

Immune and Clinical Responses in Patients with Metastatic Melanoma to CD34⁺ Progenitor-derived Dendritic Cell Vaccine¹

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ABSTRACT

Immunization to multiple defined tumor antigens for specific immune therapy of human cancer has thus far proven difficult. Eighteen *HLA A*0201*⁺ patients with metastatic melanoma received injections s.c. of CD34⁺ progenitor-derived autologous dendritic cells (DCs), which included Langerhans cells. DCs were pulsed with peptides derived from four melanoma antigens [(MelAgs) MelanA/MART-1, tyrosinase, MAGE-3, and gp100], as well as influenza matrix peptide (Flu-MP) and keyhole limpet hemocyanin (KLH) as control antigens. Overall immunological effects were assessed by comparing response profiles using marginal likelihood scores. DC injections were well tolerated except for progressive vitiligo in two patients. DCs induced an immune response to control antigens (KLH, Flu-MP) in 16 of 18 patients. An enhanced immune response to one or more MelAgs was seen in these same 16 patients, including 10 patients who responded to >2 MelAgs. The two patients failing to respond to both control and tumor antigens experienced rapid tumor progression. Of 17 patients with evaluable disease, 6 of 7 patients with immunity to two or less MelAgs had progressive disease 10 weeks after study entry, in contrast to tumor progression in only 1 of 10 patients with immunity to >2 MelAgs. Regression of >1 tumor metastases were observed in seven of these patients. The overall immunity to MelAgs after DC vaccination is associated with clinical outcome ($P = 0.015$).

INTRODUCTION

Molecular identification of human cancer antigens in the last decade (1–3) has ushered in a new era of antigen-specific cancer immunotherapy specifically targeting these antigens (4–7). However, several such approaches (*e.g.*, peptides, DNA vaccines, and viral vectors) have thus far met with little or no success in the clinic (7–9). In particular, it has proved difficult to immunize humans simultaneously with multiple tumor antigens. In the case of melanoma, in which most such antigens have been defined, the immune system may be tolerized to these “self” antigens because they are also expressed on normal tissues (*e.g.*, melanocytes). Additional obstacles may include tumor-induced tolerance and global immunosuppression in advanced cancer (7–9). Unfortunately, most human tumor vaccine studies have not included control CD8⁺ T-cell antigens (*e.g.*, viral CD8⁺ epitopes), making it difficult to distinguish among these possibilities.

DCs⁴ are antigen-presenting cells specialized to initiate and regu-

late immune responses (10, 11). Their clinical use as adjuvants has been aided by the development of methodologies to generate large numbers of these cells in culture from blood monocytes (12, 13) or CD34⁺ progenitors (14). In contrast to Mo-DCs, DCs derived from CD34⁺ cells consist of two phenotypically and functionally distinct populations (15). One subset is similar to the epidermal LCs, and the other termed “interstitial/dermal DCs” is similar to those derived from blood monocytes (15). Immune responses to these unique LC-containing preparations need to be evaluated in humans. Here we describe the safety and immunogenicity of antigen-bearing CD34-DCs in patients with stage IV melanoma.

MATERIALS AND METHODS

Study Design, Patients Characteristics, and Eligibility Criteria

Eighteen *HLA-A201*⁺ patients with metastatic melanoma received injections of CD34-DCs (Fig. 1; Table 1). Four patients (patients 1, 9, 12, and 13) had CNS involvement treated by surgery and radiation before entry into the trial, four patients (2, 4, 5, and 16) had received prior chemotherapy, and five patients (2, 4, 6, 9, and 15) had received prior biological therapy without a clinical or immune response (Table 1). Three patients (3, 13, and 20) progressed before completing the trial. Inclusion criteria were: biopsy-proven American Joint Committee on Cancer stage IV metastatic melanoma; age, ≥ 18 years; Karnofsky performance status, $>80\%$; *HLA-A*0201* phenotype; intra-dermal skin test positivity to mumps, histoplasmosis, or streptokinase antigen; normal blood CD4 and CD8 T-cell numbers by flow cytometry; and normal quantitative immunoglobulin levels. Exclusion criteria were: prior chemotherapy or biologicals <4 weeks before trial entry; untreated CNS lesions; bulky hepatic metastatic lesions; pregnancy; or concurrent corticosteroid/immunosuppressive therapy. Patients with history of asthma, venous thrombosis, congestive heart failure, autoimmune disease, or active infections, including viral hepatitis, were also excluded. All of the patients were presented with several treatment alternatives, including surgery, high-dose cytokines, chemotherapy, or alternative immunotherapy. Patients were unlikely to be cured with surgery because of the presence of visceral metastases in most patients, including CNS involvement. Patient 10 had recurrent disease close to a prior biopsy site and refused further surgery. All of the patients gave a written informed consent, and the study was approved by the Food and Drug Administration, the NCI, and the Institutional Review Board. Patients received a 6-week outpatient vaccination course with antigen-loaded CD34-DCs given s.c. every 14 days for a total of four vaccinations. DCs were administered in a dose-escalation design at the dose level per cohort of 0.1, 0.25, 0.5, and 1×10^6 DCs/kg/injection. The calculated DC dose was the actual number of CD1a⁺ and CD14⁺ cells in the cell preparation (see below).

Preparation and Administration of the DC Vaccine

Harvest of DC Progenitors. The patients received recombinant granulocyte-CSF (Neupogen) 10 $\mu\text{g}/\text{kg}/\text{day}$ s.c. for 5 days, for peripheral blood stem cell mobilization, and then underwent leukapheresis for 2 consecutive days to

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⁴ The abbreviations used are: DC, dendritic cell; LC, Langerhans cell; HPC, hematopoietic progenitor cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; FLT3-L, Flt3 ligand; TNF, tumor necrosis factor; KLH, keyhole limpet hemocyanin; SEA, staphylococcal enterotoxin A; SFC, spot-forming cell; DTH, delayed-type hyper-

sensitivity; CD34⁺-DC, CD34⁺-derived DC; CNS, central nervous system; NCI, National Cancer Institute; PBMC, peripheral blood mononuclear cell; ELISPOT, enzyme-linked immunospot; MelAg, melanoma antigen; PD, progression of measurable disease and/or new lesions; Mo-DC, monocyte-derived DC; IL, interleukin.

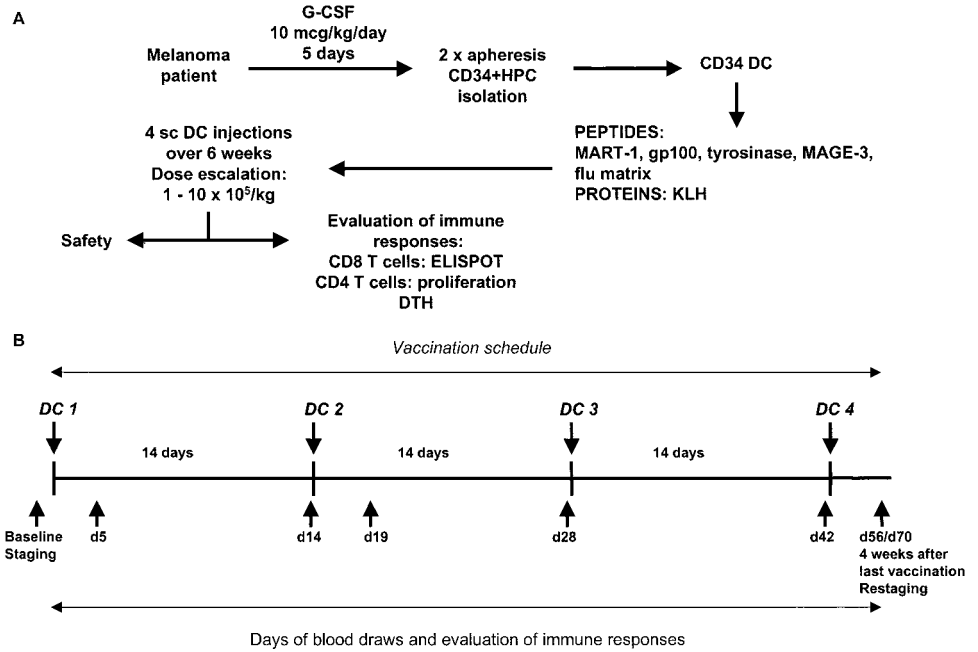


Fig. 1. Study design and vaccination schedule.

collect mobilized CD34⁺ HPCs. The cells were processed using the CEPRATE SC stem cell concentration system (CellPro Inc., Seattle, WA) to obtain an enriched population of CD34⁺ HPCs (purity, 62 ± 17%; recovery, 158 ± 133 × 10⁶ mean ± SD), which were then cryopreserved.

Preparation of DC Vaccine. All procedures were performed according to Good Laboratory Practice standards. CD34-DCs were generated from CD34⁺ HPC by culture at a concentration of 0.5 × 10⁶/ml culture medium (X-VIVO-15; BioWhittaker) supplemented with autologous serum, 10⁻⁵ M 2-β-mercaptoethanol and 1% L-glutamine. The following human recombinant cytokines, approved for clinical use, were used: GM-CSF (50 ng/ml; Immunex Corp.),

FLT3-L (100 ng/ml; Immunex Corp.), and TNF (10 ng/ml; CellPro, Inc.). Cultures were conducted in a humidified incubator at 37°C and 5% CO₂ with a separate incubator being assigned to each patient. On day 8 of culture, all of the cells were pulsed overnight with KLH (2 μg/ml; Intracell), 20% of the cells were pulsed separately with HLA-A*0201 restricted Flu-MP GILG-FVFTL₅₈₋₆₆ (2.5 μg/ml), and 80% of the cells were pulsed overnight with a mix of four HLA-A201 restricted peptides (2.5 μg/ml) derived from MelAgs (MelanA/MART-1₂₇₋₃₅: AAGIGILTV; gp100_{g209-2}: IMDQVPFSV; tyrosinase₃₆₈₋₃₇₆: YMDGTMSQV; and MAGE-3₂₇₁₋₂₇₉: FLWGPRLV). After overnight loading, all of the DCs were washed three times with sterile saline

Table 1 Patient characteristics and disease status on entry and post-DC vaccine

Pt. I.D. ^a	Age/sex	Months from diagnosis to stage IV/previous therapy	Status and measurable disease on entry	Early clinical outcome restaging at 10 wk (4 wk after last DC vaccine)
1	59/M	36/Surgery and radiation of CNS lesion	Retroperitoneal mass by CT scan (2 cm), positive PET scan	No evidence of measurable disease by CE, CT, and PET scan
2	55/M	24/Chemotherapy, IL-2, & IFN-α	Skin, LN, CNS, liver (4 cm), lung (3 cm)	PD: all sites enlarged
3	43/F	6/Surgery	Skin, LN, bones, and liver	PD: progression at all sites, early death from melanoma
4	42/F	82/Chemotherapy, IL-2, & IFN-α	PD: subcarinal LN mass by CT (6 cm)	Pulmonary nodule (2.8 cm) and subcarinal LN mass (7 cm) stable
5	45/M	10/Chemotherapy, IL-2, & IFN-α	Pulmonary nodule by CT (3.2 cm) PD: spleen nodule (3.5 cm), liver nodules (2.5 cm), and pulmonary nodules (1 cm) by CT scan	Progressive vitiligo on chest, neck, back. No new lesions Spleen nodule 1 cm by CT scan, pulmonary, and liver nodules stable
6	36/M	30/Surgery, high-dose IL-2	PD: skin nodules (1.5–2.5 cm) and LN	PD: disappearance of 3 s.c. nodules but several new lesions
8	61/F	69/Surgery and radiation	Skin nodule by CE and CT scan (3 cm), liver nodule (4.5 cm), LDH 249, AP 261, AST 71, ALT 120	Regression of skin nodule by CE and CT, liver lesion stable (4.3 cm), normalization of liver enzymes: LDH 193, AP 196, AST 26, ALT 36; progressive vitiligo chest and arms
9	44/M	10/Melanoma cell vaccine, surgery, and radiation of CNS lesion	3-cm axillary LN mass by CE	No axillary LN mass, no evidence of measurable disease by CE and CT scan
10	50/M	144/Surgery	2-cm femoral LN next to biopsy-proven LN mass	No palpable LN mass, no evidence of measurable disease by CE and CT scan
12	56/F	35/Surgery, radiation CNS lesions	Skin nodule (2 cm)	50% regression of skin nodule, no new lesions by CE and PET scan
13	50/M	1/Surgery, radiation CNS lesion	2-cm axillary LN by physical examination	PD: progression in CNS after 2 DC vaccinations
15	43/F	1/Surgery and adjuvant IFN-α	LN, lung, and spleen	PD: progression, new lesion in spleen
16	73/M	96/Chemotherapy	PD: retroperitoneal LN (8 cm), liver (10 cm)	PD: progression
17	57/F	40/Surgery	Lung nodule by CT scan (1 cm)	No evidence of progression by CT scan and CE
18	70/F	3/Surgery	Pericardial nodule by CT (2 cm), 7.5-mm lesion in vaginal wall proximal to biopsy-proven lesion	Regression of pericardial nodule by CT scan and of vaginal metastases by CE
19	66/M	6/Radiation	Parotid nodule 2 cm by PET scan.	Nonevaluable for clinical outcome (no PET scan postvaccination)
20	40/M	44/Surgery	LN, liver and chest wall	PD: progression, early death from melanoma
21	66/M	1/Surgery	Liver lesion by MRI (1.8 cm)	Liver lesion stable by MRI (1.9 cm)

^a Pt. I.D., patient identification number; CT, computed tomography; PET, positron emission tomography; LN, lymph node; LDH, lactate dehydrogenase; AP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CE, clinical examination; MRI, magnetic resonance imaging.

and were counted and resuspended in 10 ml of sterile saline containing melanoma peptides (1 $\mu\text{g}/\text{ml}$). After 2-h incubation at 22°C, the cells were centrifuged and resuspended in 9 ml of sterile saline for injection. All of the peptides were Good Manufacturing Practice (GMP) quality and were either obtained from the NCI (MelanA/MART-1, gp100, and tyrosinase) or purchased (Flu-MP and MAGE-3; MultiPeptide Systems, San Diego, CA). Vaccine release criteria included: (a) negative bacterial culture 48 h prior to DC injection; (b) negative Gram's staining after antigen pulsing; (c) DC morphology on Giemsa-stained cytopins performed 2 h before DC administration, (d) cell viability >80%; and (e) a minimum of 20% DCs (CD1a⁺ and CD14⁺) in cell preparation as determined by phenotypic analysis. The remaining cells contained DC precursors as well as cells with the ability to induce mixed lymphocyte reaction (not shown). Further quality testing of each DC batch included: (a) reactivity with a panel of monoclonal antibodies; and (b) determination of their stimulatory capacity in mixed lymphocyte reactions.

Administration of Vaccine. Vaccination was administered s.c. in three injection sites (both thighs and the upper arm). Limbs from which draining lymph nodes had been surgically removed and/or irradiated were not injected. DCs were injected using a long spinal-cord needle and were spread over a 6- to 8-cm distance.

Clinical Monitoring

Adverse events were graded according to the NCI Common Toxicity Criteria. All of the patients underwent assessment of tumor status at baseline and 4 weeks after the fourth DC vaccination (10 weeks from trial entry). Disease progression was defined as >25% increase in target lesions and/or the appearance of new lesions.

Immunological Monitoring

PBMCs samples from at least two time points before vaccination, as well as 5 and/or 14 days after each vaccination and 14 or 28 days after the fourth vaccination, were harvested and frozen. Pre- and postimmunization PBMCs were frozen in aliquots, coded, thawed and assayed together in a blinded fashion.

Antigen-specific Proliferation

PBMCs (10⁵ cells/well) were cultured in triplicate wells in the absence or presence of graded doses of KLH at 1–10 $\mu\text{g}/\text{ml}$, and as a positive control, in the presence of SEA. Assays were pulsed overnight with [³H]thymidine on day 3 (SEA) or day 5 (KLH) of culture and harvested 16 h later.

ELISPOT Assay for IFN- γ Release from Single Antigen-specific T Cells

ELISPOT assay for the detection of antigen-specific IFN- γ -producing T cells was performed as described previously (16, 17). Briefly, PBMCs (2×10^5 cells/well) were added to plates precoated with 10 $\mu\text{g}/\text{ml}$ of a primary anti-IFN- γ monoclonal antibody (Mabtech, Stockholm, Sweden) in the presence or absence of 10 $\mu\text{g}/\text{ml}$ peptide antigens. The antigens were the same HLA A*0201-restricted peptides (four melanoma peptides and Flu-MP) used in the DC vaccine. HLA A*0201-restricted gag peptide was used as a negative control, and SEA as a positive control for T-cell function. For some experiments, depending on the cell yield, influenza virus-infected PBMCs (MOI 2) were used as APCs. Antigen-specific SFCs were calculated after subtracting the background obtained with control peptide. Immune responses were scored as positive if the postimmunization measurements for antigen-specific SFCs were >2-fold higher than the baseline and >10 SFC/ 2×10^5 cells (16).

Antigen-specific Recall T-cell Responses

To evaluate the ability of antigen-specific T cells to proliferate and differentiate in culture, pre- and postimmunization PBMCs were thawed together and cocultured (2×10^5 cells/well) for 7 days with autologous mature DCs (PBMC:DC ratio, 30:1) pulsed with 1 $\mu\text{g}/\text{ml}$ peptides. After 7 days, cells were transferred to an ELISPOT plate and cultured overnight with (T-cell:APC ratio, 20:1) irradiated (3000 rads) T2 cells with or without specific antigen. Antigen-specific SFCs were calculated after subtracting the background obtained with unpulsed T2 cells.

DTH Reactions

CD34-DCs (10⁵), pulsed separately with each antigen, were injected intradermally on the patient's back and induration at the injection site was measured at 48 h.

Statistical Analysis

The sign test for discretized data were used to demonstrate the presence of specific immune response to KLH and Flu-MP (18). Because the role of different MelAgs with regard to protective immunity is not known, we integrated postvaccination responses to all four MelAgs, as measured by both direct and recall assays, into an immunity score using a nonparametric method based on the marginal likelihood approach (19, 20). To score n individuals according to their immune response profiles, one computes all rankings (permutations of numbers 1 . . . n) that are compatible with all pairwise orderings. An immune response is considered higher if it is at least as high for each of the eight variables and higher for at least one variable. A patient's immunity score is the average of the corresponding ranks among the compatible rankings minus the expected score. All of the immunized patients were included in the analysis in an "intent to treat" approach.

RESULTS

DC Vaccine. Fresh DCs were generated from granulocyte-CSF mobilized blood CD34⁺-HPCs for each vaccination. Frozen/thawed CD34⁺-HPCs cultured for 9 days with GM-CSF, TNF- α , and FLT3-L yielded MHC class I⁺, HLA-DR⁺, CD80⁺, CD86^{low}, and CD83^{low} DCs (not shown). Although CD83^{low}, these DCs are not considered immature because they are generated in the presence of TNF- α (a well-established DC maturation factor) and routinely induce proliferation of allogeneic CD8 T cells (not shown). The DCs included CD1a⁺CD14⁻ LCs as well as CD1a⁺CD14⁺ interstitial DC precursors (intDC). The LC phenotype was confirmed by confocal microscopy revealing Langerin staining in CD1a⁺ DCs (not shown; 21). The mean proportion of CD1a⁺CD14⁻ cells was $9 \pm 3\%$ (range, 4–17%; median, 9%) and that of CD1a⁺CD14⁺ cells was $32 \pm 9\%$ (range, 19–52%; median, 30%). The composition of DC vaccine for each patient is given in Table 3.

Responses to Control Antigens. DC vaccination primed KLH-specific immune responses in 16 of 18 patients (all except patients 3 and 13; $P = 0.00007$ in the exact sign test; Fig. 2A). There is no indication that higher DC doses induced greater KLH-specific proliferation. Fifteen patients (all except patients 3, 13, and 20) were evaluated for DTH after they completed the vaccination protocol (four DC vaccines). Thirteen of these patients (all except patients 1 and 19) developed DTH to KLH (median, 10 mm; range, 6–47 mm). Of the 17 patients injected with Flu-MP-pulsed DCs (all except patient 18 with a history of allergy to flu vaccine), enhancement of Flu-MP-specific memory T-cell responses by at least one assay was observed in 15 patients (all except patients 3 and 13; Table 2; Fig. 2B). The elicited T cells also recognized the naturally processed antigen from flu-infected PBMCs (not shown). The finding that all except two patients responded to both KLH and Flu-MP-pulsed DCs indicates that patients with metastatic melanoma enrolled in our study were immunocompetent.

In Vivo Expansion of Melanoma-specific Blood CD8 T Cells

Responses in Uncultured T Cells. Except for patient 8, only a few MelAg-specific IFN- γ -producing cells were detected in baseline blood samples (Table 2; Fig. 3). The response was considered as enhanced if there was a >2-fold increase and a minimum of 10 MelAg-specific ELISPOTs (after subtracting the values obtained from control wells) in postimmunization samples. After DC vaccination, enhanced responses to ≥ 1 MelAgs were detectable in uncultured T

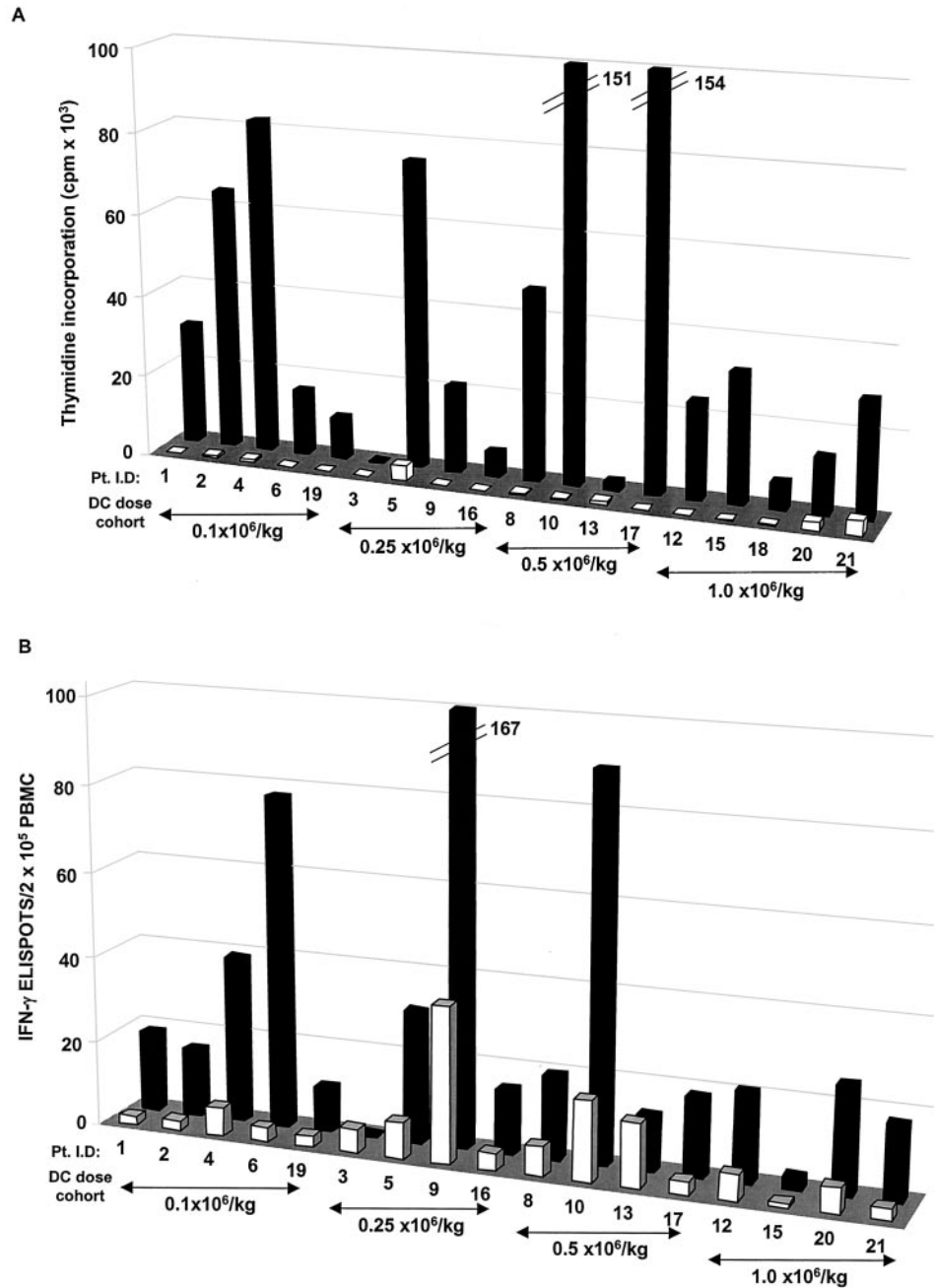


Fig. 2. Responses to control antigens. A, KLH-dependent proliferative responses: pre/postimmunization PBMCs are cultured with 10 μg/ml KLH for 5 days. KLH-specific T-cell proliferation is determined based on [³H]thymidine incorporation. B, Flu-MP responses. PBMCs obtained at baseline and after four DC vaccinations are cultured overnight with 10 μM Flu-MP peptide. Antigen-specific IFN-γ-secreting cells are quantified using an ELISPOT assay and expressed as number of IFN-γ ELISPOTS/2 × 10⁵ PBMCs. The numbers obtained in control wells are subtracted (median, 1 spot/2 × 10⁵ PBMCs; range, 0–8).

cells in 8 of 18 patients (Table 2). Five patients showed increased responses to MAGE-3, four to MelanA/MART-1, five to gp100, and seven to tyrosinase peptide (Table 2; Fig. 3A). There is no indication that higher DC doses induce greater melanoma-specific immunity. We conclude that MelAg-pulsed CD34-DCs lead to enhancement of MelAg-specific circulating effectors in melanoma patients.

Responses in Cultured T Cells. Next, we determined the capacity of blood CD8 T cells to mount melanoma-specific responses after 1-week culture with melanoma peptide-pulsed autologous DCs. Most patients had low levels of MelAg-specific memory cells in preimmunization samples (except for patient 21). However, in 14 of 15 evaluated patients, an increased response to at least one melanoma peptide was found after DC vaccination, (Table 2; Fig. 3B), and 5 of these patients (patients 1, 5, 9, 16, and 17) responded to all four melanoma peptides.

Overall Response to MelAg

Overall, enhanced immunity to ≥1 MelAg by at least one assay was observed in 16 of 18 patients after DC vaccination. Of these, enhanced immunity to two, three, or all four MelAg was seen by at least one assay in patients 3, 4, and 6, respectively. Thus, vaccination with melanoma-peptide-pulsed CD34-DCs leads to enhanced immunity to several MelAg peptides in melanoma patients.

DTH

Ten of 14 evaluated patients developed DTH to at least one peptide after repeated DC vaccination. Thus, DTH to DCs pulsed with: (a) MART-1 was observed in all 10 patients (median induration, 7.5 mm); (b) MAGE-3 in 8 of 10 patients (median induration, 9.5 mm); (c) tyrosinase in 8 of 10 patients (median induration, 8 mm); and (d)

Table 2 Number of peptide-specific IFN- γ ELISPOTS/ 10^5 PBMCs in pre/postvaccination samples: circulating effectors (direct ELISPOT, D) and memory T cells (recall ELISPOT, R)

DC dose	Pt. I.D. ^a %CD3+CD8+ ^b	Flu	MAGE	MART	Tyr	Gp100	No. of MelAgs
0.1 $\times 10^6$ /kg	1	D: 2/19	1/8	3/3	1/6	1/8	0
	16%	R: 1/276	3/40	5/59	5/45	6/40	4
		T2: 2/4	0/2	0/2	0/2	0/2	
	2	D: 2/16	1/4	1/0	0/2	0/8	0
	31%	R: 3/12	3/2	0/14	1/9	2/14	2
		T2: 0/6	0/3	4/1	3/4	1/0	
	4	D: 7/39	0/36	3/25	3/27	0/33	4
	27%	R: ND	ND	ND	ND	ND	
	6	D: 3/77	1/2	0/1	0/2	0/2	0
	25%	R: ND	1/3	ND	1/7	5/13	1
		T2: ND	10/10	ND	11/14	6/9	
	19	D: 2/10	2/6	0/1	0/4	0/1	0
36%	R: 89/164	5/20	4/14	6/8	11/14	2	
	T2: 3/1	1/1	1/4	1/1	2/2		
0.25 $\times 10^6$ /kg	3	D: 5/0	0/0	0/0	0/0	0/0	0
	ND	R: 0/0	0/0	0/0	0/0	0/0	0
		T2: 0/0	0/0	0/0	0/0	0/0	
	5	D: 8/31	1/23	10/13	5/24	1/25	3
	12%	R: 272/1019	4/178	13/55	4/110	1/59	4
		T2: 8/81	0/2	0/33	0/6	2/8	
	9	D: 37/167	0/12	0/12	0/10	0/18	4
	27%	R: 61/256	1/20	11/29	2/21	1/23	4
		T2: 14/9	6/7	7/4	6/4	6/9	
	16	D: 4/15	2/1	0/2	0/1	0/1	0
7%	R: 50/225	7/38	0/47	1/45	13/48	4	
	T2: 21/23	25/23	23/32	19/39	25/25		
0.5 $\times 10^6$ /kg	8	D: 7/19	7/20	12/39	5/15	4/5	3
	26%	R: ND	ND	ND	ND	ND	
	10	D: 19/89	1/4	4/0	2/0	0/0	0
	30%	R: 62/730	0/0	0/15	0/54	0/35	3
		T2: 1/70	0/23	0/23	0/21	0/31	
	13	D: 15/13	0/0	0/0	0/0	0/0	0
	3%	R: 9/4	3/1	1/0	1/0	5/10	0 ^c
		T2: 0/0	0/0	1/1	1/0	0/66	
	17	D: 3/18	1/9	1/12	1/10	1/7	2
	25%	R: 34/441	3/37	13/64	4/51	12/44	4
	T2: 5/12	5/18	8/19	7/23	7/27		
1.0 $\times 10^6$ /kg	12	D: 6/21	0/3	3/8	0/13	1/4	1
	17%	R: 28/100	0/0	6/20	1/36	0/36	3
		T2: 0/0	0/1	8/0	0/10	0/0	
	15	D: 1/3	0/0	1/0	0/1	0/10	1
	14%	R: 40/155	1/4	5/6	0/6	6/70	1
		T2: 25/0	17/7	37/1	3/0	3/14	
	18	D: 5/13	0/2	0/1	0/1	0/1	0
	15%	R: 35/43	5/18	13/9	0/2	0/16	2
		T2: 0/1	0/1	4/2	0/0	0/3	
	20	D: 6/25	0/0	0/0	1/0	1/2	0
	ND	R: 267/326	9/5	12/10	2/14	13/22	1
		T2: 13/4	15/8	15/3	17/3	19/9	
21	D: 3/17	1/15	1/9	1/10	1/10	3	
32%	R: 241/473	21/37	30/44	23/20	16/36	1	
	T2: 21/37	12/16	10/15	4/7	11/11		

^a Pt. I.D., patient identification number; ND, not done.

^b Percentages of CD3 + CD8 + T cells in lymphocyte gate.

^c Because of unusually high background, we did not consider this as a positive response.

gp100 in 9 of 10 patients (median induration, 8 mm). In three patients, there was reactivity to unpulsed DCs (erythema without induration (8 mm). There was no correlation between the responses in blood and DTH.

Toxicity of DC Injection

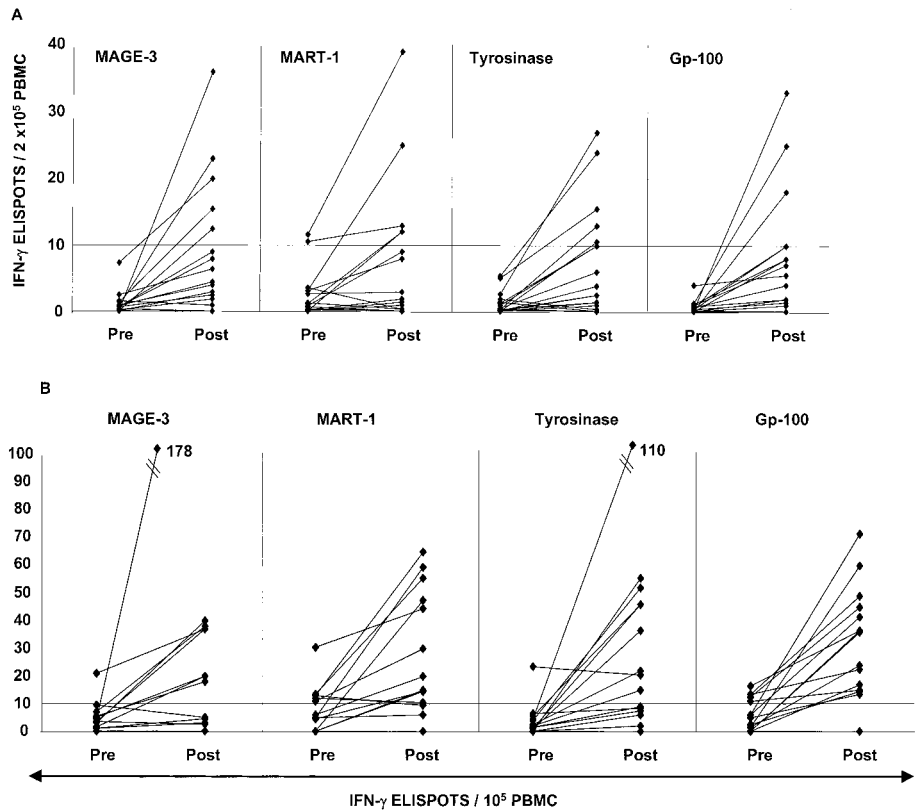
Safety and tolerability were assessed with each DC vaccination and 1 month after the fourth vaccination. No patients developed injection site erythema/irritation or systemic toxicity. Two patients (patients 4 and 8) with mild preexisting vitiligo developed progressive vitiligo during the course of DC therapy (Table 1). Rheumatoid factor and antithyroid antibodies were negative throughout the trial. The anti-nuclear antibody titer of patient 8 was negative before the first DC injection but increased to 1:80 after the fourth injection. No clinical manifestations of autoimmune disease developed in this patient.

Clinical Outcome. Seven of 17 evaluable patients experienced tumor progression (PD, Table 1). The remaining 10 patients did not progress at this time point (10 weeks from study entry). Among these, three patients (4, 17, and 21) had neither new lesions nor progression of measurable disease; four patients (5, 8, 12, and 18) with multiple lesions on entry experienced regression at one or more disease sites; and three patients (1, 9, and 10), who had only limited disease on entry, cleared any evidence of disease. Nonprogressing patients have received additional DC vaccinations on a subsequent study; therefore, we cannot assess the durability of these responses. Patient 5 has received additional immunotherapy at another institution.

Correlation of Immunological Responses and Clinical Outcome

We first used >2-fold increase and ≥ 10 antigen-specific IFN- γ ELISPOTS in the postvaccination assays as an indicator of immune

Fig. 3. Melanoma-specific responses. *A*, circulating melanoma-specific effector cells. PBMCs obtained at baseline and after four DC vaccinations are cultured overnight with each of the four melanoma peptides (*MAGE-3*, *MelanA/MART-1*, *Tyrosinase*, and *gp100* (*Gp-100*) used for vaccination (10 μM). The specific T-cell response in each of the evaluated patients is expressed as the number of IFN-γ ELISPOTS/2 × 10⁵ PBMCs. The values obtained with control gag peptide or in the wells with no peptide are subtracted (on average one spot/2 × 10⁵ PBMC; range, 0–8). *B*, expansion of MelAg-specific memory effector cells. PBMCs obtained at baseline and after four DC vaccinations are cultured for 7 days with mature melanoma-peptide-pulsed autologous Mo-DCs. The T cells are harvested and peptide induced IFN-γ release is measured in the overnight culture with melanoma-peptide pulsed T2 cells. Numbers of IFN-γ ELISPOTS/10⁵ peripheral blood lymphocytes. The values obtained with unpulsed T2 cells are subtracted; *MAGE-3*: median number of spots pre/post vaccine 1/7; range, 0–25/0–23; *MART-1*: median number of spots pre/post vaccine 5/3; range, 0–37/0–33; *Tyrosinase*: median number of spots pre/post vaccine 2/4; range, 0–19/0–39; *gp100* (*Gp-100*): median number of spots pre/post vaccine 2/9; range, 0–25/0–33.



response (16). Two patients (3 and 13) who failed to respond to either the control or MelAgs by any assay, experienced rapid tumor progression and could not complete the planned therapy. Of 17 patients with evaluable disease, 6 of 7 patients who responded to zero, one, or two MelAgs had PD on restaging 10 weeks after study entry (Table 3). In contrast, tumor progression was seen in only 1 of the 10 patients who responded to three or all four MelAgs ($P = 0.002$, Fisher exact test). Regression of >1 tumor metastases were observed in seven of these patients.

To obtain an overall assessment of antitumor immunity after the DC vaccination, we integrated data for absolute immune responses to all four antigens by direct and recall assays into a tumor immunity

score (from -8.5 to +8.5), as described earlier (19, 20). By this comprehensive analysis, tumor immunity is associated with clinical outcome ($P = 0.015$). Six of the eight patients with a negative score, but only 1 of the 9 patients with a positive score, progressed (Fig. 4). Omitting data from the *MAGE-3* epitope, which is thought to require an immunoproteasome for presentation, yields similar results ($P = 0.009$).

DISCUSSION

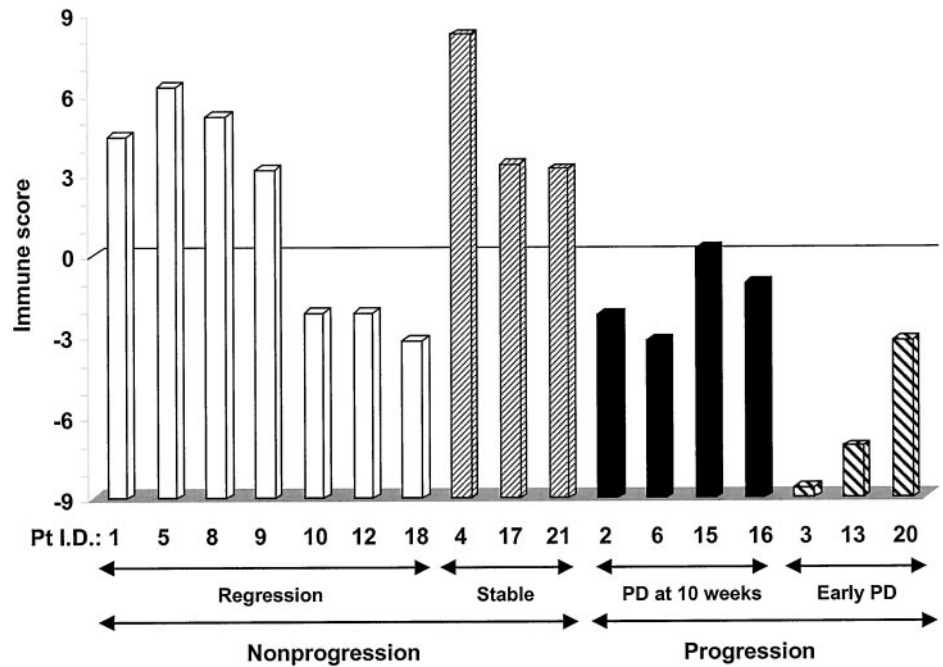
Our results indicate that vaccination of stage IV melanoma patients with antigen-pulsed CD34-DCs is well tolerated and results in en-

Table 3 DC vaccine, immune responses, and clinical outcome

Pt. I.D. ^a	DC vaccine		Immune responses				Clinical outcome
	Dose × 10 ⁶ /kg	CD1a+CD14- / CD1a±CD14+ mean % of positive cells	Control antigens		Melanoma antigens		Early evaluation at 10 wk
			FLU	KLH	n	>2 Ag	
1	0.1	4/25	Yes	Yes	4	Yes	NP
2	0.1	6/28	Yes	Yes	2	No	PD
4	0.1	7/38	Yes	Yes	4	Yes	NP
6	0.1	6/42	Yes	Yes	1	No	PD
19	0.1	10/40	Yes	Yes	2	No	NE
3	0.25	5/44	No	No	0	No	PD
5	0.25	17/20	Yes	Yes	4	Yes	NP
9	0.25	9/32	Yes	Yes	4	Yes	NP
16	0.25	7/25	Yes	Yes	4	Yes	PD
8	0.5	14/33	Yes	Yes	3	Yes	NP
10	0.5	11/42	Yes	Yes	3	Yes	NP
13	0.5	10/36	No	No	0	No	PD
17	0.5	8/27	Yes	Yes	4	Yes	NP
12	1.0	13/24	Yes	Yes	3	Yes	NP
15	1.0	10/52	Yes	Yes	1	No	PD
18	1.0	11/20	ND	Yes	2	No	NP
20	1.0	12/19	Yes	Yes	1	No	PD
21	1.0	8/30	Yes	Yes	3	Yes	NP

^a Pt. I.D., patient identification; Ag, antigen; NP, ●●●; NE, ●●●.

Fig. 4. Correlation of immune response and clinical outcome. Immune responses from both direct and recall assays for all four MelAgs were ranked and integrated into a tumor immunity score (from -8.5 to +8.5). Data are plotted as tumor immunity score (Y axis) versus clinical response (X axis). Each number on horizontal axis, the patient identification (Pt I.D.). Four groups of patients are depicted: patients with regression of one or more lesions postvaccination (Regression, white bars); patients with stable disease (Stable, thin hatched bars); patients whose disease progressed at 10 weeks (PD, black bars); and patients who experienced early progression (before evaluation at 10 weeks, Early PD, thick hatched bars).



hanced immunity to a viral antigen as well as to several MelAgs. Our findings emerged from a Phase I study designed to evaluate the tolerability of this new source of DC vaccine containing LCs. Administration of CD34-DCs leads to no major systemic toxicity. The development of progressive vitiligo in two patients came as an early demonstration that the DC vaccine may enhance immunity to the melanocyte differentiation antigens. Nevertheless, several secondary outcomes with respect to immunogenicity and clinical efficacy were also evident. Our patients, despite prior chemotherapy in some of them and advanced stage of disease, clearly showed immune competence when challenged with two non-MelAgs: the KLH protein to evaluate priming of CD4 T cells and the influenza matrix peptide to test boosting of CD8+ T cells. Immune competence in stage IV melanoma was also noted by Thurner *et al.*, demonstrating T-cell proliferation and DTH to tetanus toxoid delivered on mature Mo-DCs (22).

Injection of peptide-pulsed DCs correlates with enhanced immunity to multiple defined tumor antigens. The MelAg-specific T cells that were elicited after DC vaccine are functional and are detectable in effector T-cell assays without the need for prior *ex vivo* expansion. They are also capable of proliferation and effector function after short-term (1 week) coculture with antigen-bearing DCs, without the need for exogenous cytokines or multiple restimulations with antigen. The feasibility of eliciting immune response to multiple MelAgs suggests that tolerance to these self antigens, if present, may only be partial (23, 24).

The ability of DCs to elicit immune response to multiple tumor antigens *in vivo* may be clinically important. The development of T-cell response to multiple tumor antigens on peptide-pulsed DCs in this study was associated with a favorable early clinical outcome. Prior studies using chemical adjuvants have failed to reliably elicit immunity to MelAgs (25). Improved results were obtained more recently with the use of modified peptides combined with IL-2 (26). However, in most studies, limited clinical responses are observed, which may be attributable to targeting a limited number of epitopes.

We chose to test CD34-DCs because they are composed of two distinct DC subsets, LCs and interstitial DCs (15). This contrasts with

Mo-DCs, which are devoid of LCs (27). Mo-DCs have been shown to act as immune adjuvants in healthy volunteers and in stage IV melanoma (16, 17, 22, 28). Injection of MAGE-3-pulsed Mo-DCs were recently shown to enhance circulating MAGE-3-specific active effectors in melanoma patients (29). However, no clinical responses were observed, which may be attributable to the choice of the immunizing epitope or targeting of a single epitope. Others have reported that CD34-DCs can be more efficient than Mo-DCs in activating CTLs *in vitro* (30, 31). In the circumstances like the induction of tumor-specific CTLs, CD34-DCs could thus be advantageous. Another recent study evaluated CD34-DCs in advanced melanoma and found little clinical or immunological efficacy (32). However, the DCs were cultured in the presence of IL-4 (which inhibits LC development; Ref. 27) and administered *i.v.* Controlled studies are needed to compare the immunogenicity of this new form of DCs (and their subsets) to those derived from blood monocyte precursors.

Although these data provide encouragement for targeting MelAgs using DCs in the clinic, additional studies are needed to establish and optimize their clinical efficacy. The patients who experienced favorable clinical outcome had relatively limited disease and no history of chemotherapy, which supports the concept of testing DC vaccines earlier, *e.g.*, in a surgical adjuvant setting. Optimizing variables such as peptide loading, vaccine schedule, and DC maturation may further improve the immunogenicity of these DCs.

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