

Immunogenicity, Including Vitiligo, and Feasibility of Vaccination With Autologous *GM-CSF*-Transduced Tumor Cells in Metastatic Melanoma Patients

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

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A B S T R A C T

Purpose

To determine the feasibility, toxicity, and immunologic effects of vaccination with autologous tumor cells retrovirally transduced with the *GM-CSF* gene, we performed a phase I/II vaccination study in stage IV metastatic melanoma patients.

Patients and Methods

Sixty-four patients were randomly assigned to receive three vaccinations of high-dose or low-dose tumor cells at 3-week intervals. Tumor cell vaccine preparation succeeded for 56 patients (88%), but because of progressive disease, the well-tolerated vaccination was completed in only 28 patients. We analyzed the priming of T cells against melanoma antigens, MART-1, tyrosinase, gp100, MAGE-A1, and MAGE-A3 using human leukocyte antigen/peptide tetramers and functional assays.

Results

The high-dose vaccination induced the infiltration of T cells into the tumor tissue. Three of 14 patients receiving the high-dose vaccine showed an increase in MART-1- or gp100-specific T cells in the peripheral blood during vaccination. Six patients experienced disease-free survival for more than 5 years, and two of these patients developed vitiligo at multiple sites after vaccination. MART-1- and gp100-specific T cells were found infiltrating in vitiligo skin. Upon vaccination, the T cells acquired an effector phenotype and produced interferon- γ on specific antigenic stimulation.

Conclusion

We conclude that vaccination with *GM-CSF*-transduced autologous tumor cells has limited toxicity and can enhance T-cell activation against melanocyte differentiation antigens, which can lead to vitiligo. Whether the induction of autoimmune vitiligo may prolong disease-free survival of metastatic melanoma patients who are surgically rendered as having no evidence of disease before vaccination is worthy of further investigation.

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INTRODUCTION

Malignant melanoma is an immunogenic tumor, which has encouraged its use as tumor vaccine. Analyses of spontaneous immune responses in melanoma patients have identified a series of antigens, including melanocyte-differentiation antigens, cancer-testis antigens, and antigens from

mutated gene products,¹ which enables monitoring of vaccination-induced immune responses in patients at the level of antigen-specificity of cytotoxic T lymphocytes. Priming of antitumor immunity is improved by appropriate adjuvants, such as the hematopoietic growth factor granulocyte macrophage colony-stimulating factor

(GM-CSF).²⁻⁴ Injection of GM-CSF causes local inflammation, enhanced dendritic cell (DC) maturation, migration, and increased human leukocyte antigen (HLA) –class II expression.⁵ We have previously performed a phase I trial of subcutaneous GM-CSF injections combined with low-dose interleukin-2 (IL-2) and interferon- α (IFN- α), and observed that GM-CSF is well tolerated by patients.⁶ Other studies of GM-CSF injections in melanoma patients have confirmed the low toxicity of GM-CSF and showed occasional tumor regressions or prolonged disease-free survival of stage III or IV melanoma patients after surgical resection of disease.⁷⁻¹⁴

To ensure prolonged GM-CSF levels during vaccination, gene-modified tumor vaccines have been developed that locally produce cytokines at the site of injection.¹⁵ In the B16 murine melanoma model, Dranoff et al¹⁶ demonstrated that transduction of tumor cells with *GM-CSF* increases the immunogenicity of melanoma cells. We performed a phase I/II study in patients with stage IV melanoma using autologous tumor cells transduced with the *GM-CSF* gene. During the time course of our clinical study, Dranoff's group reported the feasibility and limited toxicity of vaccination with *GM-CSF*-transduced autologous tumor vaccines in melanoma patients with several indications for the priming of an immune response by vaccination.^{17,18}

In this article, we describe the occurrence of vitiligo and T-cell responses against known melanoma antigens in patients after *GM-CSF*-transduced tumor cell vaccination. To perform a detailed analysis of melanoma-reactive T-cell responses, we developed a set of 16 HLA/peptide tetramers to detect melanoma antigen-specific T cells in patients expressing HLA-A1, -A2, -A3, -A28, or -B7 molecules. We characterized the functional activity of the melanoma-reactive T cells by analysis of the phenotypic activation state, as well as their responsiveness to antigenic stimulation *in vitro*.

PATIENTS AND METHODS

Eligibility Criteria

Patients were eligible to enter the study if they had progressive stage IV malignant melanoma that was at least partially accessible for resection. Other criteria were Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; normal hepatic and renal function; normal hemoglobin, WBC, and platelet count; no use of systemic steroids; negative tests for hepatitis B and HIV; and magnetic resonance imaging scan showing no evidence of brain metastasis. Prior chemotherapy, immunotherapy, or radiotherapy were allowed if the patient had recovered from all toxic effects and treatment had been completed at least 4 weeks before tumor collection. The trial (M93CSF) protocol was approved by the local medical ethics committee, by the Dutch recombinant DNA advisory committee (Commissie Genetische Modificatie [COGEM]), and by the US Food and Drug Administration. Patients were reassessed after vaccine production and immediately before vaccination, and were allowed to proceed provided that the ECOG performance status was ≤ 2 . Patients with metastasis to

the CNS at that time were eligible for vaccination if they were asymptomatic, there were no signs of neurological impairment, and no tumor had exceeded 1 cm in diameter. Prior radiotherapy or chemotherapy had to be completed 3 weeks before vaccination, and the patient had to have recovered from any toxicity.

Preparation of Vaccine

Tumor for vaccine production was collected from 64 eligible patients by surgery of cutaneous or subcutaneous metastases, or metastases in lymph nodes, lung, liver, or stomach (Table 1). Tumor vaccine production was conducted under current Good Manufacturing Practices. A small portion of the tumor sample was reserved for histologic examination, and the remainder was transported under sterile conditions on wet ice to Somatix Therapy Corp (now Cell Genesys Inc, Alameda, CA). On arrival, samples of the tumor and transport medium were taken for sterility testing and the remaining tumor was mechanically dissociated and digested with collagenase to create a single-cell suspension, which was used to establish a primary culture. Aliquots of nontransduced cells were cryopreserved for delayed-type hypersensitivity (DTH) testing. The retroviral particles used to transduce the primary tumor cells were generated from the MFG-S-human (-hu) GM-CSF producer cell line. This producer cell line was created by the stable introduction, using electroporation, of a plasmid-based retroviral vector containing the human cDNA for GM-CSF into the genome of the Ψ -CRIP packaging cell line.¹⁹ In this vector, the Moloney murine leukemia virus (MoMuLV) long terminal repeat sequences are used to generate full-length RNA needed for the generation of viral particles and subgenomic mRNA analogous to the MoMuLV env mRNA, which is responsible for the expression of the *GM-CSF* transgene. The Ψ -CRIP packaging cell line is derived from NIH 3T3, and it provides the viral proteins gag, pol, and env from MoMuLV necessary for the encapsidation of the recombinant vector genome into transducing viral particles. Ψ -CRIP was specifically designed and constructed with safety features to prevent the encapsidation and mobilization of RNA molecules encoding viral structural gene products. This allows the generation of stocks of replication-deficient retroviral-transducing particles of MFG-S-huGM-CSF with a decreased frequency for generation of replication-competent retrovirus. The MFG-S-huGM-CSF producer cell line was extensively tested according to US United States Food and Drug Administration guidelines and stored as Retroviral Producer Master and Working Cell Banks. The transducing particles produced from the MFG-S-huGM-CSF Retroviral Producer Working Cell Bank in defined lots used for the transduction of the tumor cells were also tested as required. Transduction of the tumor cell cultures was performed as described previously.^{20,21} Briefly, a freshly thawed supernatant containing the viral particles from the producer cell line was layered onto primary tumor cell cultures, seeded at the first passage at a density of approximately 10^4 cells/cm², and left for 12 to 16 hours. The GM-CSF production of the transduced cultures cells was measured by enzyme-linked immunosorbent assay, and if the production was less than 40 ng/10⁶ cells/24 hours, a second round of transduction was performed. After removal of the viral supernatant, propagation of the tumor cell cultures continued for up to two additional passages to meet the required dose of cells needed for vaccination. Cultures were collected, irradiated with 150 Gy, and cryopreserved in liquid nitrogen. Transduced cells were shown to be free of replication

Table 1. Individual Data of Vaccinated Patients

Patient No.	Age (years)	Sex	Previous Therapy	Metastasis Vaccine (sites)	GM-CSF Production*	Clinical Response	Survival (months)	
							PFS	OS
Patients vaccinated with the low-dose tumor-cell vaccine								
1	59	Male	—	sub	169	PD	26	
2	28	Female	—	liver	249	NA	6	21.5
6	49	Male	CT-RT	LN	125	PD	16.5	
8	27	Male	CT-IT-RT	sub	97	NA	96+	96+
10	54	Male	—	sub	46	PD	9	
11	46	Male	RT	cut, LN	124	NA	5.5	73
20	29	Female	—	sub	180	PD	6.5	
24	36	Female	—	lung	209	PD	8	
28	70	Female	RT	LN	153	NA	34	56
29	51	Male	RT	LN	125	NA	6	90+
39	71	Male	—	cut, LN	41	PD	15	
47	36	Female	RT	cut	553	PD	13	
48	48	Female	CT	sub	503	SD	6.5	16.5
55	43	Male	CT	LN	190	NA	8	24
Patients vaccinated with the high-dose tumor-cell vaccine								
18	62	Female	RT	sub	187	PD	10.5	
19	66	Male	IT	sub	128	PD	22.5	
41	65	Male	—	LN	151	PD	18	
45	45	Female	—	LN	189	PD	10.5	
46	38	Female	—	cut	265	NA, vit	84+	84+
50	39	Male	CT	LN	271	NA	4	80
53	58	Male	—	sub	187	PD	17	
57	50	Female	RT-CT	LN	235	PD	10	
58	35	Female	IT	cut	206	PD	14	
59	58	Female	CT	stomach	689	PD	8.5	
61	51	Female	—	cut, sub	738	PD	27.5	
63	46	Male	RT	LN	373	SD	6	37
64	51	Male	—	sub	515	NA, vit	67+	67+
65	63	Male	RT	sub	154	PD	20	

Abbreviations: PFS, progression-free survival; OS, overall survival; GM-CSF, granulocyte macrophage colony-stimulating factor; CT, chemotherapy; IT, immunotherapy; RT, radiotherapy; sub, subcutaneous; cut, cutaneous; LN, lymph node; PD, progressive disease; NA, nonassessable disease; SD, stable disease; vit, patient developed vitiglio after vaccination.
 *GM-CSF production by the tumor cell vaccine (ng/10⁶ cells/24 hours).

competent retrovirus according to US United States Food and Drug Administration guidelines by bioamplification on Mus dunni cells followed by quantitative measurement of vector mobilization by immunocytochemistry. All cultured tumor cells used in the study were free of endotoxin, bacteria, and mycoplasma before use. Immediately before injection of vaccine and DTH cells, vials of tumor cells were thawed rapidly at 37°C, washed twice with Hanks' Balanced Salt Solution (HBSS; Life Sciences Invitrogen, Breda, the Netherlands), checked for viability by trypan blue exclusion, re-suspended in HBSS, and used within 20 minutes. The viability of transduced cells immediately before injection ranged from 77% to 99%. The final dose for vaccine cells was 5 × 10⁶ cells/mL (low dose) or 5 × 10⁷ cells/mL (high dose), and 5 × 10⁵ cells/mL for the DTH cells. Establishment of a primary tumor cell culture and transduction with GM-CSF was successful for 56 (88%) of 64 tumor samples. Tumor cells obtained from six patients, comprising three subcutaneous/cutaneous metastases, two lymph node metastases, and one soft tissue metastasis, failed to proliferate. For two patients, an insufficient amount of tumor was available.

Protocol Design

After re-evaluation of the disease stage in the patients before the start of the vaccination, 17 patients were withdrawn because of rapid disease progression; of the 17 withdrawn, 13 patients had

developed large or symptomatic CNS metastases. One patient was withdrawn because of refusal of treatment. As a result, 38 patients entered the vaccination protocol. The median time from the excision of tumor to the start of vaccination was 10 weeks, with a range of 6 to 26 weeks. At study entry, patients were randomly assigned to receive either 5 × 10⁶ or 5 × 10⁷ transduced tumor cells per vaccination in three vaccinations at 3-week intervals. Initially, three patients were assigned to the low-dose level and observed for a minimum of 21 days. During this period, no toxicity was observed, and the subsequent vaccinations were performed in patients who were assigned to the high- or low-dose treatment by random assignment. Random assignment, performed by the trial office of The Netherlands Cancer Institute, was continued until 14 assessable patients were accrued in both dose levels. Each vaccine was injected into the upper part of the limb, with at least 5 cm separating each injection site; limbs ipsilateral to a prior regional lymph node resection were avoided. The vaccine was administered as two intracutaneous injections and two subcutaneous injections. Vaccination with the high-dose vaccine included one extra subcutaneous injection. In parallel to the vaccine, 5 × 10⁵ DTH cells were injected intradermally. Eight patients were withdrawn after the first vaccination and two patients after the second due to progressive disease, including brain metastases in six of the 10 patients. The intended schedule of three vaccinations was thus completed in 28 patients (Table 1).

Two days after the first vaccination (day 3) and 4 days after the third vaccination (day 47), 4-mm punch biopsies were taken from the vaccination and DTH sites. At days 1 (day of vaccination), 2, 3, 8, 15, and 21 after each vaccination, the patient was examined, vital signs were recorded, local and systemic effects were noted, and toxicity was scored using the National Cancer Institute Common Toxicity Criteria. A CBC, differential, and absolute eosinophil count were measured. Serial blood samples were taken for measurement of urea, electrolytes, serum biochemistry, C-reactive protein, and replication-competent retrovirus. Serum GM-CSF levels were measured immediately before and 6, 24, 48, and 120 hours after the first vaccination. Tumor sites were evaluated weekly. Radiologic evaluation was performed before vaccination, 6 and 9 weeks after the first vaccination, and thereafter when indicated. In patients with asymptomatic small brain metastases, the magnetic resonance imaging scan was repeated before each vaccination. New accessible metastases were removed under local anesthesia from 17 patients during and/or after vaccination (Table 2). Blood samples were taken before vaccination and on days 1, 3, 22, 24, 43, 45, and 64 during vaccination. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation on a Ficoll gradient and frozen in aliquots of 5×10^6 to 10×10^6 cells in liquid nitrogen.

Pathology and Immunohistochemistry

During the study and after completing the entire protocol, metastasectomies were carried out in 17 patients (Table 2). Cryostat sections of tumor samples and biopsies were analyzed for cellular infiltration by hematoxylin and eosin staining and immunohistochemistry using antibodies against CD3, CD4, CD8 (DAKO, Glostrup, Denmark), CD1a, CD14, and CD56 (Becton Dickinson, San Jose, CA). Paraffin sections of tumor material used for vaccine preparation were stained with anti-MART-1/Melan A antibody (Ab; MART-1/Melan-A Ab-3; NeoMarkers, Fremont, CA), gp100-specific Ab (HMB45; DAKO), or tyrosinase-specific Ab (Tyrosinase Ab-1 Clone T311; NeoMarkers). All tissue sections were scored by a pathologist in a blinded fashion. Antigen expression of the tumor cells was scored as the percentage of positive cells. Infiltration of lymphocytes in tumor tissue was quantified by the relative density of infiltration (–, no infiltration; +/-, low; +, intermediate; ++, high; +++, very high density), judged by a pathologist according to standard measures.

Synthesis of HLA/Peptide Tetramers

Synthetic peptides were produced by standard fluorenylmethoxycarbonyl chemistry. Soluble allophycocyanin (APC)-labeled HLA/peptide tetramers were produced as described previously.²²

Table 2. T-Cell Infiltration in Metastases Resected During or After Vaccination

Patient No.	Site of Resected Metastasis*	Day†	Necrosis‡	T-Cell Infiltration§	
				Extent	Density
Patients vaccinated with the low dose tumor cell vaccine					
1	Small bowel	274	+	Absent	–
2	Sub	192	+	Peri	+
2	Sub	192	–	Intra	+
6	Sub	44	–	Intra	+/-
6	Sub	60	–	Intra	+
11	Sub	64	–	Absent	–
24	Sub	43	–	Peri	+
24	Ovary	170	+	Absent	–
29	LN	149	–	Peri	+
39	Sub	28	–	Peri	+/-
39	Sub	39	–	Peri	+
47	Sub	61	–	Peri	+
47	LN	61	–	Peri	+
47	Tonsil	67	–	Absent	–
48	Sub	64	–	Peri	+/-
55	Sub	150	–	Intra	+
Patients vaccinated with the high dose tumor cell vaccine					
19	Sub	201	++	Peri	+/-
41	Sub	70	–	Intra	+++
41	Sub	98	–	Intra	++
45	Sub	91	–	Peri	+/-
53	Sub	25	+	Intra	++
53	Sub	47	++	Intra	+++
53	Sub	64	+	Intra	++
59	Sub	64	–	Intra	+
61	Sub	69	+	Peri	+/-
61	Sub	121	–	Peri	+
65	Sub	155	–	Intra	+++

Abbreviations: sub, subcutaneous; peri, peritumoral T-cell infiltration; intra, intratumoral T-cell infiltration; LN, lymph node; –, no infiltration; +/-, low; +, intermediate; ++, high; +++, very high density.
 *For eight patients, infiltration was analyzed in multiple metastases.
 †Day after start of the vaccination.
 ‡Fibrosis of the tumor tissue was not observed in metastasis analyzed.
 §T-cell infiltration was determined by immunohistochemistry of CD3 expression. No apparent infiltration of T cells was found in the metastases resected before vaccination, except for patient 53, who had minimal T-cell infiltration in one metastasis before vaccination.

HLA-A28 and -B7 heavy-chain biotinylated complexes were provided by Pierre Coulie, PhD (Ludwig Institute for Cancer Research, Brussels, Belgium), and by Ton Schumacher, PhD (The Netherlands Cancer Institute, Amsterdam, the Netherlands), respectively.

Detection of Tetramer-Binding T Cells Among PBMCs

Frozen PBMCs were thawed and incubated with 10 U/mL DNase (Sigma, Zwijndrecht, the Netherlands) in Iscove's medium supplemented with 8% fetal calf serum (Life Sciences Invitrogen, Breda, the Netherlands) for 2 hour at room temperature. Cells were washed and, per staining, 1×10^6 cells were incubated with APC-conjugated HLA/peptide tetramer in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (PBSA) for 15 minutes at 37°C. After washing, the cells were incubated with fluorescein isothiocyanate (FITC) -conjugated anti-CD5 Ab, phycoerythrin (PE) -conjugated anti-CD4, anti-CD19 and anti-T-cell receptor (TCR) $\gamma\delta$ Abs (all antibodies obtained from Becton Dickinson) in PBSA for 20 minutes on ice. Cells were washed and analyzed on a FACScalibur (Becton Dickinson). Dead cells were excluded from analysis by propidium iodide staining. For phenotypic analysis, PBMCs were stained with FITC-conjugated anti-CD27 or anti-CD45RO Ab, PE-conjugated anti-CD8 β , Cy5-conjugated anti-CD45RA Abs, and APC-conjugated tetramer. Because of limited numbers of PBMCs available for analysis, the reactivity with the A2/gp100(154) tetramer was tested neither in patient 45 and patient 46 at day 43 and day 64, nor in patient 58 at days 22, 43 and 45. In patient 41, A1/tyrosinase and A3/flu tetramer reactivity was not tested at day 22, and the reactivity with the A1/flu tetramer was tested only at days 3 and 43.

Detection of Melanoma-Reactive T Cells in the Vitiligo Perilesional Skin

A 4-mm skin biopsy sampled at the perilesional skin of a vitiligo lesion of patient 46 was cultured for 1 week in Yssels medium²³ supplemented with 1% human serum and 20 U/ml recombinant human (rh) IL-2 (Proleukin, Chiron, Amsterdam, The Netherlands). Cells that migrated out of the tissue were treated with erythrocyte lysis buffer (155 mmol/L NH₄Cl; 10 mmol/L KHCO₃; 0.1 mmol/L EDTA acid, pH 7.4), washed and stained with APC-conjugated A2/gp100(154) tetramer, PE-conjugated A2/MART-1 tetramer and FITC-conjugated anti-CD8 Ab (Becton Dickinson). Cells were analyzed on a FACScalibur and dead cells were excluded from analysis by propidium iodide staining.

T-Lymphocyte Culture and Cloning

CD8⁺ T cells were isolated by a magnetically activated cell sorter using anti-CD8 Ab-coated beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and cultured at a ratio of 1:2 with irradiated (40 Gy) autologous CD8⁻ cells that were preloaded with 100 μ mol/L of each peptide in Yssels medium²³ supplemented with 1% human serum and 20 U/mL rhIL-2. Cultures were restimulated weekly with irradiated, peptide-loaded autologous CD8⁻ cells. Antigen-specific T cells were detected in the cultures by tetramer staining. Limiting dilution of tetramer-positive T cells was performed by fluorescence-activated cell sorting (FACStar Plus, Becton Dickinson) at 100 cells/well with 30, 10, or 3 cells, or 1 cell per well densities in round-bottom, 96-well plates. Sorted cells were stimulated biweekly with 5×10^4 /well irradiated allogeneic PBMCs, 0.5×10^4 cells/well JY, 100 ng/mL phytohemagglutinin (HA16, Murex, Dartford, UK) and 20 U/mL rhIL-2 until growing cultures were visible for cloning efficiency determi-

nation. Clones were tested for specific tetramer binding and for specific target-cell recognition in ⁵¹Cr release assays, as described previously.²⁴

T-Cell Activation Assays

T-cell activation was determined by the production of IFN- γ upon stimulation, using the intracellular IFN- γ staining kit (BD Pharmingen, Heidelberg, Germany). Per stimulation, 1×10^6 PBMCs were stimulated with 1 μ mol/L peptide, Brefeldin A (Golgiplug 1:1000 dilution, BD Pharmingen) and 20 U/ml rhIL-2 for 5 hour at 37°C. Positive control incubations for activation were performed in 10 ng/mL phorbol 12-myristate-13-acetate and 2 μ mol/L ionomycin. Cells were washed and incubated with APC-conjugated tetramers and PE-conjugated anti-CD8 β Ab (Coulter Immunotech, Marseille, France). Subsequently, the cells were fixed, permeabilized, and stained with anti-IFN- γ Ab, according to the manufacturer's protocol (BD Pharmingen).

RESULTS

Feasibility and Toxicity of the Vaccination

Twenty-eight stage IV metastatic melanoma patients received three vaccinations with autologous tumor cells that were retrovirally transduced with a retroviral vector encoding the human GM-CSF gene in a phase I/II clinical study. Table 1 shows the individual patient demographics, previous therapy, vaccination dose, and GM-CSF production by the vaccine. Vaccine preparation succeeded for the majority (56) of the original 64 eligible patients in the study (88%), and the treatment was well tolerated. Because of the advanced disease stages of melanomas that were included in our study, 39 (61%) of 64 patients entered the vaccination protocol, of whom only 28 patients completed three vaccinations. Therefore, possible selection of slow-growing disease cannot be excluded.

During the vaccination, no significant changes in vital signs or hepatic, renal, or other organ function were observed. Two patients developed a generalized urticarial rash 10 days after the second injection. They had associated facial and periorbital edema, which resolved with antihistamines. One week after a test dose of 0.5×10^6 nontransduced cells, the patients received the third vaccination, with antihistamines, and experienced no further adverse effects. Systemic toxicity included fever of less than grade 2 in 30% of vaccinations and mild to moderate headache in 33%. Fatigue and generalized pruritus occurred in 20% of patients. These toxicities did not correlate with the dose level of the vaccine, nor were the effects cumulative. Pruritus at the site of vaccination was the most common toxicity, observed with 85% of the vaccinations. The occasionally observed edema and local induration at the site of vaccination caused discomfort.

Clinical Responses and Vitiligo Development

Long-term survival of more than 5 years was observed for six patients (patients 8, 11, 29, 46, 50, and 64; Table 1).

Three of these patients have remained free of disease (patients 8, 46, and 64) and one patient (patient 29) had a subcutaneous metastasis removed after 6 months and has remained free of disease since. Two patients (patients 48 and 63) had stable disease with a duration of 6 months. Nonassessable disease was noted in 9 patients because all tumor sites either had been removed by surgery for vaccine preparation or had disappeared after previous radiotherapy (Table 1). Relapse occurred in 6 of the 9 patients with nonassessable disease after 4 to 34 months (median, 6 months).

Two patients (patients 46 and 64) of the high-dose tumor-cell vaccine group developed vitiligo lesions, an autoimmune disease directed against the melanocytes, at multiple sites a few months after vaccination. These patients experienced long-term disease-free survival of more than 84 and 67 months, respectively (Table 1).

Effector-Cell Recruitment

Metastases were resected from 17 patients during or after vaccination, of which all patients who received the high-dose tumor cell vaccine showed a clear infiltration of T cells and plasma cells at the tumor site (Table 2; Figs

1A and 1B). This infiltration was absent in the metastases that were resected before vaccination, except for patient 53, who had minimal T-cell infiltration in one metastasis before vaccination. The presence of necrosis in the resected metastases did not, however, correlate with the presence of infiltrating T cells. Infiltration of T cells into the tumor was found in four of seven patients analyzed who received the high-dose vaccine and in three of 10 patients analyzed who received the low-dose vaccine (Table 2). Peritumoral infiltration was present in three and six patients of each group, respectively. Moreover, T-cell infiltration was found at a higher density in the patients receiving the high-dose vaccine as compared with patients receiving the low-dose vaccine, suggesting a dose effect of the vaccination.

At the vaccination site, we observed an increased infiltration of CD4⁺ and CD8⁺ T cells (Figs 1C, 1D, and 1E), CD1a⁺ DCs or Langerhans cells (Fig 1F) and CD14⁺ monocytes or macrophages (Fig 1G) after the third vaccination in all patients. Neither residual tumor cells nor natural killer cells (CD56⁺) were found at the vaccination site. DTH reactions to nontransduced tumor cells became positive in all patients after vaccination. These reactions were characterized by infiltration of T cells, mainly CD4⁺ T cells, DCs, and macrophages.

The vaccination induced eosinophilia in the peripheral blood in all patients with peak levels between 1 and 3 days after each vaccination (Fig 2). Patients receiving the high-dose vaccine (Fig 2B) developed more eosinophilia than patients receiving the low-dose vaccine (Fig 2A). GM-CSF was not found in the serum after vaccination.

Detection of T Cells Reactive With Melanoma Antigens Using 11 Different HLA/Peptide Tetramers

We generated a set of 11 HLA/peptide tetramers that consisted of HLA-A1, -A2, -A3, -A28 or -B7 molecules and antigenic peptides from melanoma antigens, MART-1, gp100, tyrosinase, MAGE-A1 or MAGE-A3 (Table 3). In addition, five control tetramers detecting a T-cell population reactive with an antigen that is not shared by melanoma were generated for each HLA allele (ie, influenza [flu] virus peptide presented by HLA-A1, -A2 or -A3 molecules, a MUM-3 peptide and a CD20 peptide binding to HLA-A28 and HLA-B7, respectively). We tested the integrity of specific TCR staining of 15 tetramers by a positive staining of established T-cell clones with the concordant TCR reactivity and a negative staining on a control T-cell clone with identical HLA restriction. In addition, the binding of tetramers to the T-cell receptor was further validated by specific inhibition of tetramer binding to the T cells after preincubation with the anti-CD3 antibody SPV-T3b and goat antimouse immunoglobulin G (P. Weder and R.M. Luiten, unpublished results).

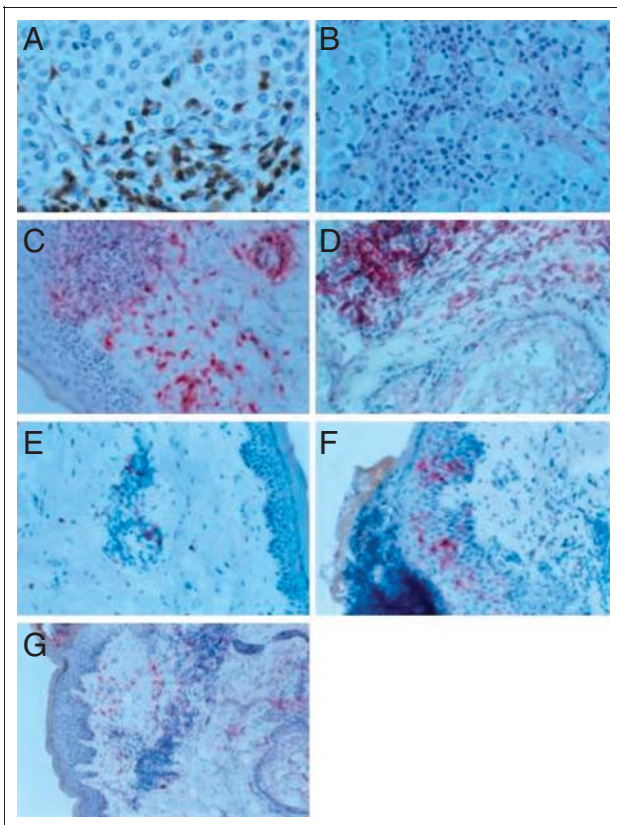


Fig 1. Cellular infiltration. Metastases were stained with (A) anti-CD3 antibody (Ab) or (B) hematoxylin and eosin. Sections of biopsies of the vaccination site after the third vaccination at day 47 were stained with (C) anti-CD3 Ab, (D) anti-CD4 Ab, (E) anti-CD8, (F) anti-CD1a, or (G) anti-CD14. Pictures are representative of all biopsies analyzed.

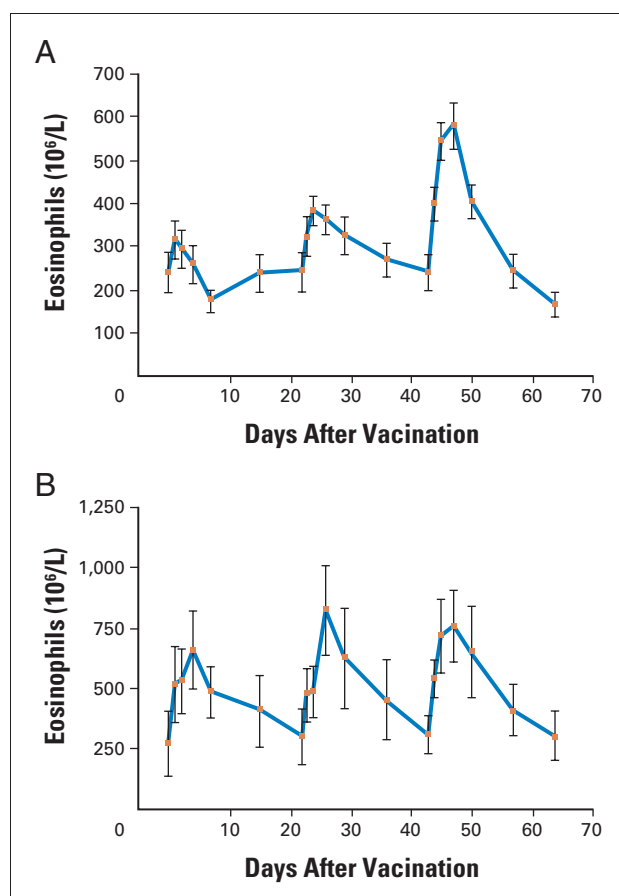


Fig 2. Characteristics of blood eosinophilia on vaccination. Eosinophil counts in the peripheral blood of vaccinated patients were recorded at 1- to 5-day intervals during and after vaccination. Graphs show the average eosinophil counts and standard deviation of the patients receiving (A) the low-dose vaccine, and (B) the high-dose vaccine.

PBMCs for immune analysis were available for 25 of 28 patients who completed vaccination. Twenty-four (96%) of these patients expressed one or more HLA alleles for which tetramers were available (Table 4). In general, this set of tetramers can be used to monitor T-cell responses to multiple epitopes expressed in melanoma in 88% of the white patient population. To avoid the risk of a decreased tetramer staining intensity by simultaneous staining with an anti-CD8 α antibody,²⁵ we chose to perform the incubations with tetramers in combination with an anti-CD5 Ab and excluding the cells that expressed CD4⁻ CD19⁻ TCR- $\gamma\delta$ ⁻. The CD5-high CD4⁻/CD19⁻/TCR- $\gamma\delta$ ⁻ population comprised more than 95% CD8⁻ cells, which was confirmed in a parallel staining with anti-CD8 Ab (Fig 3).

T-Cell Responses in Vaccinated Patients

The level of melanoma-reactive T cells increased during vaccination in three patients receiving the high-dose vaccine (patients 46, 45, and 58; Fig 4). Patient 46 showed an increase in T cells that are specific for gp100(154) or MART-1 peptide bound to HLA-A2, whereas the percent-

age of T cells reactive with A2/MAGE-A3, A2/gp100(280) or A3/MAGE-A1 tetramers remained unchanged. This patient developed vitiligo a few months after the vaccination. The other vitiligo patient (patient 64) showed low levels of A2/MART-1- and A2/tyrosinase-specific T cells, which did not increase during vaccination (Fig 4). Tumor tissue was not available of this patient to test the antigen expression profile, which leaves the possibility that antigens other than MART-1, gp100, or tyrosinase were expressed by the tumor vaccine and triggered the autoimmunity. In patients 45 and 58 of the high-dose vaccine group, we found an increase in A2/MART-1- or A2/gp100(154)-specific T cells (Fig 4), respectively. Immunohistochemical analysis showed, however, that 50% to 90% of the tumor cells of these patients did not express MART-1 or gp100 antigens (Table 4). Despite antitumor immunity, these antigen-negative tumor cells may have expanded, leading to disease progression. Patient 48, who received the low-dose vaccine, showed a minor increase in A3/MAGE-A1-specific T cells and experienced a period of stable disease.

In the other 19 patients analyzed, we found low or undetectable levels of melanoma-reactive T cells that did not increase consistently during or after vaccination. Two representative examples (patients 19 and 41) of the analyses in these patients are shown in Figure 4. Patients 8, 11, and 50 experienced long-term survival after vaccination, but no vitiligo development. Because of the HLA type of these patients (Table 4), T-cell responses could only be monitored by tetramer composed of MAGE-A1 peptides bound to HLA-A3, -A28 or -B7, respectively. T-cell reactivity to the MAGE-A1 antigen was not enhanced by the vaccination in these patients, which does not exclude that T-cell reactivity to other melanoma antigens may have occurred.

T-Cell Activation and Phenotype

To determine the functionality of the melanoma-reactive T cells in the vaccinated patients, we analyzed the phenotype *ex vivo* as well as antigen-specific T-cell activation. The A2/MART-1-reactive T cells of patient 46 consisted of CD27⁺ CD45RA⁺-naive cells, CD45RA⁻-activated/-memory cells, and CD27⁻ CD45RA⁺-effector cells (Fig 5A).²⁶ During vaccination, the number of CD45RO⁻ CD45RA⁺ cells had increased from 10% at day 1 to 21% at day 45. This accumulation in CD45RA⁺ cells represents an increase in effector-cell population, given that the number of CD27⁺ cells had not changed. The MART-1-reactive T cells from patient 46 produced IFN- γ on *ex vivo* stimulation with the A2/MART-1 peptide, and not by control A2/flu-virus peptide stimulation (Fig 5B), indicating MART-1-specific T-cell activation. To evaluate whether these T cells were involved in the vitiligo development, we analyzed the T cells isolated from a biopsy taken from the perilesional vitiligo skin. HLA-A2-/MART-1-reactive T cells were present at a high percentage (3.05%)

Table 3. Overview and Validation of the HLA-Peptide Tetramers

HLA Allele	Antigen	Peptide	Position	Reference	Validation*
HLA-A1	Tyrosinase	KCDICTDEY	243-251	59	+
	MAGE-A1	EADPTGHSY	161-169	60	+
	MAGE-A3	EVDPYGHLY	168-176	61	+
	Influenza	CTELKLSDY	44-52	62	+
HLA-A2	MART-1 gp100	ELAGIGILTV	26-35 (mod. 27A>L)	63	+
		KTWGQYWQV	154-162	64	+
		YLEPGPVTA	280-288	65	+
	tyrosinase	YMDGTMSQV	369-377	66,67	+
	MAGE-A3	FLWGPRLV	271-279	68	+
	Influenza	GILGFVFTL	58-66	69	+
HLA-A3	MAGE-A1	SLFRAVYTK	96-104	70	+
	Influenza	ILRGSVAHK	265-273	59	+†
HLA-A28	MAGE-A1	EVDYDGREHSA	222-231	70	+
	MUM-3	EAFIQPITR	ND	71	+
HLA-B7	MAGE-A1	RVRFFFPSL	289-297	72	+
	CD20	RPKSNIVLL	222-230	73	NA

Abbreviations: HLA, human leukocyte antigen; mod, modified; ND, not determined; NA, cytotoxic T lymphocyte clone not available.
 *Validation of tetramer binding on cytotoxic T lymphocyte clone.
 †Tetramer binding validated on interferon- γ -producing T-cell population in a culture of peripheral blood mononuclear cells stimulated with influenza peptide.

as well as a low level of A2/gp100(154)-specific T cells (0.44%; Fig 5C), indicating that MART-1- and gp100-reactive T cells had migrated to the skin.

We tested whether the melanocyte antigen-specific T cells that we found in several non-responding patients were activated by the vaccination without significant expansion. The phenotype of T cells reactive with the tetramers, A1/MAGE-A3, A1/tyrosinase, A3/MAGE-A1 or A2/gp100(154), in the PBMCs of six patients

(6, 19, 24, 41, 47, and 58) was identical before and during vaccination, and did not differ greatly from the phenotype of T cells reactive with flu tetramers, which were not targeted by the vaccine (Fig 6A). To further evaluate whether the absence of activated/effector T cells against tyrosinase, gp100, or MAGE antigens in nonresponding patients was due to a generalized T-cell suppression or involved only the melanoma-reactive T-cell pool, we compared the melanoma-reactive T cells with the influenza

Table 4. HLA Type and Antigen Expression in the Tumor of the Melanoma Patients Who Have Completed the Schedule of Three Vaccinations

Patient No.	HLA Type*			Tetramers†	Antigen Expression in the Tumor‡			
	HLA-A	HLA-B	HLA-C		MART-1	Tyrosinase	gp100	
1	A1	A26	B27	B57	4	100	90	40
2	A2	A29	B18	B35	6	50	70	20
6	A1	A3	B8	B35	6	80	90	90
8	A3	A25	B56	B57	2	100	100	100
10	A3	A28	B44		4	100	100	80
11	A11	A28	B8	B63	2	100	100	90
18	A11	A3	B62	B17	2	100	100	90
19	A3	A26	B7	B14	4	100	100	100
20	A11	A28	B8	B38	2	100	70	80
24	A1	A11	B44 (12)	B55 (22)	4	0	0	0
28	A3	A24	B12	B35	2	100	100	100
29	A2	A31	B39	B52	6	100	80	100
39	A2	A26	B27	B60(40)	6	100	100	100
41	A1	A3	B51	B60	6	100	90	0
45	A2		B37	B60 (40)	6	60	50	10
46	A2	A3	B18	B62 (15)	8	100	100	90
47	A1	A3	B7	B8	8	100	40	10
48	A3		B44 (12)	B42	2	100	50	70
50	A24 (9)	A29 (19)	B7	B58 (17)	2	100	100	100
53	A24 (9)	A28	B14	B62 (15)	2	100	100	80
55	A24	A31	B13	B63 (15)	0	NT	NT	NT
57	A1	A3	B7	B8	8	70	20	0
58	A2	A29 (19)	B37	B44 (12)	6	10	10	10
59	A2	A3	B18	B37	8	100	100	80
64	A2	A23	B49	B57	6	NT	NT	NT

NOTE. Boldfacing indicates the HLA alleles of the tetramers used. Numbers in parentheses represent the broad HLA type into which the HLA allele can be classified.
 Abbreviations: HLA, human leukocyte antigen; NT, not tested.
 †No. of tetramers used for immunomonitoring
 ‡Values indicate the percentage of antigen-positive tumor cells in the tumor tissue.

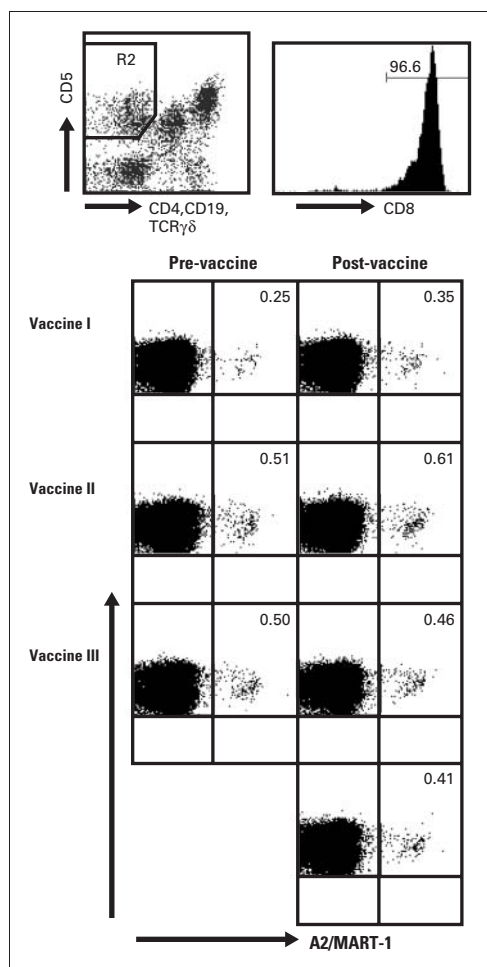


Fig 3. Detection of T cells reactive with melanoma antigens. Reactivity of peripheral blood mononuclear cells of patient 46 with A2/MART-1 tetramer in the CD5-high, CD4⁻, CD19⁻, TCR- $\gamma\delta$ ⁻ population, consisting of > 95% CD8⁺ T cells (histogram). The numbers in the dot plots indicate the percentage of tetramer-reactive cells of CD5-high, CD4⁻, CD19⁻, TCR- $\gamma\delta$ ⁻ population. No reactivity with tetramers was observed in the CD5⁻, CD4⁻, CD19⁻, TCR $\gamma\delta$ ⁻ population.

virus-specific T cells of patients 19, 41, and 58 for the responsiveness to peptide stimulation in vitro (Table 5). T cells that are reactive with MAGE-A1, MAGE-A3, gp100, or tyrosinase peptides did not proliferate upon peptide stimulation, whereas influenza virus-specific T cells did grow out. The limited growth capacity of the melanoma-reactive T cells was also evident from the significantly lower cloning efficiency of MAGE-A1-, MAGE-A3-, or tyrosinase-specific T cells, as compared to influenza virus-specific T cells (data not shown). Furthermore, MAGE-A1-reactive T cells of patient 19 did not produce IFN- γ upon specific peptide stimulation in culture (Fig 6B). The unresponsiveness of melanoma-reactive T cells in these patients even to highly stimulating conditions in vitro may have restricted the potential vaccination effect in these patients and is worthy of further investigation.

DISCUSSION

The phase I/II study described herein demonstrates that vaccination of metastatic melanoma patients with autologous GM-CSF-secreting tumor cells was well tolerated and can lead to vitiligo development. Although the vaccine preparation succeeded for the majority of patients (56 [88%] of 64), the feasibility was limited to 39 (61%) of 64 patients because of the advanced disease status of stage IV melanoma patients in combination with the manufacture time of the vaccine. The time lag of 10 weeks may, however, pose fewer problems when the vaccine is applied in patients with earlier disease stages.

We conclude that the vaccination can enhance melanoma immunity on the basis of the following findings. The vaccination induced infiltration of T cells into metastases that arose during treatment. The vaccination sites were infiltrated by T cells, macrophages, DCs, and eosinophils, and increased levels of melanoma antigen-specific T cells were found in the peripheral blood. Two patients developed vitiligo after vaccination, indicating that GM-CSF-transduced tumor-cell vaccination may even break tolerance to self-antigens expressed on melanoma cells and melanocytes. Because spontaneous vitiligo occurs in less than 0.1% of stage IV melanoma patients (G.C. de Gast, unpublished observations), the observed incidence of vitiligo in two (14%) of 14 patients in the high-dose vaccine group can be considered a vaccination effect. We found T cells that are specific for melanocyte-differentiation antigens, shared between normal melanocytes and melanoma cells, in vitiligo skin and in the peripheral blood. These T cells became more activated toward effector cells during vaccination and produced IFN- γ on specific peptide stimulation. The tumor of vitiligo patient 46 homogeneously expressed MART-1 and gp100 at the time of excision for vaccine preparation (Table 4), which suggests that the in vivo effective T-cell response, as judged by the autoimmunity, played a role in preventing the relapse of the melanoma (Table 1). These results are in agreement with earlier observations of a possible correlation between autoimmunity and relapse-free survival of advanced melanoma patients.²⁷

In other studies of GM-CSF-transduced tumor-cell vaccination in melanoma patients, no vitiligo development was observed.^{17,18} Whether vitiligo develops during immunotherapy depends on the antigen specificity of the primed T cells. Melanoma regression in combination with vitiligo development was observed on adoptive transfer of MART-1- and gp100-specific T cells,^{28,29} or on vaccination with DCs pulsed with gp100, MART-1, or tyrosinase peptides.^{30,31} Furthermore, blocking T-cell regulation with antibodies against CTLA-4 during gp100-peptide vaccination enhanced breakage of tolerance to the gp100 antigen, resulting in melanoma regression and vitiligo.³² In the

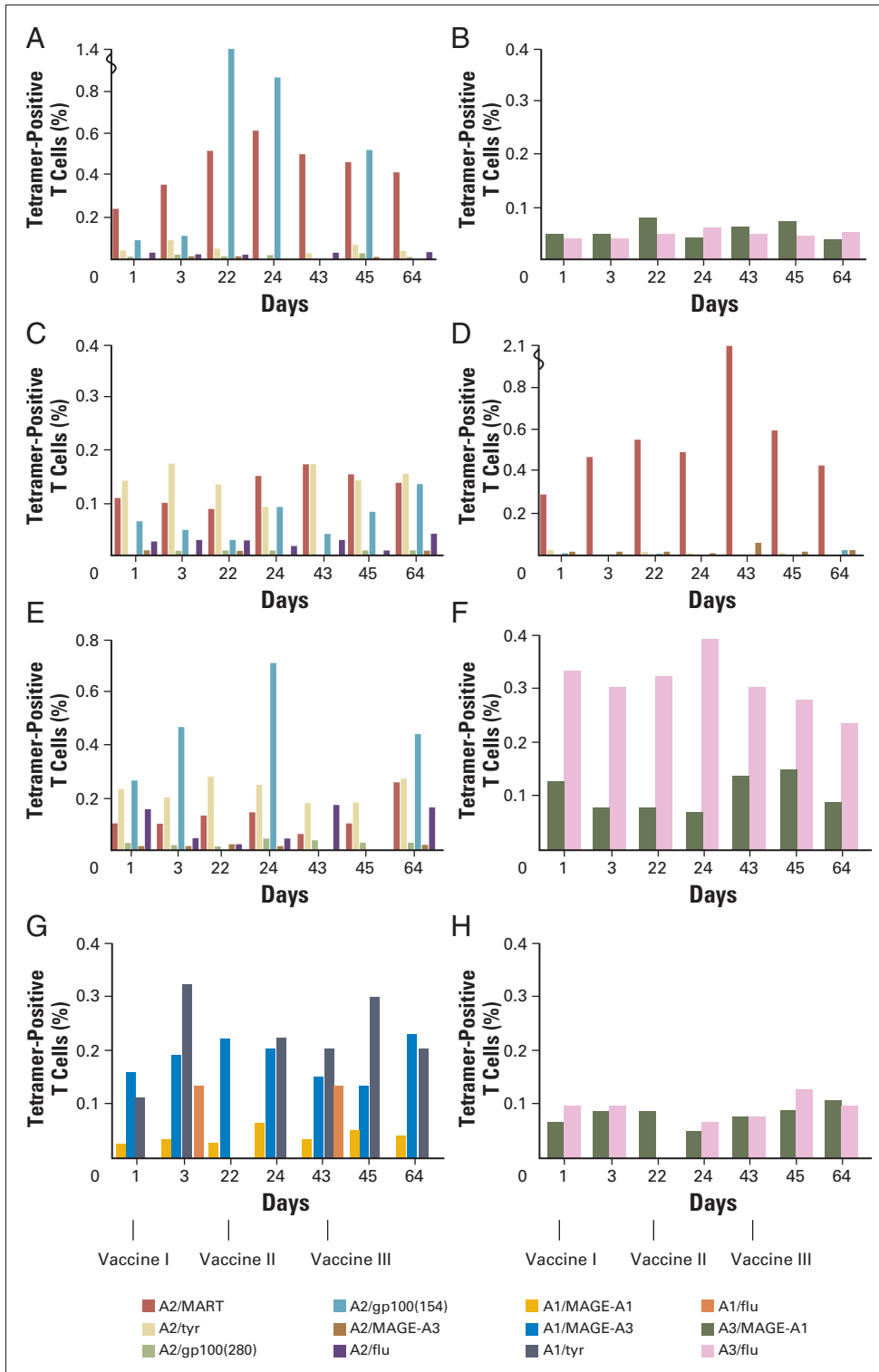


Fig 4. Melanoma-reactive T cells in the peripheral blood. T-cell response during and after vaccination of patients 46 and 64 who developed vitiligo after vaccination and of patients 45, 58, 19, and 41, using HLA-A1, A2 or A3 tetramers. (A) Patient 46, HLA-A2; (B) patient 46, HLA-A3; (C) patient 64, HLA-A2; (D) patient 45, HLA-A2; (E) patient 58, HLA-A2; (F) patient 19, HLA-A3; (G) patient 41, HLA-A1; and (H) patient 41, HLA-A3. HLA, human leukocyte antigen; tyr, tyrosinase; flu, influenza virus.

GM-CSF-transduced melanoma vaccination study of Soiffer et al,^{17,33} the antigen specificity of the T-cell responses was characterized to be against the melanoma inhibitor of apoptosis protein (ML-IAP). ML-IAP is not expressed in normal melanocytes³⁴ and, therefore, will not cause auto-

immunity towards melanocytes and depigmentation. We have investigated the T-cell responses against known melanoma antigens, and found that the development of vitiligo and disease-free survival after vaccination coincided with increased levels of activated MART-1- and

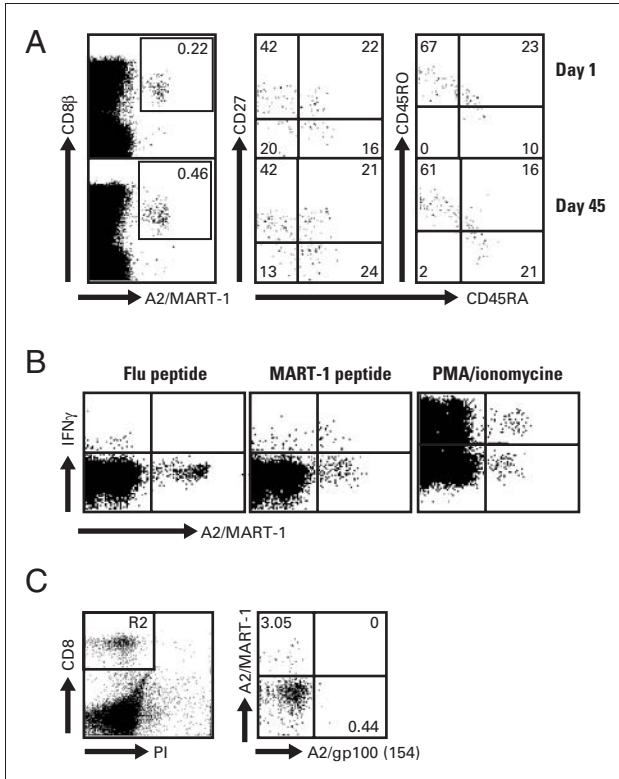


Fig 5. Autoimmunity in vitiligo patient 46. (A) Percentage of cells expressing CD45RA, CD27, or CD45RO of A2/MART-1-tetramer⁺ T cells at days 1 and 45. (B) Intracellular interferon- γ staining of A2/MART-1-tetramer⁺ T cells with A2/flu or MART-1 peptide, or PMA/ionomycin. (C) A2/MART-1 and A2/gp100(154) tetramer⁺, viable (PI⁻) CD8⁺ T cells in perilesional vitiligo skin. Flu, influenza virus; PMA, phorbol 12-myristate-13-acetate; PI, propidium iodide.

gp100-specific T cells in the periphery and infiltrating in vitiligo. These results suggest that the GM-CSF-transduced tumor cell vaccination can activate T cells to exert lytic activity against normal melanocytes as well as against residual melanoma cells in vivo.

Antitumor immune responses were mostly found in patients treated with the high-dose vaccine, which indicates that the high-dose vaccine was more effective than the low-dose vaccine. In patients who did not respond to the vaccination, we observed that the melanoma antigen-specific T cells were not activated to grow or produce cytokine on ex vivo antigenic stimulation, which may be due to the advanced disease stage. Nonresponsiveness of T cells toward tumors has been observed frequently in a variety of cancer patients^{35,36} and should be overcome to achieve effective antitumor immunity. Several mechanisms that explain this lack of responsiveness have been described. For example, most tumor antigens are self-antigens to which tolerance exists. Moreover, tumor antigens are not presented in an inflammatory environment, which may lead to the induction of T-cell tolerance. GM-CSF has a major effect on the activation and function of DCs, and may provide the required stimulus for the

DCs to prime T cells against tumor antigens. On the other hand, anergy to self-antigens may persist as a result of the continuous level of self-antigen presentation. Vitiligo development was observed in two of the three patients in the high-dose vaccine group with nonassessable disease during vaccination. In these patients, the melanocyte-differentiation antigens (shared among tumor cells and melanocytes) were present at a lower level because of the absence of tumor tissue. This temporary lower level of antigen may have facilitated the T cells' regaining their

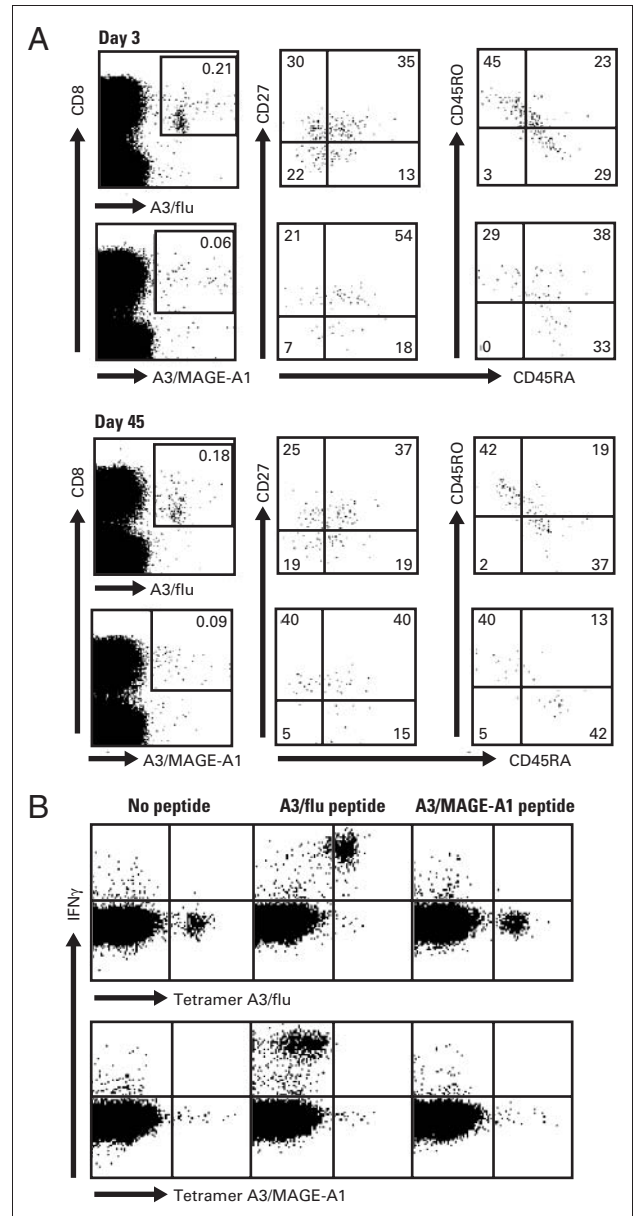


Fig 6. Activation of melanoma-reactive T cells during vaccination. (A) Phenotype of A3/flu- (upper panel) or A3/MAGE-A1-tetramer⁺ T cells (lower panel) of patient 19 at day 3 (above) and 45 (below), representative of all 6 patients analyzed. (B) Interferon- γ production by A3/flu- or A3/MAGE-A1-tetramer⁺ T cells of patient 19 stimulated with A3/flu peptide, A3/MAGE-A1 peptide, or without peptide. Flu, influenza virus.

Table 5. Responsiveness of Melanoma-Reactive CTL to In Vitro Stimulation With Peptide

Experiment	Patient No.	Peptide Stim	Tetramer	% of Tetramer ⁺ -Positive Cells of the CD8 β ⁺ Cells*					
				Day 0†	Stim 1†	Stim 2	Stim 3	Stim 4	Stim 5
C	19	A3/flu + A3/MAGE-A1	A3/flu	0.29	2.43	22.92	NT	NT	NT
			A3/MAGE-A1	0.08	0.09	0.08	0.28	0.04	NT
D	41	A3/flu + A1/tyr + A1/MAGE-A3	A3/flu	0.07	1.22	3.40	NT	NT	NT
			A1/tyr	0.23	0.20	0.25	0.22	0.37	0.30
			A1/MAGE-A3	0.20	0.24	0.31	0.13	0.12	0.11
E	41	A3/flu + A3/MAGE-A1	A1/flu	0.02	0.60	1.91	NT	NT	NT
			A3/MAGE-A1	0.07	0.07	0.09	0.28	0.05	NT
F	58	A2/flu + A2/gp100(154)	A2/flu	0.04	6.29	3.66	NT	NT	NT
			A2/gp100(154)	0.47	0.22	0.05	0.07	0.03	NT
H	58	A2/tyr + A2/gp100(280)	A2/tyr	0.20	0.29	0.05	0.15	0.03	NT
			A2/gp100(280)	0.02	0.03	0.00	0.03	0.01	NT

Abbreviations: CTL, cytotoxic T lymphocyte; stim, stimulation; flu, influenza virus; NT, not tested; tyr, tyrosinase.

*Tetramer-positive cells were measured at day 0 and 1 week after each stimulation (stim 1, stim 2, etc). At stim 3, 4, and 5, flu peptides were excluded from the peptide mixture that was used to stimulate the cultures to avoid overgrowth of the flu-reactive T cells.

responsiveness to these self-antigens, as was described for CD4⁺ T cells in a TCR-transgenic murine model.³⁷ These results suggest that the tumor cell vaccinations performed here are most effective in breaking tolerance to self-antigens and inducing autoimmunity when applied in a minimal residual disease setting. To achieve activation of melanoma-reactive T cells in the nonresponding patients of this study, in whom tumor tissue was present during vaccination, may, therefore, require more than three vaccinations. Indeed, the patients who responded to *GM-CSF*-transduced melanoma vaccines described by Soiffer et al received 6 or more weekly or biweekly vaccinations.^{17,18} They did not observe melanoma immunity in patients who were vaccinated with only three doses every 28 days, which vaccination schedule is most comparable to our study. Likewise, the clinical and immunologic effects in other clinical trials of cytokine-secreting tumor cell vaccines were observed only in patients who were subjected to frequent vaccinations at short time intervals,^{18,21,38-44} or following adoptive transfer of primed lymph node cells.⁴⁵

We describe here the long-term survival of six (21%) of 28 vaccinated stage IV melanoma patients for more than 5 years. Other vaccination trials of stage III or IV melanoma patients with various types of vaccines, including peptides, protein, DCs, tumor cell lysates, autologous tumor cells, or cytokine-transduced tumor cells, have reported 3-year survival rates ranging from 5% to 30%.^{17,18,38,46-49} Chapman et al⁵⁰ observed an even higher 3-year survival rate of 71% after vaccination with anti-GD3 ganglioside antibody. However, many clinical trials have not yet reached a long-term follow-up,^{30,39,51-55} and studies in which advanced melanoma patients are followed for more than 5 years are, therefore, less abundant. Dinitrophenyl-haptenated tumor cell vaccination resulted in 30% survival after 5 years.⁵⁶ A vaccination study with tumor cells mixed with *GM-CSF* revealed a 5-year survival of 23%,⁵⁷ which is comparable to

our study. It is, however, intriguing to note that in our study all patients (three of three) in the high-dose vaccination group that entered the vaccination with nonassessable disease experienced prolonged survival, as well as half of the patients (three of six) with nonassessable disease receiving the low-dose vaccine (Table 1). This observation compares beneficially to the reported the 5-year survival of 21% of patients with resected stage III or IV melanoma without vaccination, or 41% after allogeneic tumor-cell vaccination.⁵⁸ Moreover, Spittle et al¹⁰ have reported that *GM-CSF* administration can prolong survival by 4 years in 50% of resected stage III or IV melanoma patients. Although the numbers in our study are too low for statistical conclusions, our results suggest that *GM-CSF*-transduced tumor cell vaccination may be most effective in prolonging survival in an adjuvant setting.

Together, the results of our study show that the *GM-CSF*-transduced autologous tumor cell vaccination can activate melanoma-specific T-cell responses and autoimmunity in stage IV metastatic melanoma patients. Whether the induction of autoimmune vitiligo may prolong disease-free survival of metastatic melanoma patients who are surgically rendered as having no evidence of disease before vaccination is worthy of further investigation.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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