumor lysate-pulsed DCs admixed with 50% of KLH-pulsed DCs. The DC suspension was adjusted to a total

volume of 0.5 ml of PBS. For the 10^8 DC dose, the cell suspension was divided into five separate syringes each containing 0.5 ml for intradermal administration.

Flow Cytometric Analysis of DCs

An aliquot of the vaccine preparation was retained to examine the expression of extracellular markers. The following monoclonal antibodies were purchased from Caltag Laboratories (Burlingame, CA): FITC-conjugated CD45, CD83, CD86, mouse IgG1, and mouse IgG2b; PE-conjugated CD14, mouse IgG1, and mouse IgG2a. PE-conjugated CD40 and HLA-DR were purchased from PharMingen (San Diego, CA). Vaccine cells were washed with FACS buffer (PBS + 2% fetal bovine serum, 0.1% sodium azide) and counted. One million cells in 100 μ l were added to culture tubes containing 1 μ g of each labeled antibody. Cells were incubated on ice for 40 min, washed two times with FACS buffer, and then suspended in PBS + 1% paraformaldehyde and stored at 4°C before FACS analysis.

Immune Monitoring of PBMCs

PBMCs were harvested pretreatment at the time of leukapheresis for DC generation and 1 month after the third vaccination when tumor response assessment was determined. All of the assays were done in batch on cryopreserved PBMCs. Cells were used soon after thawing. Cell viabilities ranged from 67 to 98% between patients, but for a given patient, viabilities between pre- and posttreatment samples were within 15%. The following assays were performed by either the Immune Monitoring Core of the University of Michigan Comprehensive Cancer Center or at Stanford University.

Proliferation Assay. Cryopreserved PBMCs were thawed, washed, and suspended in X-VIVO 15 medium supplemented with 1% human AB serum (BioWhittaker), 2 mM glutamine, 100 units of penicillin, 10 mg/ml streptomycin, and 50 μ M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). Viability was assessed by trypan blue exclusion, and cell concentrations were adjusted to 6×10^6 /ml. Cells were added to 96-well, round-bottomed plates (Falcon-BD, Franklin Lakes, NJ) in 100- μ l volumes and incubated in a final volume of 200 μ l with either medium alone, KLH (40 μ g/ml), or tumor lysate (prepared to deliver lysate at tumor cell equivalence) for a total of 6 days at 37°C, 5% CO₂. Phytohemagglutinin (Sigma Chemical Co.) was added to some of the wells as a positive control on day 3. The cultures were pulsed with 1 μ Ci/well of [³H]thymidine (ICN, Costa Mesa, CA) on day 5 and incubated overnight before harvest onto glass-fiber filter plates (Millipore, Bedford, MA). Data were collected on a TopCount NXT scintillation counter (Meriden, CT). A SI was calculated:

$$SI = \frac{\text{Avg. cpm of antigen-stimulated culture}}{\text{Avg. cpm of unstimulated culture}}$$

ELISPOT Assay. One day prior to assay, unfiltered plates (Whatman, Clifton, NJ) were prepared by adding 75 μ l/well of anti-IFN- γ monoclonal capture antibody (Pierce-Endogen, Rockford, IL) adjusted at 4 μ g/ml in sterile 0.1 M carbonate buffer (pH 9.5) and incubated at 4°C overnight. The day of assay, the plates were washed twice with sterile

PBS (Mediatech, Herndon, VA) and then blocked for ≥ 1 h with X-VIVO 15 medium with 10% AB serum. PBMCs were prepared as above and adjusted to 1×10^7 /ml. One hundred μ l of PBMCs were added to each well and incubated with antigen as above. Negative controls for the assay were unstimulated PBMCs. Background counts for these samples were quite low and were subtracted from the counts generated from stimulated cultures. Positive controls were stimulated with phytohemagglutinin. Cultures were incubated undisturbed at 37°C, 5% CO₂ for 24 h.

After 24 h, cells were removed, and the plates were washed three times with PBS and then three times with Tris-buffered saline (0.05 M, pH 7.5) TBS-Tween 20 (TBS-T). Biotinylated secondary antibody (Pierce-Endogen; 2 μ g/ml) in TBS-T plus 10% fetal bovine serum (Life Technologies, Inc.) was added, and the plates were incubated at room temperature for 2 h, followed by six TBS-T washes. Streptavidin-alkaline phosphatase (Sigma Chemical Co.) was added and incubated for 1 h at room temperature, followed by eight TBS-T washes. Plates were developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate substrate (Sigma Chemical Co.) for 15–45 min and then rinsed extensively with deionized water and allowed to dry. Quantitative analysis of positive spots was performed on an ImmunoSpot Series 1 Analyzer (Cellular Technology Ltd., Cleveland, OH).

MHC/Peptide Tetramer Analysis. Production of MHC/peptide tetramers was described in detail elsewhere (6). Plasmids encoding the A2-BSP fusion protein and human β 2-microglobulin were kind gifts of Dr. John Altman (Emory University, Atlanta, GA). These proteins were expressed in *Escherichia coli* and refolded *in vitro* with the specific peptide ligand. The properly folded MHC-peptide complexes were extensively purified using fast protein liquid chromatography and anion exchange and biotinylated on a single lysine within the BSP using the BirA enzyme (Avidity, Denver, CO). Tetramers were produced by mixing the biotinylated MHC-peptide complexes with either PE-conjugated avidin or APC-conjugated avidin (Molecular Probes) at a molar ratio of 4:1. Tetramers were validated by staining against a CTL line or clone specific for HLA-A2 in association with the peptide of interest. Specificity was demonstrated by the lack of staining of irrelevant CTLs. By titrating positive CTLs into PBMCs from normal controls, we established our limit of detection to be as low as 0.01% of CD8⁺ cells. Each tetramer reagent was titered individually and used at the optimum concentration, generally 10–50 μ g/ml.

Three-color FACS analyses were performed using a Becton Dickinson FACScan (Mountain View, CA). For phenotypic analysis of antigen-specific T-cell populations, 10-color FACS analysis was performed on a 12-parameter FACS instrument developed by the Stanford Shared FACS Facility. Data were compensated, analyzed, and presented using FlowJo (Tree Star, Inc., San Carlos, CA). Except as listed, all fluorescein- or PE-conjugated as well as unconjugated monoclonal antibodies were obtained from PharMingen (San Diego, CA). Anti-CD8-FITC, anti-CD4-Cy5PE, anti-CD13-Cy5PE, and anti-CD19-Cy5PE antibodies were purchased from Caltag (South San Francisco, CA).

Table 1 Patient characteristics, therapy, and response

Patient no.	Age	Sex	Diagnosis	Sites of disease	Prior systemic treatments	DC dose	Response
1	63	F	Melanoma	Retroperitoneal LN ^a	None	10 ⁶	Minor response after 3 vaccines, partial response after 3 more vaccines; CNS relapse after 8th vaccine
2	65	M	Melanoma	Liver, axillary, and retroperitoneal LNs	None	10 ⁶	Progressed
3	55	M	Melanoma	Abdominal LNs	None	10 ⁶	Progressed
4	40	F	Melanoma	Lung, liver, inguinal LNs, subcutis	IFN- α	10 ⁶	Progressed
5	66	M	Melanoma	Lung, adrenal, axillary LNs	None	10 ⁶	Progressed
6	50	M	Colorectal cancer	Liver	Chemotherapy	10 ⁷	Progressed
7	69	F	Melanoma	Lung, liver, subcutis	None	10 ⁷	Stable after 3 vaccines, progressed after 6th vaccine
8	73	M	Melanoma	Lung	None	10 ⁷	Progressed
9	41	M	Melanoma	Retroperitoneal and pelvic LNs	IFN- α	10 ⁷	Stable after 6 vaccines, minor response after 9th vaccine, progressed after 12th vaccine
10	40	M	Melanoma	Mesenteric LNs	IFN- α IL-2	10 ⁷	Progressed
11	47	M	Colorectal cancer	Liver, retroperitoneal LNs	Chemotherapy	10 ⁷	Stable after 3 vaccines, progressed after 6th vaccine
12	49	M	Melanoma	Lung, axillary LNs	IFN- α	10 ⁸	Progressed
13	66	F	Melanoma	Lung, breast	None	10 ⁸	Stable after 3 vaccines, progressed after 6th vaccine
14	36	F	Neuroblastoma	Lung, thoracoabdominal wall mass	Chemotherapy	10 ^{8b}	Stable after 3 vaccines, progressed after 6th vaccine

^a LN, lymph node; CNS, central nervous system.

^b First 3 vaccines were at 10⁷/dose; subsequent vaccines at 10⁸/dose.

DTH Testing

In addition to *in vitro* immune monitoring, we assessed patients for *in vivo* immune reactivity to KLH and autologous tumor by DTH testing. For KLH reactivity, patients were given intradermal inoculations of 2, 20, and 200 μ g of KLH in 0.2-ml volumes of PBS. Induration was measured 48 h later in two perpendicular diameters. For autologous tumor reactivity, patients were assessed before treatment and 1 month after treatment with irradiated autologous tumor cells (6,000 cGy) at 10⁴, 10⁵, and 10⁶ doses *i.d.* Induration was measured in a similar fashion as KLH. Positive DTH reactions were scored if the average perpendicular measurements exceeded 5 mm.

Light Microscopic and Immunohistochemical Analysis of Vaccine Sites

In select patients, vaccine sites and adjacent skin were excised 1 week after the third vaccine for histological analysis. Besides routine light microscopic assessment of formalin-fixed, paraffin-embedded sections of either tissue excised from the vaccine site or adjacent non-injected normal skin, stained with H&E, immunopathological examination was also performed. Serial sections of the paraffin blocks were cut onto glass slides and immunostained using a highly sensitive avidin-biotin immunoperoxidase technique with diaminobenzidine to produce a positive brown reaction product. For detection of some antigens, tissue sections were microwaved in PBS for antigen retrieval before addition of primary antibodies. The following antibodies were used to visualize DCs: LN3 mAb (Dako, Carpinteria, CA) was used to detect HLA-DR and rabbit antisera was used to

detect factor XIIIa (Calbiochem-Novabiochem, San Diego, CA). DCs were also distinguished by their morphology. Rabbit anti-S100 was used to detect melanoma cells (Dako) and anti-MAC387 mAb used to detect macrophages. Lymphocytes were detected by using an anti-CD3 pan T cell mAb, as well as anti-CD4 and anti-CD8 mAbs (Becton Dickinson, Mountain View, CA).

RESULTS

Patient Characteristics. A total of 14 patients received all three vaccinations and had adequate follow-up to monitor toxicity, immune response, or tumor response. An additional 10 patients were entered into the study who progressed and did not complete all of the protocol end points. The study required autologous tumor harvest, DC generation, three vaccinations, and a follow-up leukapheresis 1 month after the last vaccine. In general, these study requirements involved a period of 3.5–4 months. Among the 10 inevaluable patients, 1 patient with colorectal cancer did not receive any vaccines because of the onset of brain metastases after tumor harvest. The remaining patients (3 with breast tumors and 6 with melanoma) progressed during the course of therapy and became ineligible. The characteristics of the 14 evaluable patients are summarized in Table 1. There were 5, 6, and 3 patients in groups where 10⁶, 10⁷, and 10⁸ DCs were administered, respectively. The histological diagnoses included melanoma ($n = 11$), colorectal cancer ($n = 2$), and 1 patient with neuroblastoma. The median age of the patients was 53 years (range, 36–73 years).

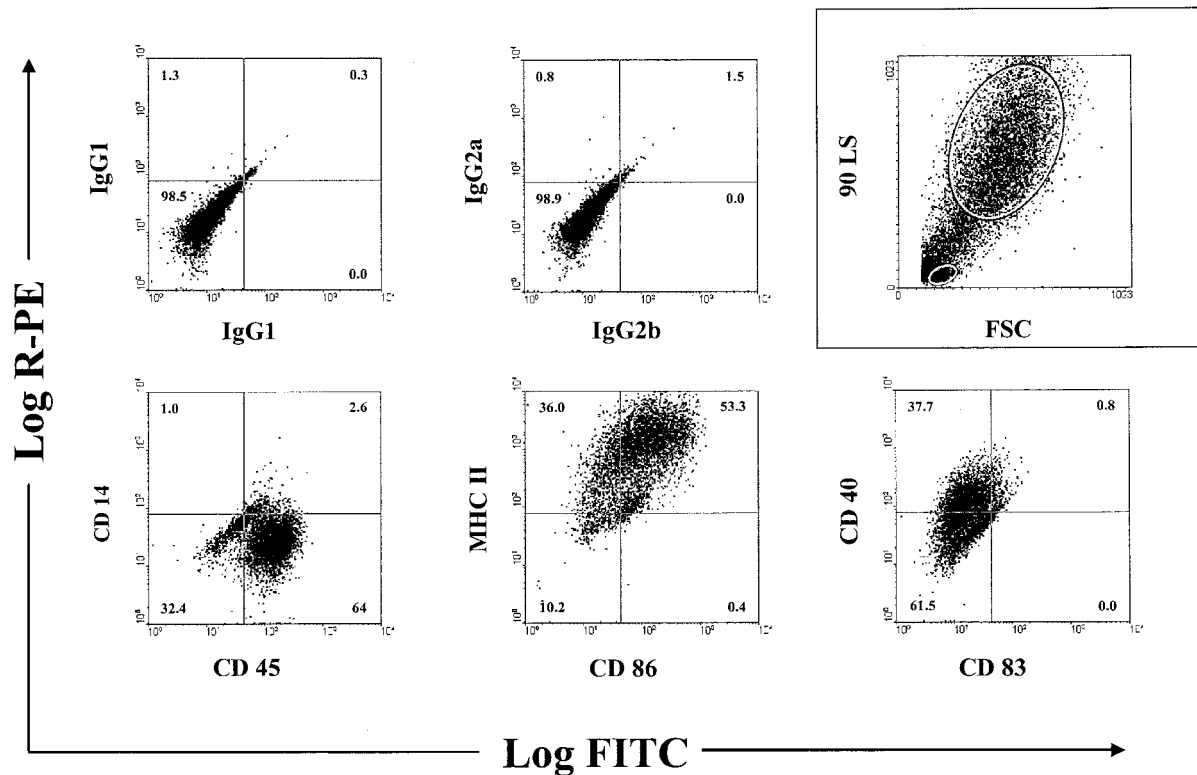


Fig. 1 Two-color FACS analysis of tumor lysate-pulsed DCs. Upper two left panels, background antibody staining controls. Upper right panel, scatterplot and gated areas (ovals) of cells evaluated. The DCs were positive for HLA-DR, CD86, CD40, and CD45. Markers for monocytes (CD14) and mature DC (CD83) were low.

DC Culture Results and Phenotype. PBMCs were cultured in serum-free medium containing IL-4 and GM-CSF for 6 days after plastic adherence and removal of nonadherent cells. At the end of that period, the yield of cells compared with the starting PBMC population was a mean (SE) of $4.5 \pm 0.6\%$ (range, 2.2–10.4%; $n = 17$ cultures). The cells were readjusted in cell numbers and subsequently pulsed with KLH or autologous tumor lysate. The yield of cells after antigen pulsation was a mean (SE) of $70 \pm 7.4\%$ (range, 27–100%; $n = 12$ cultures). FACS analysis of the DC phenotypic profile from a representative culture is illustrated in Fig. 1. Among 5 patients undergoing 15 DC cultures, the mean (SE) percentage of positively stained cells for various markers was: CD14, 18 ± 3 ; CD45, 59 ± 5 ; CD40, 12 ± 4 ; CD83, 17 ± 4 ; CD86, 68 ± 6 ; and HLA-DR, 62 ± 5 . The expression of CD83 was low, indicating the relative immature status of the DCs.

Toxicity. There were no significant toxicities associated with the vaccine administrations. Two of 5 patients who received a dose of 10^6 DCs experienced grade 1 local toxicity at the vaccine site. Grade 2 local toxicity was observed in 1 patient from each cohort that received 10^7 and 10^8 DCs, respectively. No patients experienced local tumor growth at the site of the vaccine injection.

A panel of autoimmune parameters was used before and 1 month after the completion of three vaccinations. Table 2 summarizes the results of the tests. Overall, there was no evidence of autoimmunity as assessed by these tests. One patient (no. 5)

Table 2 Autoimmune parameters after DC vaccination

DC dose	No. of patients with abnormal values/Total no. of patients ^a					
	RF	ANA	DNA binding	CH50	T4	TSH
10^6	0/4	0/4	0/4	0/4	0/4	1/4 ^b
10^7	0/2	1/2 ^c	0/2	0/2	0/2	0/2
10^8	0/3	0/3	0/3	0/3	0/3	0/3
Total	0/9	1/9	0/9	0/9	0/9	1/9

^a Autoimmune parameters consist of: rheumatoid factor (RF); ANA; DNA binding; total complement (CH50); total T4 or free T4; and TSH.

^b One patient with elevated TSH level (10.38 uu/ml; nl. range, 0.30–6.50 uu/ml).

^c One patient had a positive ANA with a titer of 1:80.

with the elevated TSH value after vaccination had an elevated TSH value before vaccination. One patient (no. 9) with a positive ANA after vaccination had a negative assessment before treatment; however, the posttreatment ANA level had a low titer (1:80). Interestingly, patient 10 experienced the onset of vitiligo after the vaccinations and demonstrated normal posttreatment autoimmune parameters and no evidence of melanoma regression.

Characterization of Vaccine Sites. In two different patients, the vaccine sites were excised after the last vaccination, and the intradermal reaction to the injection of lysate-pulsed

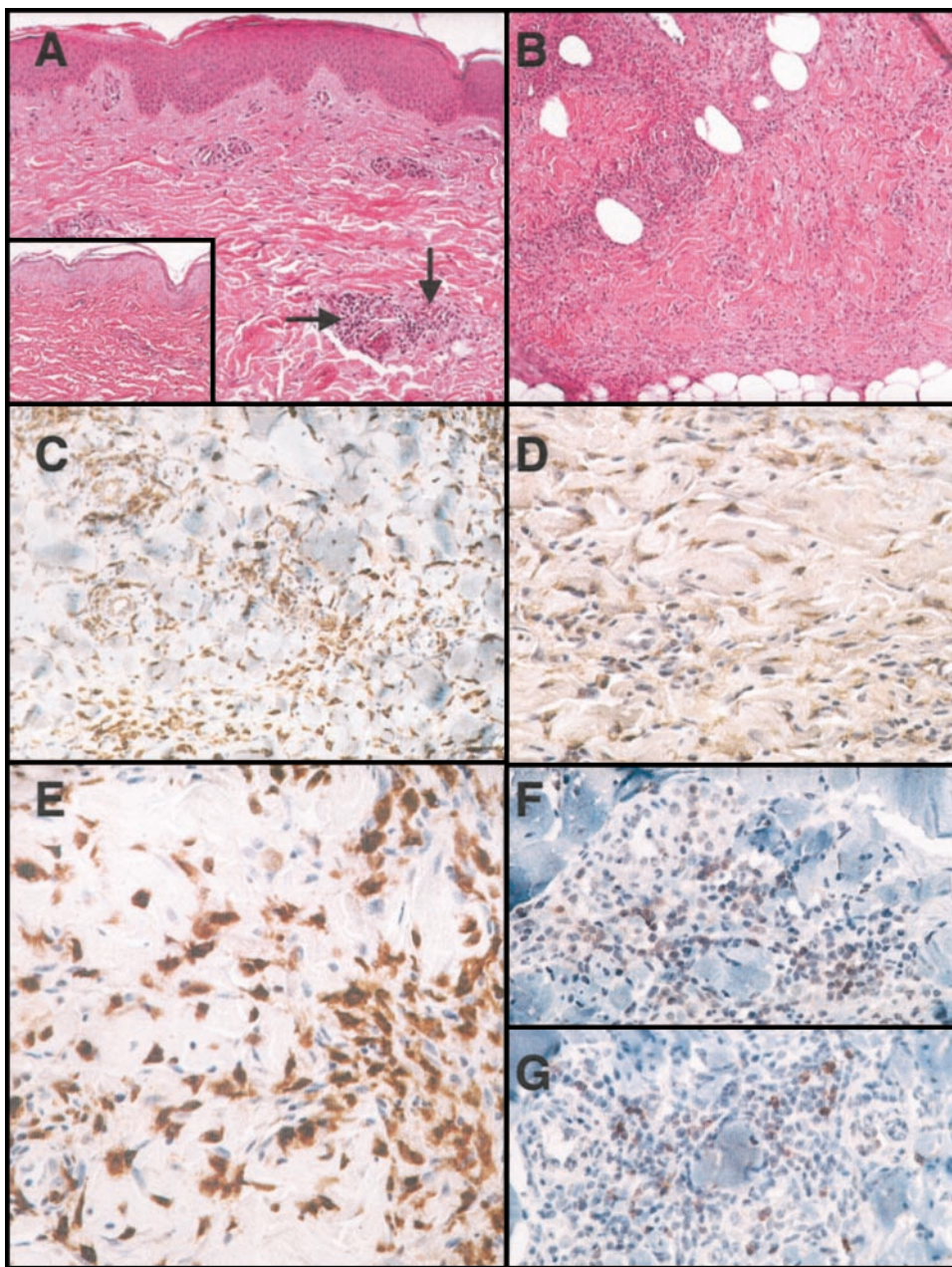


Fig. 2 Normal skin (*A*, *inset*) and skin vaccine site (*A–G*). Routine light microscopic appearance comparing normal skin with skin injected with tumor lysate-pulsed DCs reveals an unremarkable appearing epidermis but increased superficial perivascular lymphocytic infiltrate (*A*, *arrows*). In the deep dermis, the vaccine site contains an increased perivascular and interstitial mononuclear cell infiltrate (*B*). Immunophenotypic analysis of the vaccine site confirms increased interstitial density of DCs expressing HLA-DR encircling collagen bundles (*C*), which are also factor XIIIa positive (*D*), and MAC387 positive (*E*). The accompanying lymphoid infiltrate includes a predominance of CD4⁺ T cells (*F*) compared with CD8⁺ T cells (*G*).

DCs was examined microscopically. In addition, adjacent normal, non-injected skin was also biopsied from the same individual. To detect DCs in the dermis, a panel of antibodies were used based on our previous experience in detecting dermal DCs, which represent professional antigen-presenting cells (7, 8). Compared with normal skin (*Fig. 2A*, *inset*), both vaccine sites were characterized by an unremarkable appearing epidermis, but

routine light microscopy revealed a mild superficial perivascular lymphocytic infiltrate (*Fig. 2A*), accompanied by a more intense interstitial mononuclear cell infiltrate in the mid and deep dermis, extending into a widened interlobular fibrous septae (*Fig. 2B*). The dermal inflammation included an admixture of lymphocytes, eosinophils, DCs, and occasional plasma cells.

To further characterize the mononuclear cells, immunohis-

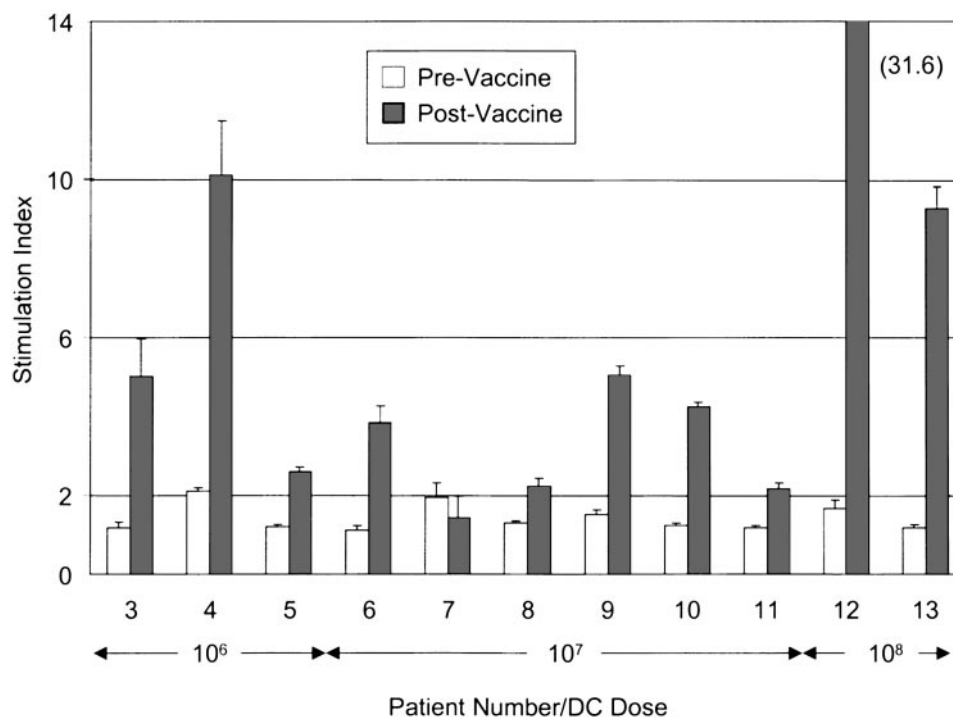


Fig. 3 Proliferation SI of PBMCs before and after vaccination to KLH antigen. The mean (bars, SE) medium control cpm for pre- and posttreatment PBMCs were 1295 (309) and 667 (118), respectively. The ranges of cpm for pre- and posttreatment PBMCs in response to KLH were 129-3959 and (864-10,229), respectively.

tochemical staining revealed, using the LN3 antibody to detect HLA-DR, a striking increase in the number of interstitial DCs in the mid and deep dermis was apparent (Fig. 2C). These HLA-DR-positive cells included hypertrophied dermal DCs interdigitating between collagen bundles, as well as activated appearing lymphocytes. These DCs were also expressed factor XIIIa (Fig. 2D) and were S100 negative (data not shown), which is characteristic of dermal DCs. In addition, these dermal DCs were also strongly and diffusely expressing MAC387 (Fig. 2E).

With regard to the lymphoid component, these CD3⁺ lymphocytes were present in both perivascular and interstitial dermis. Subset analysis revealed approximately twice as many CD4⁺ T cells (Fig. 2F) compared with the relative number of CD8⁺ T cells (Fig. 2G).

Immune Reactivity of PBMCs to KLH. At the time of initial leukapheresis to generate DCs and 1 month after the completion of the third vaccination, patients had PBMCs collected for immune function analyses. A proliferation assay was used to assess the reactivity of PBMCs to KLH protein. Fig. 3 summarizes the results obtained in 11 patients where pre- and posttreatment samples were available. There was a significant difference in the change of pre- versus postvaccine stimulation indices over the three doses by ANOVA ($P = 0.02$).

Another assay that was used to assess KLH reactivity was the ELISPOT assay for IFN- γ production. This assay involves the determination of the frequency of T cells (CD4⁺ and CD8⁺) that secrete IFN- γ in response to antigen exposure. In our study, we have arbitrarily defined an induction of an immune response as a 3-fold increase in the number of IFN- γ spots (with a

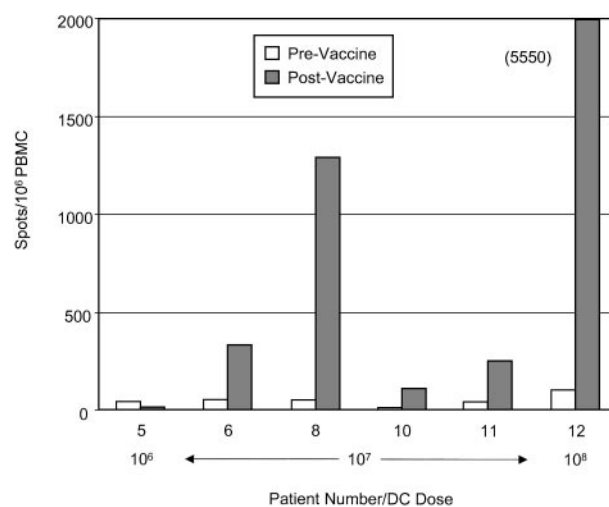


Fig. 4 ELISPOT analysis of IFN- γ secretion of PBMCs to KLH before and after vaccination.

minimum of 50 countable spots) compared with pretreatment levels, with each patient serving as his/her own control. Fig. 4 summarizes the data from 6 patients where adequate numbers of PBMCs were available. In 5 of 6 patients, we were able to document the induction of KLH reactivity as defined by our criteria. The one patient who did not have a KLH IFN- γ response received the lowest vaccine dose (10^6 cells/dose).

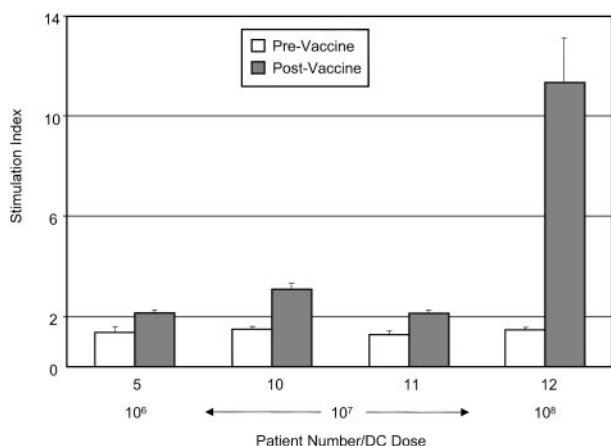


Fig. 5 Proliferation SI of PBMCs before and after vaccination to tumor lysate. The means (bars, SE) medium control cpm for pre- and post-treatment PBMCs were 2325 (1194) and 1482 (625), respectively. The ranges of cpm for pre- and posttreatment PBMCs in response to tumor lysate were 956-8278 and 1952-7845, respectively.

Immune Function of PBMCs to Autologous Tumor Lysate. In a similar fashion, immune reactivity of PBMCs to autologous tumor lysate was assessed using the proliferation and ELISPOT assays. Fig. 5 illustrates the results from 4 patients. Although all 4 patients manifested increased proliferation indices to autologous tumor lysate posttreatment compared with pretreatment, only 1 patient (no. 12), who received the highest DC dose (10^8 cells/dose), showed marked increase after vaccination.

The limited amount of cryopreserved autologous tumor cells restricted the ability to perform these assays in the whole group of patients. We felt that the ELISPOT assay would be the more informative functional test to perform. The ELISPOT data documenting PBMC response in 7 patients to autologous tumor lysate are summarized in Fig. 6. Using the criteria defined above for vaccine induction of immune response, 3 of 7 patients displayed reactivity to autologous tumor lysate. These 3 patients received either 10^7 or 10^8 DCs/dose as their vaccination regimen.

MHC Peptide-Tetramer Binding of PBMCs. Melanoma patient 9, who was HLA-A2 positive, was assessed for the frequency of CD8 T cells in PBMCs that expressed T-cell receptors specific for selected melanoma-associated peptides (*i.e.*, MART-1₁₂₇₋₃₅, tyrosine₃₆₈₋₃₇₆, and gp100 G209-2 M). Before treatment, the patient's PBMCs showed no detectable circulating tetramer-positive T cells; the low background staining level was used for gating purposes. After the sixth or ninth immunization, however, the patient's PBMCs showed significant levels of melanoma-specific CTLs against MART-1₁₂₇₋₃₅ and gp100 G209-2 M at frequencies approaching 1:1000 (Fig. 7). By contrast, the patient's PBMCs remained negative for reactivity to the peptide tyrosine₃₆₈₋₃₇₆. By 10-color FACS analysis, the tetramer-binding cells were found to have a memory CTL phenotype: CD45RO⁺, CD45RA⁻, CD28⁺, CD27⁺, CD11a⁺, CD44⁺, CD8b⁺, CD57⁻, CD38⁻, CD16⁻, and CD56⁻.

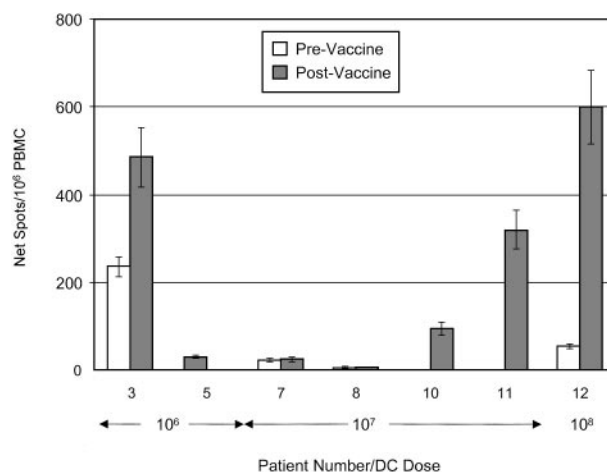


Fig. 6 ELISPOT analysis of IFN- γ secretion of PBMCs to tumor lysate before and after vaccination.

DTH Reactivity to KLH and Autologous Tumor. DTH reactivity was assessed for KLH and autologous tumor in patients 1 month after receiving the third vaccination. In our early experience with KLH DTH testing, we assessed the baseline reactivity of 3 patients before vaccination. These patients were therefore excluded from the immune functional analysis of PBMCs to KLH described earlier. Among these three patients, there was no measurable KLH DTH reactivity at the 2- and 20- μ g test doses. Two of the 3 patients had 10-mm diameter indurations at the 200- μ g inoculation site. An average of 5 mm of induction is conventionally considered a positive DTH response. Table 3 summarizes the KLH DTH reactivity after vaccination in 9 patients. At the 2- and 20- μ g test dose, there were 2 and 5 patients who had a positive KLH DTH. At the 200- μ g test dose, 8 of 9 patients had a positive KLH DTH.

The DTH reactivity of autologous tumor is summarized in Table 4, which lists both pre- and posttreatment assessments. Using the 5-mm measurement as a positive DTH response, 4 of 10 patients exhibited autologous tumor DTH reactivity at the posttreatment assessment. Three of these 4 patients had pretreatment assessments performed and were found to be negative. Patient 10, who developed vitiligo after immunization, developed a vigorous DTH to autologous tumor; however, his tumor went on to progress.

Tumor Responses. Although the primary end point of this Phase I trial was toxicity, we also evaluated clinical response in patients. The clinical outcomes of the patients are summarized in Table 1. One patient with melanoma had a partial response and went on to receive additional vaccines until relapse occurred in the brain. Another patient was stable over the course of six vaccines and went on to have a minor response for a short duration. Because of the limited number of patients in this study, no correlations of clinical outcome to immunological function assays could be assessed.

DISCUSSION

The capacity of DCs to activate naive T cells to antigen is a unique trait not shared by other antigen-presenting cells.

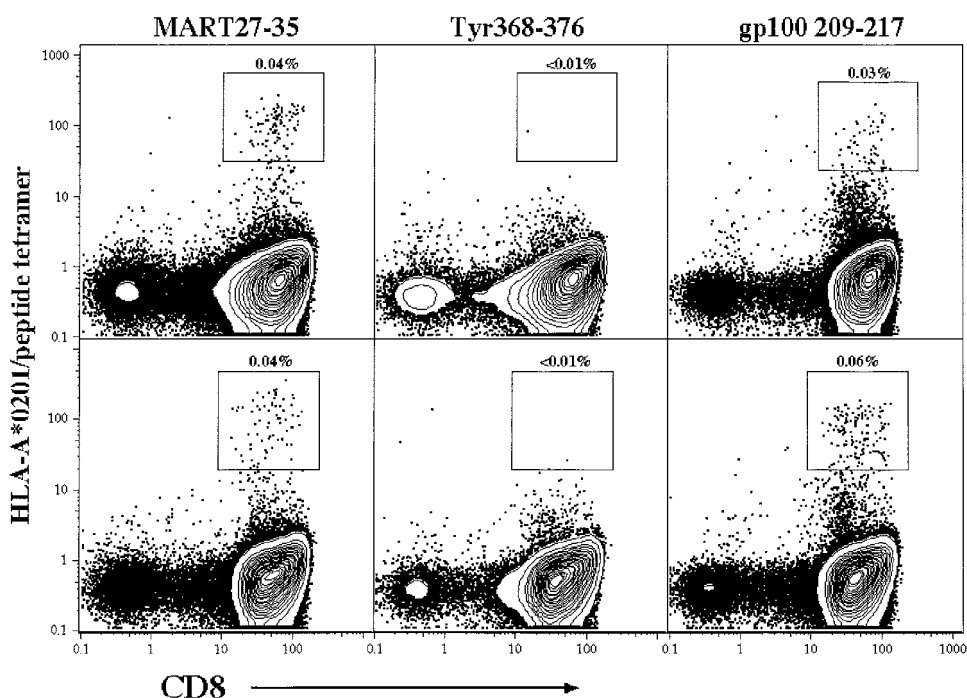


Fig. 7 MHC-peptide tetramer analysis of CD8⁺ T cells from patient 9 with metastatic melanoma for T-cell receptors that bind to MART-1₂₇₋₃₅, tyrosine₃₆₈₋₃₇₆, and gp100 G209-2 M epitopes. *Top panels*, analysis of PBMCs after the sixth vaccine; *lower panels*, analysis after the ninth vaccine.

Table 3 DTH to KLH after vaccination

Patient	DC dose	KLH (μg) ^a		
		2	20	200
3	10 ⁶	10	12	14
4	10 ⁶	5	7.5	15
5	10 ⁶	0	13	20
6	10 ⁷	0	0	7
7	10 ⁷	0	3	5
8	10 ⁷	0	0	0
9	10 ⁷	0	3	14
11	10 ⁷	0	5	10
13	10 ⁸	0	15	25

^a DTH is recorded as the average of two perpendicular diameters in mm.

Hence, they have come under intense scrutiny as reagents in cancer therapy. In this regard, several clinical studies have been reported using DCs as vaccines (3). The use of autologous tumor lysate to pulse DCs represents a minority of these reported studies (9–11). The advantage of tumor lysate-pulsed DCs is that all possible antigens expressed by a tumor cell will be incorporated in the vaccine, which may be more capable of inducing tumor rejection responses compared with single defined antigen-pulsed DCs. In animal studies performed in our laboratory, tumor lysate-pulsed DCs were more effective than single peptide-pulsed DCs in mediating tumor regression (12).

Among the three clinical reports using autologous tumor lysate-pulsed DCs, intranodal (9), i.v. (10), and s.c. (11) routes of administration were used compared with the intradermal route used in our study. An important question that needs to be addressed is which route of administration is most effective in clinical therapy. We postulate that a key step involved with

Table 4 DTH to autologous tumor

Patient	DC dose	Prevaccine ^a			Postvaccine ^a		
		10 ⁴	10 ⁵	10 ⁶	10 ⁴	10 ⁵	10 ⁶
3	10 ⁶	0	0	0	0	0	14
4	10 ⁶	0	0	0	14	15	14
5	10 ⁶	0	0	0	0	0	14
7	10 ⁷	ND ^b	ND	ND	0	0	0
8	10 ⁷	ND	ND	ND	0	0	0
9	10 ⁷	0	0	0	0	0	0
10	10 ⁷	0	0	3	13	14	14
11	10 ⁷	ND	ND	ND	0	0	3
14	10 ⁷	ND	ND	ND	0	6	6
13	10 ⁸	0	0	4	0	0	0

^a DTH is recorded as the average of two perpendicular diameters in mm to varying amounts of irradiated tumor cells (10⁴, 10⁵, 10⁶).

^b ND, not done.

T-cell sensitization after vaccination is DC antigen presentation in regional draining lymph nodes. Morse *et al.* (13) reported that only the intradermal route of human DC inoculation resulted in the migration of cells to lymph nodes; and that the i.v. and s.c. routes were ineffective in nodal localization. Thomas *et al.* (14) reported that immature human DCs migrate rapidly to draining lymph nodes after intradermal inoculation. The phenotype of DCs we used was similar and represented an immature form. Fong *et al.* (15) have reported that the route of administration of antigen-pulsed DCs given in patients affected the type of T-cell responses measured in the PBMCs. In their report, intradermal and intralymphatic routes were superior to i.v. administration in eliciting IFN- γ production to antigen. If lymph nodes are requisite organs for DCs to present antigen to T cells, then one alternative approach would be to deliver DCs by direct intran-

odal injection. Lambert *et al.* (16) used a murine model to document that intranodal inoculation of antigen-pulsed DCs was superior to intradermal/s.c. or i.v. routes to induce cellular immunity. Nestle *et al.* (9) has reported that the intranodal injection of tumor lysate-pulsed DCs was associated with measurable tumor responses in patients with advanced melanoma. In future clinical studies, we plan to compare intradermal *versus* intranodal administration of DCs as a method to induce antitumor immune responses.

Another important area of investigation is defining the appropriate maturational state of DCs that should be used in clinical therapy. Immature DCs are highly effective in taking up antigen for subsequent processing compared with mature DCs. By contrast, mature DCs are more efficient in stimulating T cells. There are a variety of ways to mature DCs *in vitro*. These include exposure of DCs to CD40L, various cytokines, bacterial or viral products, calcium ionophores, and others (3, 17). Larson *et al.* (18) observed that mature DCs were superior to immature DCs in eliciting antigen-specific IFN- γ production by CD8⁺ effector cells *in vitro*. This same group reported that antigen-pulsed mature DCs may inhibit effector T-cell function after s.c. inoculation (19). In our study, we have been able to generate T-cell responses to KLH and tumor antigen with a relatively low percentage of mature DCs (*i.e.*, mean, 17%) given i.d. The use of tumor lysate enriched in heat shock proteins has been shown to induce maturation of DCs (20) and may account for the presence of mature DCs in our vaccine preparation. Furthermore, the route of administration (*i.e.*, intradermal *versus* s.c.) may be critical. It is possible that additional *in vivo* maturation of DCs occurred after intradermal administration. This is supported by a study reported by Barrett-Boyes *et al.* (21), who found that the intradermal inoculation of immature DCs undergo spontaneous maturation during the course of migration to the draining lymph nodes. We plan to assess the relative efficacy of immature *versus* mature DCs to elicit tumor-reactive cellular responses in future clinical trials.

We examined the local immune response induced by the vaccine in 2 patients. On the basis of the microscopic analysis of the vaccine site, it appeared that the local injection of lysate-pulsed DCs included not only an increased density of dermal DCs but was also accompanied by the infiltration of T lymphocytes and to a lesser extent eosinophils and plasma cells. These findings are similar to our previous report in which we characterized the vaccine site of human subjects receiving autologous GM-CSF-transduced and lethally irradiated melanoma cells (8). It is currently unclear in the present study whether the increased density of dermal DCs resulted from the intradermal injection or represents DCs recruited along with the T cells and other inflammatory cells in response to the vaccination, or both. We favor the former possibility, because dermal DCs recruited into cutaneous sites of inflammation are generally MAC387 negative. Otherwise, the immunophenotype of these dermal DCs, which express factor XIIIa and HLA-DR but are S100 negative, are typical for this subset of professional antigen-presenting cells (7). The local accumulation of T cells admixed with the DCs demonstrated a relative increase in CD4⁺ T cells over the CD8⁺ T-cell subsets. Because almost all of the T cells also expressed HLA-DR, this would suggest a local activation for the T cells within the dermal compartment of the skin receiving the

vaccination. The significance of the eosinophils or plasma cells is currently unknown, but such cells were also identified in the vaccine sites excised in our earlier clinical trial using GM-CSF-transduced tumor cells injected i.d. (8).

An important aspect of all these immunotherapy trials is the need for relevant immunological monitoring. Surrogate monitoring assays are considered necessary to provide leads for optimizing various immunotherapeutic approaches (22, 23). To date, however, there is no specific correlative assay that would predict for *in vivo* tumor rejection responses. Nevertheless, from a significant body of experimental evidence, T-cell responses to tumor antigen by way of IFN- γ release appears to be associated with conditions that favor tumor regression (24–26). Hence, cellular assays involving IFN- γ elaboration to tumor antigen would seem to be a relevant surrogate measurement for tumor response. We have found the ELISPOT assay to be a useful quantitative assay for this purpose. In our study, we were able to measure both reactivity of PBMCs to KLH and tumor lysate induced by the DC vaccination. MHC-peptide tetramer analysis of CD8 T-cell responses to defined antigen epitopes is also a highly sensitive method. Using this assay, we found that autologous tumor lysate-pulsed DC vaccines could induce T-cell responses to more than one defined epitope. It is unknown what the functional significance of T-cell responses as measured by MHC-peptide tetramers represent with respect to tumor rejection. This is because in some studies, sorted MHC-tetramer binding T cells were found to be functionally anergic in the tumor setting (6).

Another monitoring tool investigators have used is DTH testing to antigen as a method to demonstrate cellular immunity. We documented the induction of DTH to KLH in the majority of patients and in a smaller percentage to autologous tumor cells. Although some investigators (27, 28) have reported that DTH to autologous tumor is one of the better predictors of potential tumor response, our Phase I study was not designed to assess the correlative significance of DTH reactivity with tumor responses. One problem with DTH testing, however, is the potential variability of reading from one observer to another.

KLH was incorporated into the trial as a surrogate marker antigen for the assessment of cell-mediated responses from the DC vaccinations in advanced cancer patients. This foreign protein has also been found to be a useful adjuvant for providing additional help in tumor vaccines (29–31). In animal models, we have reported that the provision of KLH and tumor lysate-pulsed DCs could enhance antitumor immunity compared with tumor lysate-pulsed DCs alone by a mechanism that involved host-derived CD4⁺ TH₁ cells (32).

As with other clinical studies using DCs (3, 9, 11), the treatment regimen was well tolerated. There was minimal local toxicity and no significant clinical problems associated with autoimmunity. We believe that this is an important observation because our vaccine approach made use of a crude tumor lysate preparation, which could have allowed the pulsed DCs to uncover self autoantigens. On the basis of preclinical studies reported by our group, we will examine the concomitant administration of IL-2 with tumor lysate pulsed DCs (33). In our animal models, the administration of IL-2 plus tumor lysate/

KLH-pulsed DCs had a synergistic antitumor effect compared with either alone.

In summary, our results in this Phase I trial demonstrated the feasibility and safety of administering tumor lysate-pulsed DCs. We found that a relatively immature population of antigen-pulsed DCs given *i.d.* can induce antigen reactivity in T cells. Moreover, we were able to observe IFN- γ production by circulating T cells in response to autologous tumor cells in a significant portion of patients after vaccination. These findings warrant further investigations to optimize this approach to enhance tumor rejection responses.

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