Boosting T Cell-Mediated Immunity to Tyrosinase by Vaccinia Virus-Transduced, CD34+-Derived Dendritic Cell Vaccination: A Phase I Trial in Metastatic Melanoma

Massimo Di Nicola,1 Carmelo Carlo-Stella,1,8 Roberta Mortarini,2 Paola Baldassari,2 Anna Guidetti,1 Gian Francesco Gallino,3 Michele Del Vecchio,4 Fernando Ravagnani,5 Michele Magni,1 Paul Chaplin,9 Natale Cascinelli,6 Giorgio Parmiani,7 Alessandro M. Gianni,1,8 and Andrea Anichini2

1“Cristina Gandini” Bone Marrow Transplantation Unit, 2Human Tumor Immunobiology Unit, 3Colo-Rectal Surgery Unit, 4Medical Oncology B Unit, 5Laboratory Medicine Unit, 6Scientific Direction, and 7Human Tumor Immunotherapy Unit, Istituto Nazionale Tumori, Milan, Italy; 8Chair of Medical Oncology, University of Milan, Milan, Italy; and 9Bavarian Nordic GmbH, Martinsried, Germany

ABSTRACT

Purpose: Six American Joint Committee on Cancer stage IV melanoma patients were enrolled into a Phase I study of vaccination with autologous CD34+-derived dendritic cells transduced with a modified vaccinia Ankara virus encoding human tyrosinase gene (MVA-hTyr).

Experimental Design: Patients received a first intravenous injection of 1 × 10^8 MVA-hTyr-transduced dendritic cells, followed by three s.c. injections at a 14-day interval.

Results: Treatment was well tolerated, except for low-grade fever (three of six patients), mild erythema at injection site (five of six), and vitiligo (two of six). A partial response, involving shrinkage of an s.c. nodule, later surgically re-moved, was observed in 1 patient, who then remained disease-free (>850 days). By human lymphocyte antigen tetramer analysis, significant and often long-lasting increases in frequency of T cells directed to tyrosinase368–376 but not to gp100299–217 were documented in periphery of 4 of 5 HLA-A*0201+ patients, a few days after vaccine administration. In addition, maturation phenotype of tyrosinase-specific T cell shifted toward the T effector memory/T

terminally differentiate stages (CCR7–CD45RA–/+) in synchrony with the T-cell frequency peaks. By enzyme-linked immunospot in peripheral blood of five HLA-A*0201+ patients, we found that the vaccine could induce interferon γ-releasing effector cells directed to HLA-A*0201/tyrosi-nase368–376 and to vaccinia virus HLA-A*0201/HLA-184–192 epitopes. Moreover, an interferon γ response after vaccination was elicited even against the HLA-DRB1–1501/tyrosi-nase386–406 epitope in one out of two HLA-A* DRB1–01501+ patients.

Conclusions: These results indicate that vaccination with MVA-hTyr–transduced dendritic cells is well tolerated, can possibly produce clinical responses, and activates tyrosinase- and vaccinia virus-specific T cells in vivo. These data suggest a broad utility of the MVA vector for targeting tumor-associated antigens to dendritic cells for tumor immunotherapy.

INTRODUCTION

Advanced melanoma is an aggressive malignancy with poor prognosis with current standard therapies (1, 2). In American Joint Committee on Cancer stage IV, the median survival is ~6 months, and only a minority of these patients, eligible for surgical resection of metastases, have a median survival of 15–31% at 2 years (1, 2). These results underscore the need for new therapeutic approaches for advanced melanoma and explain the efforts toward development of innovative immunologic therapies aimed at inducing/boosting T cell-mediated response to tumor-associated antigens (TAA) by vaccination (3, 4). In the attempt to achieve this goal, several trials have been carried out by administering to patients dendritic cells loaded with tumor antigen-derived peptides (reviewed in Refs. 5, 6). However, in such approach, the need for matching the human lymphocyte antigen genotype of the patient with the human lymphocyte antigen allele bound by the tumor antigen-derived peptides has imposed a significant restraint on patient enrollment criteria. Alternatively, TAA transfer to dendritic cells can be achieved by transfection with cDNA, RNAs, encoding tumor antigen, or by infection with recombinant viruses (7–10), thus leading to expression in dendritic cells of all of the potential T cell epitopes encoded by a given TAA (11).

With respect to vector selection for TAA transfer to dendritic cells, vaccinia virus, a member of the poxvirus family, represents an attractive candidate, because it is not oncogenic and does not integrate into the host genome (12). In particular, the modified vaccinia Ankara strain, a replication-defective form, has lost the capacity to infect productively mammalian cells while remaining effective to achieve expression of heterologous genes (13). Modified vaccinia Ankara has an excellent safety profile in humans (14), and its immunologic efficacy, as gene transfer tool, has been proven in vitro by the successful
induction of TAA-specific responses after T cell coculture with modified vaccinia Ankara-infected dendritic cells (15). In addition, by administration of recombinant modified vaccinia Ankara, either alone or in DNA-modified vaccinia Ankara prime-boost combinations, significant induction of T cell-mediated immunity has been elicited in vivo, in humans, against HIV-1 nef or against Plasmodium falciparum telomeric repeat amplification protocol proteins (16, 17).

In this study recombinant modified vaccinia Ankara encoding tyrosinase, a melanoma-associated antigen recognized by both CD4+ and CD8+ T cells (11), was used to infect CD34+-derived dendritic cells from metastatic melanoma patients. The transduced antigen-presenting cells were then administered to six stage IV melanoma patients in a Phase I trial of vaccination. The results indicated that this vaccination strategy was safe and highly effective in promoting/boosting T cell-mediated responses to the melanoma-associated antigen.

MATERIALS AND METHODS

Patients and Trial Design. Patients with stage III or stage IV malignant melanoma refractory to standard therapeutic regimens were enrolled in a nonrandomized, noncontrolled, single-center, open label Phase I study. The protocol was approved by the Ethical and Scientific Committees of Istituto Nazionale Tumori (Milan, Italy) and by the Gene Therapy Committee of the Italian Ministry of Health. Written informed consent was obtained from each patient before enrolling in the study. Trial design and objectives, as well as eligibility and exclusion criteria, were described extensively elsewhere (18). Briefly, the trial was designed to enroll 6 patients with the primary objective of defining the safety and toxicity of injections of autologous CD34+-derived dendritic cells transduced ex vivo with a recombinant nonreplicating vaccinia vector encoding the human tyrosinase gene (DCs/MVA-hTyr). Secondary objective was to determine whether immunization with DCs/MVA-hTyr vaccine could promote tyrosinase-specific immune responses. Enrolled patients were selected on the basis of measurable, histologically confirmed, malignant melanoma stage IV or high-risk stage III and whose metastatic tumor expressed the tyrosinase antigen as evaluated by immunohistochemistry. Serologic and molecular human lymphocyte antigen typing was performed as described (19).

Mobilization of CD34+ Progenitor Cells in Peripheral Blood and Dendritic Cell Differentiation. To mobilize peripheral blood CD34+ progenitor cells, patients received daily s.c. injections of glycosylated recombinant human granulocyte colony-stimulating factor (15 μg/kg body weight; Myelostim; Italfarmaco, Milano, Italy) for 5 days. Two 150-ml samples of nonheparinized venous blood, collected on days 5 and 6 of the CD34+ mobilization phase, were used as source of serum for autologous dendritic cell cultures. Large-scale collection of peripheral blood progenitor cells was carried out by leukapheresis using a continuous flow blood cell separator (COBE-SPECTRA, Lakewood, CO). Harvested peripheral blood progenitor cells from the six enrolled patients contained 205 × 10^6 ± 20 × 10^6 CD34+ cells. CD34+ progenitor cells were then positively selected using clinical grade ClineMACS Instrument (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; model no. 151–01). The yield of CD34+ selected cells, after ClineMACS processing, was 164 × 10^6 ± 30 × 10^6 with an average purity of 95% ± 2. Purified CD34+ cells were cultured with 10% autologous serum in Iscove’s modified Dulbecco’s medium (Cambrex, Milan, Italy) supplemented with granulocyte macrophage colony-stimulating factor (50 ng/ml; Myelogen/Leucomax, Schering-Plough/Sandoz, Milan, Italy), tumor necrosis factor α (10 ng/ml; Knoll, Ludwigshafen, Germany), recombinant human stem cell factor (50 ng/ml; Amgen, Thousand Oaks, CA), and Flk-2/Fli-3 ligand (50 ng/ml; Immunex, Seattle, WA), as described (18). Generation of dendritic cells was estimated after 12 days of culture by evaluation of CD1a+ CD14+ cells (Langherans cells) and CD1a+ CD14+ cells (interstitial dendritic cells) through two-color flow cytometry using a FACScalibur instrument (Becton-Dickinson, San Jose, CA) and the Cell Quest software (Becton-Dickinson). At day 12 immunophenotype indicated a mean percentage of 25.5% CD1a+ CD14+ dendritic cells (range, 18–40%) and 19.5% CD1a− CD14+ dendritic cells (range, 10–30%). Both the CD1a+ CD14+ and the CD1a− CD14+ fractions showed a mature phenotype, as assessed by coexpression of CD40, CD80, CD83, CD86, and HLA-DR. Harvested dendritic cells were divided into 4 aliquots of 140 × 10^6 cells, resuspended in 50 ml of cold cryoprotectant mixture (90% autologous serum; 10% DMSO) and then frozen into 100-ml polyolefin freezing bags (Delmed, New Brunswick, NJ).

Vaccinia Vector and Dendritic Cell Transduction. Wild-type modified vaccinia Ankara vector, MVAP11LZ vector expressing the Escherichia coli lacZ gene, and MVA-hTyr vector carrying the gene for human tyrosinase were supplied from Bavarian Nordic GmbH (Martinsried, Germany). The MVA-hTyr vector, containing the entire tyrosinase coding sequence, inserted into deletion 2 site of modified vaccinia Ankara genome, was generated and characterized as described previously (15). DCs/MVA-hTyr vaccine was prepared by infection of dendritic cells with the MVA-hTyr vector as described recently in the published trial protocol (18). Briefly, in a level II containment laboratory, after thawing, 100 × 10^6 viable dendritic cells were cultured overnight at 37 °C, 5% CO2 in a 150-cm^2 tissue culture flask with 30 ml of cold cryoprotective mixture (90% autologous serum; 10% DMSO) and then frozen into 100-ml polyolefin freezing bags (Delmed, New Brunswick, NJ).
Presentation of Tyrosinase Epitopes by MVA-hTyr–Transduced Dendritic Cells to Cytotoxic T-Lymphocytes. HLA-A*0201+ dendritic cells, isolated from CD34+ progenitors and transduced or not with MVA-hTyr or with MVA-P11LZ were assessed for susceptibility to cell-mediated lysis by tyrosinase368–376 or Melan-A/Mart-126–35 specific cytotoxic T-lymphocyte (CTL) clones. CTL clones to tyrosinase (CTL-C4 and CTL-C11) or to Melan-A/Mart-1 (CTL-A83) were isolated from HLA-A*0201+ melanoma patients after mixed lymphocyte-tumor culture as described (20). Dendritic cells at days 12–14 of culture were infected with vaccinia vectors at a multiplicity of infection of 5 and, after 3 h, were washed and labeled with 51Cr as described (19). MVA-hTyr–transduced and MVA-P11LZ-transduced dendritic cells were then seeded at 1 x 10^3/well in 96-well round-bottomed plates and incubated for an additional 8 h at 37°C. Effector cells were then added to infected and noninfected dendritic cells. As a control, in the same assay, CTLs were also tested against an aliquot of 51Cr-labeled dendritic cells loaded or not with 10 μg/ml Melan-A/Mart-12–35 or tyrosinase368–376 peptides. Lysis by peptide-specific CTL clones and by LAK cells [T-cell lines generated by culturing healthy donor peripheral blood lymphocytes (PBLs) for 2 weeks with 1000 units/ml of interleukin 2] was then assessed in a 4-h 51Cr release assay at an effector:target ratio of 2.5:1.

Peripheral Blood Lymphocytes and Human Lymphocyte Antigen Tetramer Analysis. Lymphocytes were isolated from peripheral blood as described (19). Three pretherapy blood samples were collected immediately preceding the first vaccination. Subsequent blood samples were obtained on a weekly basis during vaccination (until day 42), and then once every 1–2 months, according to patient status and tumor progression. Phycoerythrin-labeled tetramers of HLA-A*0201-containing peptides from gp100269–217 (21) and tyrosinase368–376 (22) were purchased from Proimmune Ltd. (Oxford, United Kingdom). Tetramers were titrated against peptide-specific T-cell lines to minimize background staining whereas preserving mean fluorescence intensity of positive cells and then used at the final dilution of 1:200 of the stock solution (23). Two x 10^6 cells were stained for 15 min with phycoerythrin-labeled tetramers at 37°C and then stained for 30 min on ice with anti-CD8 coupled to PerCP (BD PharMingen, San Diego, CA). The lower limit of detection for tetramer analysis was set at 0.01% of CD8+ cells (i.e., 1/10,000 CD8+ cells) based on analysis of PBL from HLA-A*0201+ healthy controls (24). Maturation phenotype of tetramer+CD8+ T cells was performed by staining lymphocytes with phycoerythrin tetramers and then with anti-CD8, anti-CCR7, and anti-CD45RA (BD PharMingen) as described (23). Acquisition and analysis by flow cytometry were carried out by a dual-laser FACScalibur cytometerfluorimeter (Becton Dickinson) using the CellQuest software.

Interferon γ Enzyme-Linked Immunospot. The interferon γ enzyme-linked immunospot assay was performed in 96-well Multiscrut hemagglutinin plates (Millipore, Bedford, MA). To this end, plates were coated at 4°C overnight with 15 μg/ml of 1-D1K anti-interferon γ monoclonal antibody (Mabtech, Stockholm, Sweden) in NaHCO3 0.1 m (pH 9.6) solution. Plates were seeded with 1 x 10^5 cells/well of uncultured lymphocytes from PBLs of HLA-A*0201+ vaccinated patients with 2 x 10^4 cells/well of a peptide-loaded or empty HLA-A*0201+ T2 cells in RPMI 1640, 10% pooled human serum. The following peptides (PRIMM s.r.l.; San Raffaele Biomedical Science Park, Milan, Italy) were used: tyrosinase368–376 and modified vaccinia Ankara envelope protein H3L184–192 (25). As a control, the response of uncultured lymphocytes to T2 cells loaded with influenza-matrix388–406 peptide (in HLA-A*0201+ patients) was also assessed. For detection of CD8+ T cells in response to empty antigen-presenting cells was <10% of the number of spots obtained in response to peptide-loaded antigen-presenting cells and was subtracted from the latter value.

Statistical Analysis and Immunologic Response Criteria. Comparison of different blood samples for the number of interferon-γ producing cells in enzyme-linked immunospot was carried out by analysis of variance followed by Student-Newman-Keuls multiple comparison test. Pre-postvaccine tetramer frequency values were analyzed with Wilcoxon signed rank tests. The main criteria for immunologic response were: a) evidence for significant increase in frequency of tetramer+ T cells or in frequency of cytokine-secreting cells in response to tyrosinase or modified vaccinia Ankara H3L epitopes, at any time point during or after vaccination, compared with pretherapy values; b) lack of significant increase in frequency of T cells directed to control antigens not targeted by vaccination (gp100269–217 peptide for tetramer analysis and Flu-Matrix388–406 peptide for enzyme-linked immunospot) in the same blood samples evaluated for response to tyrosinase and MVA-H3L epitopes.

RESULTS
MVA-hTyr–Transduced Dendritic Cells Process and Present Tyrosinase Epitopes to CTL Clones. To assess whether dendritic cell infection with MVA-hTyr vector resulted in presentation of tyrosinase epitopes, HLA-A*0201+ dendritic cells were tested for lysis by tyrosinase-specific HLA-A*0201-restricted CTL clones, after infection with MVA-hTyr. Two different CTL clones specific for the tyrosinase368–376 peptide lysed CD34+-derived dendritic cells after infection with MVA-hTyr, but not after infection with a control modified vaccinia Ankara vector coding for LacZ (Table 1). Moreover, extent of lysis of MVA-hTyr–transduced dendritic cells, by tyrosinase-
Vaccination of Melanoma Patients

(CTL-A83) recognized the HLA-A*0201/H11001 positive control of target susceptibility to cell-mediated lysis. To assess for lysis by CTL clones to tyrosinase 368 were assessed for lysis by CTL clones on an MVA-infected peptide-loaded dendritic cells (Table 1). Similar results were obtained by testing the same CTL clones on an MVA-infected peptide-loaded HLA-A*0201. Moreover, the results suggest that dendritic cell transduction with modified vaccinia Ankara vectors (Table 1). These results are in agreement with those obtained by infecting monocyte-derived dendritic cells with MVA-hTyr (15) and indicate that MVA-hTyr transduction of dendritic cells leads to efficient expression of tyrosinase-specific epitopes recognized by CTLs in association with HLA-A*0201. Moreover, the results suggest that dendritic cell transduction with modified vaccinia Ankara vector is as efficient as exogenous peptide loading in the generation of human lymphocyte antigen-tyrosinase peptide complexes recognized by T cells.

**Clinical Results.** Six American Joint Committee on Cancer stage IV melanoma patients were enrolled into a Phase I trial between July 2000 and July 2001. All but one patient (patient 6) had extensive metastatic disease, and the main characteristics are listed in Table 2. Five patients were HLA-A*0201+. All of the patients completed the four planned vaccinations consisting of injections of $10^6$ MVA-hTyr–transduced dendritic cells at 2-week intervals. The treatment was well tolerated. Grade 1 erythema without induration and itching at injection sites was observed consistently after each subcutaneous vaccination in five of six patients and resolved without sequelae over the subsequent 48–72 h. Mild fever was observed in three of six patients 12 h after vaccination but disappeared without specific treatment. Two patients developed localized (patient 1) or generalized (patient 3) vitiligo during the course of dendritic cell therapy. Patient 3 developed multiple areas of vitiligo on both arms and on the back. Each area measured $<1$ cm of diameter. No significant hepatic, renal, pulmonary, cardiac, hematologic, gastrointestinal, or neurologic toxicities attributable to treatment were observed. In five patients (patients 1–5) disease was stable during therapy, but progressed between 1 and 9 months after the end of the vaccination program. Eventually, patients 3, 4, and 5

### Table 1 Recognition of MVA-hTyr–transduced dendritic cells by tyrosinase-specific CTL clones

<table>
<thead>
<tr>
<th>CD34+–derived HLA-A*0201+ dendritic cells</th>
<th>HLA-A*0201+ lymphoblastoid cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected with MVA vector</td>
<td>Loaded with peptide</td>
</tr>
<tr>
<td>Effectors</td>
<td>hTyr</td>
</tr>
<tr>
<td>CTL-C4</td>
<td>2.0†</td>
</tr>
<tr>
<td>CTL-C11</td>
<td>2.6</td>
</tr>
<tr>
<td>CTL-A83</td>
<td>0.0</td>
</tr>
<tr>
<td>LAK cells</td>
<td>35.6</td>
</tr>
</tbody>
</table>

*CD34+-derived dendritic cells and an HLA-A*0201+ lymphoblastoid cell lines infected with vaccinia vectors or loaded with synthetic peptides were assessed for lysis by CTL clones to tyrosinase (15) or to Melan-A/Mart-126-35 (CTL-A83) or by LAK cells (as positive control of target susceptibility to cell-mediated lysis).

† Results expressed as percentage of lysis at 2.5:1 effector:target ratio in a 4 h Cr release assay.

### Table 2 Patients characteristics and response to treatment

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>HLA-A*0201</th>
<th>AJCC stage</th>
<th>Age/Sex</th>
<th>Tumor site</th>
<th>Previous treatment</th>
<th>Toxicity/grade*</th>
<th>Vitiligo</th>
<th>During vaccination</th>
<th>Follow-up</th>
<th>Survival (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No</td>
<td>IV</td>
<td>50/M</td>
<td>s.c. nodules</td>
<td>Surgery</td>
<td>Fever/I erythema/I</td>
<td>Yes</td>
<td>SD</td>
<td>+1m PRO (sc nodules)</td>
<td>40+</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>IV</td>
<td>41/F</td>
<td>Liver</td>
<td>Surgery DTIC IFNα</td>
<td>No</td>
<td>No</td>
<td>SD</td>
<td>+9m PRO (Lung)</td>
<td>38+</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>IV</td>
<td>64/M</td>
<td>Lungs and Suprarenal</td>
<td>Surgery</td>
<td>Erythema/I</td>
<td>Yes</td>
<td>SD</td>
<td>+1m PRO (Lung)</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>IV</td>
<td>67/F</td>
<td>Liver</td>
<td>CVD Surgery CVD IFNα</td>
<td>Fever/I erythema/I</td>
<td>No</td>
<td>SD</td>
<td>+3m PRO (Liver)</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Yes</td>
<td>IV</td>
<td>50/F</td>
<td>Liver and s.c. nodules</td>
<td>Surgery</td>
<td>Erythema/I</td>
<td>No</td>
<td>SD</td>
<td>+2m PRO (Lung)</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>Yes</td>
<td>IV</td>
<td>60/F</td>
<td>s.c. node</td>
<td>Surgery</td>
<td>Fever/I erythema/I</td>
<td>No</td>
<td>PR</td>
<td>NED</td>
<td>29+</td>
</tr>
</tbody>
</table>

*Abbreviations: DTIC, dacarbazine; CVD, cisplatin, vinblastine, dacarbazine; CDDP, cisplatin; SD, stable disease; NED, no evidence of disease (after subcutaneous nodule resection); PR, partial response; PRO, progression; AJCC, American Joint Committee on Cancer.

*National Cancer Institute common toxicity criteria.
died of progressive disease. Patients 1 and 2 have been treated with chemotherapy and are still alive with disease at 40 and 38 months after end of vaccination program, respectively. Patient 6, the only patient with a reduced tumor load (Table 2) and no previous chemo-immune-hormonal therapy, experienced a partial response of a subcutaneous nodule beginning at the third vaccination. The nodule shrank from 5.6 × 5 cm to 3 × 2.5 cm after the fourth vaccination. One week after the fourth vaccination, the nodule was surgically removed due to increasing risk of superficial rupture. The patient has remained disease-free ever since (follow up >29 months).

**Frequency and Maturation Phenotype of Tyrosinase-Specific T Cells in Vaccinated Patients.** To evaluate the impact of vaccination on the frequency of tyrosinase-specific T cells in peripheral blood, human lymphocyte antigen tetramer analysis was carried in five vaccinated patients that expressed the HLA-A*0201 allele. To this end, tetramers of HLA-A*0201 containing the tyrosinase368–376 peptide were used. As a control, all of the blood samples from these five patients were also characterized with an HLA-A*0201 tetramer directed to gp100209–217, a melanoma-associated antigen not targeted by the immunization with modified vaccinia Ankara-transfected dendritic cells. In four of five patients (patients 2, 4, 5, and 6) significant increases in tyrosinase368–376-specific T cells, compared with pretherapy values, were documented ($P < 0.001$ for the median postvaccine increases compared with pretherapy values). Such increases generally occurred a few days after each vaccine administration (Fig. 1). In some instances, an augmented T-cell frequency to tyrosinase was observed even in blood samples taken after the end of therapy (see for example patient 2 after day 50 and patient 6 at day 560). By contrast, tetramer$^+$ T cells directed to gp100209–217 did not show such increases in most patients (Fig. 1) and even showed instances of reduction of frequency during therapy (patients 2 and 5; Fig. 1).

To evaluate whether vaccination could impact on maturation phenotype of circulating tyrosinase-specific T cells, the tetramer$^+$ T cells from the three patients with the most marked increases (patients 2, 4, and 5) during therapy were additionally...
characterized for the expression of CCR7 and CD45RA, two markers of T-cell differentiation along the T naïve → T central memory → T effector memory → T terminally differentiated pathway (27). Interestingly, in these three patients the proportion of tyrosinase-specific tetramer T cells at the T EM (namely CCR7 - CD45RA +) or T TD (namely CCR7 + CD45RA +) stages increased in synchrony with the peaks of T-cell frequency (Table 3). Such shift toward a more differentiated phenotype was not observed in gp100-specific T cells from patient 2 (Table 3) nor in the bulk CD8 T-cell fraction of these three patients (data not shown). In patient 6, at day 49, a subcutaneous nodule, showing partial regression, was surgically removed and analyzed for frequency and maturation phenotype of tyrosinase-specific T cells. Tetramer T cells from such tissue showed a higher frequency and a more differentiated phenotype (mostly TCM and T EM ) compared with tyrosinase-specific T cells taken at the same day from peripheral blood (Fig. 2).

Taken together, the tetramer analysis suggests that vaccination with MVA-hTyr–transduced dendritic cells can affect frequency and differentiation phenotype of HLA-A*0201-restricted T cells directed to tyrosinase epitopes.

### Table 3  Frequency and maturation phenotype of tetramer T cells in peripheral blood of vaccinated patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Day</th>
<th>Specificity of HLA-A2 tetramer</th>
<th>Frequency</th>
<th>Phenotype (% Pos. cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tyrosinase&lt;sub&gt;368–376&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td></td>
<td>0.21</td>
<td>T EM: 9, T TD: 31</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td></td>
<td>0.76</td>
<td>T EM: 15, T TD: 44</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td></td>
<td>0.35</td>
<td>T EM: 13, T TD: 22</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td></td>
<td>0.57</td>
<td>T EM: 16, T TD: 36</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td></td>
<td>0.50</td>
<td>T EM: 10, T TD: 26</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td></td>
<td>0.18</td>
<td>T EM: 10, T TD: 34</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>Gp100&lt;sub&gt;209–217&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td></td>
<td>0.94</td>
<td>T EM: 13, T TD: 50</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td></td>
<td>0.43</td>
<td>T EM: 11, T TD: 47</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td></td>
<td>0.40</td>
<td>T EM: 13, T TD: 39</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td></td>
<td>0.44</td>
<td>T EM: 14, T TD: 36</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td></td>
<td>0.18</td>
<td>T EM: 16, T TD: 42</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>Tyrosinase&lt;sub&gt;368–376&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td></td>
<td>0.16</td>
<td>T EM: 42, T TD: 22</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td></td>
<td>0.66</td>
<td>T EM: 52, T TD: 22</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td></td>
<td>0.37</td>
<td>T EM: 38, T TD: 41</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td></td>
<td>0.39</td>
<td>T EM: 31, T TD: 38</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td></td>
<td>0.62</td>
<td>T EM: 55, T TD: 17</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>Tyrosinase&lt;sub&gt;368–376&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td></td>
<td>0.18</td>
<td>T EM: 15, T TD: 33</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td></td>
<td>0.26</td>
<td>T EM: 16, T TD: 26</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td></td>
<td>0.24</td>
<td>T EM: 24, T TD: 35</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td></td>
<td>0.24</td>
<td>T EM: 17, T TD: 41</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td></td>
<td>0.33</td>
<td>T EM: 19, T TD: 35</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td></td>
<td>0.24</td>
<td>T EM: 25, T TD: 30</td>
</tr>
</tbody>
</table>

**NOTE.** Peripheral blood lymphocytes from patients 2, 4, and 5 were stained with tetramers to tyrosinase<sub>368–376</sub> or gp100<sub>209–217</sub> and with monoclonal antibodies to CD8, CCR7, and CD45RA.

*Frequency of tetramer T cells is reported as proportion of tetramer T cells/CD8 T cells. Maturation phenotype is reported as percentage of the tetramer T cells expressing a T EM (CCR7 - CD45RA +) or T TD (CCR7 + CD45RA +) phenotype. The highest frequency values for tyrosinase-specific T cells compared with pretherapy samples were reported in bold.*

---

**Peptide-Specific T Cells Responses to Tyrosinase- and MVA-Encoded Epitopes in Vaccinated Patients.** Circulating tyrosinase-specific T cells with a memory phenotype (T EM ) and exerting effector functions (interferon γ release and cytotoxicity) without activation have been described in melanoma patients (28). However, tetramer staining, even when coupled to analysis of T-cell maturation phenotype, does not necessarily...
correlate with functional assays in evaluating frequency of antigen-specific T cells (24). In fact, it has been shown that a large fraction of Tet+ T cell may not produce cytokines (29) even when at the TEM stages (30), a finding that in some patients may be linked to T-cell anergy (31). Therefore, to assess whether vaccination could impact not only on frequency and maturation phenotype but even on function of antigen-specific circulating T cells an interferon-γ enzyme-linked immunospot was performed on uncultured lymphocytes isolated from the enrolled patients. In patients 2–6 (HLA-A*0201+) we evaluated interferon-γ secretion in response to tyrosinase366–377 peptide and in response to a recently identified (25) HLA-A*0201-restricted peptide encoded by the modified vaccinia Ankara envelope protein H3L (peptide H3L386–406). In addition, in patients 1 and 6 expressing the human lymphocyte antigen class II allele HLA-DRB1*0101 we evaluated interferon-γ response to the HLA-DR15-restricted tyrosinase386–406 peptide (26). In all of the HLA-A*0201+ patients, after the beginning of vaccination, we detected transient but significant increases of circulating T cells releasing interferon-γ in response to both tyrosinase and modified vaccinia Ankara encoded epitopes (Fig. 3A). In three patients (2, 5, and 6), the interferon-γ releasing cells remained at higher frequency in peripheral blood compared with pretherapy values, even after the end of vaccination. Moreover, the concurrent response to the MVA-encoded epitope (H3L184–192), indicating vector immunogenicity, did not inhibit the tyrosinase-specific response detected during or after vaccination (Fig. 3A). The interferon-γ response by enzyme-linked immunospot to a control antigen (Influenza-Matrix peptide58–66) and to PHA in these patients did not indicate significant changes in pre-versus post-therapy samples (data not shown). A response to the HLA-class II-restricted epitope of tyrosinase was documented in uncultured PBL (and confirmed in preactivated lymphocytes from the same blood samples) of one of the two HLA-DR15+ patients. In patient 1, we could detect an increase in tyrosinase386–406-specific interferon-γ-secreting T cells at the third and fourth immunization as well as after the end of vaccination (Fig. 3B). Such boosting of interferon-γ-secreting cells was not observed in the other HLA-DR15+ patient (patient 6; data not shown). Taken together, these results indicate that vaccination with MVA-hTyr–transduced dendritic cells can boost circulating effector cells able to release interferon-γ in response to human lymphocyte antigen class I- and class II-restricted epitopes of tyrosinase and in response to human lymphocyte antigen class I-restricted epitopes of the vaccinia vector.

**DISCUSSION**

The results of this Phase I study indicated that vaccination of metastatic melanoma patients with CD34+ cell-derived dendritic cells transduced with MVA-hTyr is feasible, safe, well tolerated, and can possibly induce clinical responses. Immunologic monitoring indicated that administration of MVA-hTyr–transduced dendritic cells could promote T cell-mediated immunity to tyrosinase, as documented by analysis of frequency, maturation stage, and function of circulating tyrosinase-specific T cells. Moreover, a modified vaccinia Ankara-specific T cell-mediated response was also induced, but vector immunogenicity was not detrimental to the response against the target antigen of vaccination.

Our study is the first clinical trial with dendritic cells derived from CD34+ hematopoietic progenitor cells engineered to express human tyrosinase gene by transduction with a recombinant modified vaccinia Ankara vector. CD34+–derived dendritic cells have attracted considerable interest as effective natural adjuvants for immunotherapy since the early reports on their generation from human progenitor cells (32, 33). The subsequent evidence on feasibility of massive ex vivo generation of CD34+–derived dendritic cells (34), and on their ability to prime antigen-specific CD4+ and CD8+ T cells (35–37) has opened the way to the clinical use of these professional antigen-presenting cells. CD34+–derived dendritic cells after loading with tumor antigens have been used recently in the vaccination of melanoma and myeloma patients (38–41). In these clinical studies, both humoral and cell-mediated immunity to the target antigens have been promoted in at least some of the treated patients. In all of the previously published studies tumor antigen transfer to CD34+–derived dendritic cells was accomplished by exogenous loading with synthetic peptides (39, 40), whereas vector-mediated gene transfer, as carried out in this study, has not been attempted previously. As shown by the in vitro evaluation of MVA-hTyr–transduced dendritic cells, the infection with the vaccinia vector led to expression of tyrosinase epitopes, as documented by specific recognition of dendritic cells by CTL clones directed to tyrosinase368–376. Moreover, transduced dendritic cells were recognized as efficiently as peptide-loaded dendritic cells, by the tyrosinase-specific CTL clones, indicating that modified vaccinia Ankara infection is an effective strategy to promote antigen expression and processing, as well as human lymphocyte antigen-peptide complex presentation at the cell surface of dendritic cells.

In comparison with peptide loading, expression in dendritic cells of the whole tumor antigen gene has the additional advantage of enabling generation of several different T-cell epitopes from the same tumor antigen protein. This may allow the targeting of the same tumor antigen in patients with different human lymphocyte antigen genotypes compared with the stringent human lymphocyte antigen requirements of peptide vaccination. Moreover, if a tumor antigen can encode for human lymphocyte antigen class I and class II-restricted epitopes, then the expression into antigen-presenting cells of the whole TAA gene may lead to activation of both the CD4+ and CD8+ arms of T cell-mediated immunity. This possibility has been verified in vitro by transfection of human dendritic cells with RNA coding for Her-2, Muc-1, hTert, and MAGE-A3 (8–10) or by dendritic cell infection with vaccinia virus encoding tyrosinase (11). In our study, in addition to HLA-A2-restricted responses to tyrosinase, we also found, in one of two patients, evidence for HLA-DR15-restricted response to this tumor antigen after vaccination. A human lymphocyte antigen class-II-restricted response to a target antigen after vaccination has been documented recently in melanoma patients treated with monocye-derived dendritic cells loaded with an HLA-DP4-restricted MAGE-3 peptide (42) but, to our knowledge, our results represent the first example of induction, in vivo, of human lymphocyte antigen class I- and human lymphocyte antigen class-II-restricted responses against a TAA protein after vaccination.
Fig. 3 Interferon (IFN) γ enzyme-linked immunospot for T cells directed to tyrosinase and MVA epitopes in peripheral blood of vaccinated patients. A, uncultured peripheral blood lymphocytes of HLA-A*0201+ patients 2–6 were evaluated by enzyme-linked immunospot for the frequency of cells releasing IFN-γ in response to tyrosinase366–376 (●) or MVA-H3L184–192 (○) peptides. B, uncultured (□) or preactivated (■) lymphocytes from peripheral blood of patient 1 were evaluated for frequency of cells releasing IFN-γ in response to the HLA-DR15-restricted tyrosinase386–406 peptide. In each graph, all of the values above the horizontal line were significantly different from pretherapy (day 0) with a P at least <0.05 (analysis of variance followed by Student-Newman-Keuls test).
with vector-transduced dendritic cells. These results provide strong support to the use of modified vaccinia Ankara-infected dendritic cells as an effective vaccine strategy to boost human lymphocyte antigen class I- and class II-restricted responses to tumor antigens, even in tumors different from melanoma.

In the six patients enrolled in the trial, frequency of tyrosinase-specific T cells in pretherapy peripheral blood samples was consistent with the highest values found previously by us in a large \((n = 75)\) panel of untreated patients. However, such frequency was much lower than described in a few patients by other authors. Interestingly, all of the patients enrolled in the present study had been selected for evidence of tyrosinase expression in their metastatic tumors, a condition that might have promoted development of T cell-mediated immunity to tyrosinase before vaccination. In agreement with this possibility, the maturation phenotype of tyrosinase-specific T cells found in pretherapy samples did contain variable proportions of antigen-specific T cells with a \(T_{EM}\) or \(T_{TD}\) phenotype.

The interferon \(\gamma\) enzyme-linked immunospot provided evidence for a vaccine-induced T cell-mediated response not only against tyrosinase epitopes, but also against an modified vaccinia Ankara-encoded T-cell epitope, indicating immunogenicity of the modified vaccinia Ankara vector. However, the modified vaccinia Ankara-specific response was not detrimental to the response against the target antigen of vaccination. In fact, the tyrosinase-specific effector cells, releasing interferon \(\gamma\), could be detected in some patients even a few months after vaccination together with an ongoing response to modified vaccinia Ankara-encoded H3L peptide. Interestingly, in a study aimed at evaluating MVA-hTyr-transduced dendritic cells as antigen-presenting cells \(in vitro\), Drexler et al. (15) found induction of tyrosinase-specific T cells, as well as generation of CTLs against dendritic cells infected with wild-type modified vaccinia Ankara, in PBLs of HLA-A\(^*\)0201 \+ melanoma patients.

The latter response was interpreted by these authors as likely to be vaccinia specific due to fact that these patients had been vaccinated against smallpox in their childhood and that the vaccinia-specific T-cell memory has been shown to be long-lived (43). Thus, despite its immunogenicity, modified vaccinia Ankara may lack one of the drawbacks of other vectors, such as the adenoviruses, of which the efficacy in treatment schedules based on multiple injections may be undermined by a strong and pre-existing immunity to the vector itself (44).

At the clinical level, one partial response was observed in patient 6, characterized by limited disease at beginning of vaccination, suggesting that the treatment may be effective in instances of reduced tumor load. In this patient, a subcutaneous nodule showed a partial shrinkage and was eventually removed to prevent possible rupture risk, and the patient is still disease free 29 months after vaccination. No major adverse events were observed but two patients, after the third vaccination, developed vitiligo. However, no ocular or aural consequences were observed, suggesting that autoimmune activated by targeting self-antigens of the melanocyte lineage is a clinically acceptable side effect of the vaccination strategy based on modified vaccinia Ankara-transduced dendritic cells. Interestingly, one of the two instances of vitiligo after vaccination occurred in a patient (patient 1) showing a transient response in interferon \(\gamma\) enzyme-linked immunospot against a human lymphocyte antigen class II-restricted epitope of tyrosinase, suggesting that these two events may be related. In support of this possibility, Overwijk et al. (45) have reported in a murine model of vaccination against melanoma differentiation antigens that development of vitiligo was dependent on activation of CD4\(^+\) T lymphocyte-dependent immunity. On the other hand, it is to be pointed out that vitiligo can be elicited even by adoptive cell transfer with CD8\(^+\) human lymphocyte antigen class I-restricted T cells directed to differentiation epitopes of the melanocyte lineage (46).

In conclusion, active immunotherapy with MVA-hTyr-transduced CD34\(^+\) cell derived-dendritic cells represents a novel approach for melanoma immunotherapy. Our data provide evidence on safety and \(in vivo\) bioactivity of MVA-hTyr-transduced dendritic cells in patients with metastatic melanoma, although the proof for clinical benefit remains to be established in future clinical trials.

ACKNOWLEDGMENTS

We thank Paolo Longoni, Marco Milanesi, Alessandra Molla, and Claudia Vegetti for excellent technical assistance. We are grateful to Drs. Alfonso Marchianò, Carlo Spreafico, Daniele Morelli, and Gabrina Tragni (Istituto Nazionale Tumori, Milan, Italy) for their contribution to clinical management of patients and Dr. Salvatore Siena (Niguarda Hospital, Milan, Italy) for referring one patient. We would also like to thank Nathaly Arndtz (Bavarian Nordic GmbH, Martinsried, Germany), Drs. Gerard Sutter and Ingo Drexler (Institute for Molecular Virology, GSF - National Research Center, Munich, Germany), and Dr. Filippo De Braud (European Oncology Institute, Milan, Italy).

REFERENCES

11. Yee C, Gilbert MJ, Riddell SR, et al. Isolation of tyrosinase-specific CD8\(^+\) and CD4\(^+\) T cell clones from the peripheral blood of melanoma
Boosting T Cell-Mediated Immunity to Tyrosinase by Vaccinia Virus-Transduced, CD34⁺-Derived Dendritic Cell Vaccination: A Phase I Trial in Metastatic Melanoma

Massimo Di Nicola, Carmelo Carlo-Stella, Roberta Mortarini, et al.