Long Overall Survival After Dendritic Cell Vaccination in Metastatic Uveal Melanoma Patients


PURPOSE: To assess the safety and efficacy of dendritic cell vaccination in metastatic uveal melanoma.

METHODS: We analyzed 14 patients with metastatic uveal melanoma treated with dendritic cell vaccination. Patients with metastatic uveal melanoma received at least 3 vaccinations with autologous dendritic cells, professional antigen-presenting cells loaded with melanoma antigens gp100 and tyrosinase. The main outcome measures were safety, immunologic response, and overall survival.

RESULTS: Tumor-specific immune responses were induced with dendritic cell vaccination in 4 (29%) of 14 patients. Dendritic cell-vaccinated patients showed a median overall survival with metastatic disease of 19.2 months, relatively long compared with that reported in the literature. No severe treatment-related toxicities (common toxicity criteria grade 3 or 4) were observed.

CONCLUSIONS: Dendritic cell vaccination is feasible and safe in metastatic uveal melanoma. Dendritic cell-based immunotherapy is potent to enhance the host’s antitumor immunity against uveal melanoma in approximately one third of patients. Compared with other prospective studies with similar inclusion criteria, dendritic cell vaccination may be associated with longer than average overall survival in patients with metastatic uveal melanoma. (Am J Ophthalmol 2014;158:939–947. © 2014 The Authors. Published by Elsevier Inc. All rights reserved. This is an open access article under the CC BY-NC-ND license [http://creativecommons.org/licenses/by-nc-nd/3.0/].)

Uveal melanoma is the most common primary intraocular malignancy in adults with an annual incidence of 4 to 10 per 1 million in the white population, although representing only 3% of all melanoma cases.1,2 Uveal melanoma arises from melanocytes residing in the uveal tract of the eye that have migrated out of the neural crest. Approximately 90% of uveal melanoma arise in the choroid, 6% in the ciliary body, and 4% in the iris.1 In up to 50% of the patients with primary uveal melanoma, metastatic disease ultimately develops, which occurs by hematogenous dissemination; the median time from initial diagnosis of uveal melanoma until detection of metastatic disease ranges from 2 to 5 years.1,3,4 Currently, there is no effective systemic treatment for metastasis to improve overall survival,5 resulting inevitably in tumor-related death when metastasis occurs, with the minor exceptions of a small proportion of patients who have successful curative surgery of metastasis or patients with spontaneous regression of metastatic disease.

Prognostic factors to identify patients with primary uveal melanoma at risk for metastatic disease include clinical (tumor location, tumor size, age), histologic (cell type, vascular pattern, mitotic count, extraocular extension), and genetic (chromosomal aberrations, expression profiling, gene mutations) parameters, partially included in the American Joint Committee on Cancer classification of uveal melanoma.9–11 Over the past few decades, treatment of the primary tumor has changed drastically because several forms of radiotherapy have replaced enucleation as the preferred treatment of the primary tumor, depending on size and location of the tumor and patient preference. However, despite the improvements in diagnosis and the development of eye-conserving treatments, none of these treatment methods prevents the development of metastases. The relative 5-year survival rates have not increased over the past decades, fluctuating at approximately 70% to 80%.3,12–14

Only up to 2% of patients have detectable metastasis when their primary uveal melanoma is diagnosed15; most patients have a long disease-free interval before metastasis becomes clinically evident.5 In uveal melanoma, liver metastases are seen most frequently (90% to 95%), and it is
often the sole site of metastatic disease. Other common sites of metastases, mostly in the presence of liver metastases, are lungs (25%), bone (15%), skin (10%), and lymph nodes (10%); in contrast to cutaneous melanoma, uveal melanoma infrequently metastasizes to the brain. After metastasis develops, overall survival mainly is independent of previously mentioned prognostic factors if one is identifying patients with primary uveal melanoma at risk for metastatic disease. Presence of symptomatic disease, metastatic extensiveness, and metastatic-free interval may correlate with survival time. Nevertheless, median survival is short, typically less than 9 months, with a poor 1-year survival rate (10% to 40%). The small group of patients in whom metastases are confined to extrabhepatic locations have a significantly longer median survival, approximately 19 to 28 months.

Several locoregional treatment options can be considered in selected patients with metastasis confined to the liver, including surgery, isolated hepatic perfusion, or radiofrequency ablation. Although prolonged survival has been reported after surgical resection of liver metastasis, this may be the result in part of selection bias. To date, treatment options for metastatic uveal melanoma are limited, and compelling evidence that any systemic therapy, including chemotherapy, improves overall survival is lacking. Disease stabilization is described in several patients receiving ipilimumab, which recently has shown survival benefit in metastatic cutaneous melanoma patients. However, data are based on a limited number of patients. Therefore, effective therapies resulting in meaningful clinical benefit are required urgently, and immunotherapy may be a promising treatment method.

Immune-based therapies aim to induce antitumor immunity. Despite uveal melanoma developing in the immune-privileged environment of the eye, immune cells have been found within uveal melanoma, including dendritic cells and T cells. Dendritic cells are antigen-presenting cells with the unique capacity to activate naïve antigen-specific T cells, and hence are suitable for inducing immunologic antitumor responses (Figure 1). Dendritic cell-based immunotherapy has shown promising results in cutaneous melanoma patients. Although uveal and cutaneous melanoma are different biologically, cutaneous melanoma and uveal melanoma share many antigenic features, including tumor antigens, providing a rationale for the application of dendritic cell-based therapies in uveal melanoma. The tumor antigens used in our dendritic cell vaccination studies for metastatic melanoma patients, gp100 and tyrosinase, are both expressed in most human uveal melanoma tumor cells and thus constitute an appropriate target for immunotherapy in uveal melanoma.

Our research group has performed several prospective dendritic cell vaccination studies in patients with melanoma, of which most consisted of patients with cutaneous melanoma. We here present data on the subset of metastatic uveal melanoma patients who were enrolled in these studies.
(available at AJO.com), a detailed description on dendritic cell culture is provided.

**ANALYSES OF IMMUNOLOGIC RESPONSES:** To assess the immune response against control and tumor peptides generated in vaccinated patients, peripheral blood was drawn and delayed-type hypersensitivity challenges were performed. In the Supplemental Methods (available at AJO.com), a detailed description of immunomonitoring tests is provided.

**FLUORESCENT IN SITU HYBRIDIZATION ANALYSIS:** Fresh tumor material from enucleated eyes containing uveal melanoma were cultured routinely for karyotyping and were used directly for fluorescent in situ hybridization (FISH) analysis of chromosome 3 as previously described. Dual-color FISH was performed with the following probes: P103.5 (centromere 3), RP11-64F6 (3q25), and RP11-1059N10 (5q12). Chromosome 5 is rarely involved in genetic changes in uveal melanoma and was used as a control for aneuploidy, truncation, and cutting artifacts. The concentration for centromeric probe was 5 ng per slide, whereas for the bacterial artificial chromosome probes, 50 to 75 ng per slide was used. After hybridization and washing, the slides were counterstained with 4', 6-diamidino-2-phenylindole and mounted in antifade solution (Dabco-Vectashield 1:1; Vector Laboratories, Burlingame, California, USA). Signals were counted in 300 interphase nuclei. Scoring for deletion (>20% of the nuclei with 1 signal) or amplification (>10% of the nuclei with 3 signals or more) was adapted from the available literature. Using FISH analysis, we subdivided the variation in chromosome 3 into the following categories: monosomy 3 (loss of 1 copy), disomy 3 (normal copy numbers [2 copies]), and hyperdiploidy (gain of 1 copy).

**STATISTICAL ANALYSIS:** Overall survival was calculated from the date of leukapheresis to death. Patients who did not die during the follow-up period were censored at the time of last follow-up. The Kaplan-Meier method was used to obtain estimates of median survival times and to generate survival curves. IBM SPSS Statistics (SPSS version 20.0) software (SPSS, Inc., Chicago, Illinois, USA) was used for statistical analysis.
RESULTS

• **PATIENT CHARACTERISTICS:** Fourteen uveal melanoma patients with metastatic disease were enrolled in dendritic cell vaccination studies. Patient characteristics are shown in Table 1. The mean age was 52 years; 9 patients were men and 5 were women. One patient had metastases confined to extrahepatic locations. All other patients had liver metastases, of which the liver was the sole site of metastasis in 5 patients. Six patients had received prior treatment for their metastatic disease, mostly consisting of surgery or dacarbazine (chemotherapy). Lactate dehydrogenase, (if elevated, a negative prognostic factor in metastatic uveal melanoma), was elevated at baseline in 3 of 14 patients. Median time between diagnosis of the primary tumor and metastatic disease was 20.4 months. Four patients had synchronous metastasis at presentation (Table 2).

All tumors were confirmed histopathologically as uveal melanoma. Histopathologic examination results of the primary tumor were available in 9 patients who were treated with enucleation. Based on cell type, 8 primary tumors were classified as epithelioid or mixed and 1 as spindle. The median largest tumor diameter of the primary tumor was 13 mm. One tumor was located in the ciliary body (VI-DE3) and 11 were located in the choroid (2 unknown primary location in the ciliary body or choroid). In 12 of 14 patients, metastatic disease was confirmed by histopathologic analysis. All uveal melanoma tumor cells tested, 6 primary tumors and 8 metastases, showed positive results for gp100 expression. Additionally, 11 of 12 uveal melanoma tumor cells tested also expressed tyrosinase.

• **IMMUNOLOGIC RESPONSES:** To test the capacity of the patients in this study to generate an immune response with vaccination, dendritic cells were loaded with a control antigen. Peripheral blood mononuclear cells collected after each vaccination were analyzed for the presence of control antigen-specific T cells, indicative of tumor recognition by T-helper and CD8+ T cells, tetramer analysis. In 7 of 13 patients tested, control antigen-specific IgG antibodies were detected after vaccination (Table 3). These results indicate that the vaccine induced de novo immune responses. To determine the presence of tumor antigen-specific CD4+ and CD8+ T cells, tetramer analyses for 1 tyrosinase and 2 gp100 epitopes were performed after 3 vaccinations. In peripheral blood, tetramer-positive CD4+ T cells, indicative of tumor recognition by T-helper cells, could be seen in 1 of 2 HLA-DRB*01:04-positive patients tested, which were also detectable in the blood before dendritic cell vaccination.

• **CYTOGENETIC RESULTS:** Uveal melanomas of 11 patients were analyzed for chromosomal changes by using cytogenetic and FISH analyses and were classified for gain and loss in chromosome 3 (Table 1). Analyses were performed on primary tumors in 5 patients, on metastases in 4 patients, and on both in 2 patients. Not enough tumor material was available to analyze the remaining 3 patients. Clonal chromosomal abnormalities were present in 8 of 11 tumors tested. Seven tumors showed monosomy 3, 3 patients showed disomy, and 1 patient had a tumor showing hyperdiploidy of chromosome 3. No discrepancies were seen in the patients where both the primary tumor and a metastasis were tested.

### TABLE 1. Characteristics of Dendritic Cell-Vaccinated Patients With Metastatic Uveal Melanoma

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age (y)</th>
<th>LTD (mm)</th>
<th>Chromosome 3</th>
<th>Treatment of Primary</th>
<th>No. of Metastases</th>
<th>Site of Metastatic Disease</th>
<th>LDH (U/L)</th>
<th>LDM (mm)</th>
<th>Prior Treatment for Metastases</th>
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<tr>
<td>I-C14</td>
<td>M</td>
<td>54</td>
<td>14</td>
<td>Disomy</td>
<td>E</td>
<td>1</td>
<td>Liver</td>
<td>312</td>
<td>33</td>
<td>No</td>
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<tr>
<td>III-B7</td>
<td>M</td>
<td>54</td>
<td>13</td>
<td>Monosomy</td>
<td>E</td>
<td>2</td>
<td>Liver</td>
<td>277</td>
<td>16</td>
<td>No</td>
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<tr>
<td>III-B8</td>
<td>M</td>
<td>40</td>
<td>13</td>
<td>Monosomy</td>
<td>E</td>
<td>3</td>
<td>Liver</td>
<td>1289</td>
<td></td>
<td>Diffuse</td>
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<tr>
<td>IV-A4</td>
<td>M</td>
<td>51</td>
<td>13</td>
<td>Monosomy</td>
<td>E</td>
<td>&gt;5</td>
<td>Liver</td>
<td>417</td>
<td>41</td>
<td>No</td>
</tr>
<tr>
<td>IV-A10</td>
<td>F</td>
<td>54</td>
<td>n.a.</td>
<td>Monosomy</td>
<td>No</td>
<td>&gt;5</td>
<td>Liver, lung</td>
<td>432</td>
<td>17</td>
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</tr>
<tr>
<td>IV-B11</td>
<td>M</td>
<td>65</td>
<td>n.a.</td>
<td>n.t.</td>
<td>RT</td>
<td>&gt;10</td>
<td>Liver, lymph node, lung</td>
<td>640</td>
<td>182</td>
<td>C1</td>
</tr>
<tr>
<td>IV-D3</td>
<td>F</td>
<td>42</td>
<td>n.a.</td>
<td>Monosomy</td>
<td>RT/L</td>
<td>&gt;10</td>
<td>Liver, lymph node, lung, pancreas</td>
<td>344</td>
<td>19</td>
<td>S, C1</td>
</tr>
<tr>
<td>V-A3</td>
<td>M</td>
<td>52</td>
<td>n.a.</td>
<td>n.t.</td>
<td>Ru</td>
<td>&gt;5</td>
<td>Liver, bone</td>
<td>517</td>
<td>56</td>
<td>RFA, P/T</td>
</tr>
<tr>
<td>VI-B6</td>
<td>M</td>
<td>53</td>
<td>15</td>
<td>Disomy</td>
<td>E, RT</td>
<td>&gt;5</td>
<td>Liver, lung, bone, soft tissue</td>
<td>434</td>
<td>40</td>
<td>S</td>
</tr>
<tr>
<td>VI-DE3</td>
<td>M</td>
<td>62</td>
<td>23</td>
<td>Hyperdiploidy</td>
<td>E, RT</td>
<td>&gt;5</td>
<td>Liver</td>
<td>360</td>
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<td>No</td>
</tr>
<tr>
<td>VI-DE4</td>
<td>M</td>
<td>35</td>
<td>16</td>
<td>n.t.</td>
<td>E</td>
<td>&gt;5</td>
<td>Liver, lymph node, soft tissue</td>
<td>320</td>
<td>47</td>
<td>No</td>
</tr>
<tr>
<td>VIII-A1</td>
<td>F</td>
<td>49</td>
<td>12</td>
<td>Monosomy</td>
<td>E</td>
<td>&gt;5</td>
<td>Liver</td>
<td>424</td>
<td>16</td>
<td>No</td>
</tr>
<tr>
<td>VIII-A4</td>
<td>F</td>
<td>46</td>
<td>12</td>
<td>Disomy</td>
<td>Ru/Th</td>
<td>&gt;5</td>
<td>Skin, large intestine, soft tissue</td>
<td>440</td>
<td>25</td>
<td>S</td>
</tr>
<tr>
<td>VIII-DE2</td>
<td>F</td>
<td>70</td>
<td>12</td>
<td>Monosomy</td>
<td>E</td>
<td>&gt;10</td>
<td>Liver, lymph node, lung, adrenal gland</td>
<td>447</td>
<td>53</td>
<td>C1</td>
</tr>
</tbody>
</table>

C1 = chemotherapy (DTIC/dacarbazine); E = enucleation; F = female; L = laser; LDH = lactate dehydrogenase (upper limit of normal, 450 U/L); LTD = diameter of the largest measurable metastasis; LTD = largest tumor diameter of primary uveal melanoma; M = male; n.a. = not available; n.t. = not tested; P/T = pazopanib/topotecan; RFA = radiofrequency ablation; RT = radiotherapy; Ru = ruthenium; S = surgery; Th = thermotherapy.
In 3 patients (protocol VI), blood mononuclear cells were restimulated in vitro over 2 weeks with the 3 antigenic peptides, before screening all microcultures for the presence of CD8⁺ tetramer-positive cells. This procedure allowed estimation of the frequencies of tumor antigen-specific CD8⁺ T cells in blood that proliferate in vitro in response to tumor antigen. Two patients showed a significant increase (≥5-fold) of the frequency of gp100-specific CD8⁺ T cells.

Antigen-specific CD8⁺ T cells were detected in delayed-type hypersensitivity skin tests in 2 of 11 HLA-A*02:01-positive patients (Figure 2; Table 3). In patient IV-B11, functionality of the antigen-specific CD8⁺ T cells was tested, and they proved to be fully functional.
and to produce high levels of interleukin-2 and interferon-γ on antigen-specific stimulation.

**CLINICAL OUTCOME:** All patients received at least 3 vaccinations (1 cycle), and 1 patient did not have a skin test because of rapid progressive disease. Ten patients showed stable disease at the first evaluation point, 3 months after start of vaccination, but 7 patients progressed before a second cycle was started after 6 months according to protocol. One patient received a second cycle of vaccinations, and 2 patients received all 3 vaccination cycles and had stable disease up to 28 months. Seven (50%) patients survived more than 2 years after start of dendritic cell vaccination for metastatic uveal melanoma. Thus far, 12 patients had M1a substage (diameter of the largest metastasis of 3.0 cm or less), 6 patients had M1b substage (diameter of the largest metastasis between 3.1 and 8.0 cm), and 2 patients had M1c substage (diameter of largest metastasis more than 8.1 cm). Our patients showed a median overall survival of 29 months for M1a, 22.5 months for M1b, and 6 months for M1c.

**SIDE EFFECTS:** No severe toxicity (grade 3 or 4) occurred. The vaccine-related side effects observed in the vaccinated patients were grade 1 fatigue (5 patients), flu-like symptoms (8 patients), and erythema at the intradermal injection site (6 patients).

**DISCUSSION**

THE INTRINSIC RESISTANCE OF UVEAL MELANOMA TO CONVENTIONAL SYSTEMIC THERAPIES HAS MADE THE TREATMENT OF METASTATIC UVEAL MELANOMA A TOUGH CHALLENGE. THE DEVELOPMENT OF UVEAL MELANOMA AT AN IMMUNE-PRIVILEGED SITE, THE EYE, MADE IT QUESTIONABLE IF IMMUNOTHERAPY WOULD BE A SUITABLE TREATMENT METHOD. THE LACK OF PROPER IMMUNE SURVEILLANCE IN THE EYE CAN LEAD TO CHARACTERISTICS THAT MAKE TUMOR CELLS MORE SUSCEPTIBLE FOR RECOGNITION BY THE IMMUNE SYSTEM WHEN CELLS DISSEminate SYSTEMICALLY, FOR EXAMPLE, HIGH EXPRESSION OF TUMOR-SPECIFIC ANTIGENS, AS
well as less susceptible, for example, resistance to interferon-γ-induced upregulation of major histocompatibility complex class II molecules. At present, accumulating evidence shows that uveal melanoma tumor cells can be lysed by CD8+ T cells in vitro and by T cells adoptively transferred in a mouse model, indicating the susceptibility of uveal melanoma for immunotherapy.

In our study, we vaccinated metastatic uveal melanoma patients with autologous, mature dendritic cells to induce or strengthen a tumor-specific immune response. First, we showed that dendritic cell vaccination in metastatic uveal melanoma is feasible and safe, as shown in more than 200 patients with cutaneous melanoma. Second, the control antigen-specific T-cell proliferation indicated that the vaccine effectively induced de novo immune responses in all patients. Tumor-specific CD8+ T cells were detected in 29% of patients in peripheral blood or in antigen-challenged skin sites. Our previous findings in metastatic melanoma patients, of which most had cutaneous melanoma, showed a similar immunologic response rate (32%) and demonstrated that the presence of tumor-specific T cells after dendritic cell vaccination correlates with clinical outcome. The cohort is too small to confirm these data in metastatic uveal melanoma patients.

Obviously, our study has several limitations. First, this study consists of a small cohort, mainly because of rarity of the tumor and selection on HLA-A*02:01 phenotype in most protocols (approximately 50% of the white population). The latter was necessary because the selected peptides only bind HLA-A*02:01. We do not expect that this has influenced our results, because HLA-A*02:01 phenotype has shown no correlation with survival. Other factors were more likely to be of influence on overall survival, for example, excluding patients with World Health Organization performance status of 2 or more. However, patients were not excluded based on anatomic site of metastasis, number of metastases, or metastasis-free interval, all known to be prognostic factors in metastatic uveal melanoma.

The primary end point of the dendritic cell vaccination studies was safety and feasibility; however, the data on overall survival appeared interesting. The median overall survival of the vaccinated patients was 19.2 months, calculated from the day of leukapheresis instead of from diagnosis of metastasis, as is done in unselected case series. Overall survival from date of diagnosis of metastatic disease in our dendritic cell vaccinated patients was 30.3 months. According to the American Joint Committee on Cancer Staging Manual, median overall survival is 17 months for M1a, 9 months for M1b, and 4.5 months for M1c. Our patients showed a median overall survival of 29 months for M1a, 22.5 months for M1b, and 6 months for M1c. No large difference in overall survival was seen in patients who received prior therapy for metastatic disease to treatment-naïve patients. Comparing our results on survival with other published series, the observed median overall survival of 19.2 months in dendritic cell-vaccinated patients not only exceeded the overall survival as reported in studies using systemic treatment (range, 5.2 to 15.3 months), but also the overall survival in almost all studies in more selected metastatic uveal melanoma patients treated with local therapies of the liver (range, 5.2 to 24 months), such as surgical resection of liver metastasis, hepatic artery chemoembolization, and hepatic artery infusion chemotherapy. These invasive therapies excluded patients with extrahepatic metastasis and high World Health Organization performance status, that is, more strict inclusion criteria, and consequently included patients with more favorable prognostic factors. Further comparison with a cohort of patients with a similar proportion of pretreated patients (12 of 20 patients) and selection criteria, treated with treosulfan and gemcitabine, showed a similar median overall survival (19.2 vs 17 months). Although our results do not allow definite conclusions about clinical outcome, the immunologic responses, previously shown to correlate with clinical outcome, and the observed long overall survival in our cohort of metastatic uveal melanoma patients seem promising. Additionally, the minimal toxicity associated with dendritic cell vaccination compares favorably with other treatment methods.

As to metastatic patients, the high tumor burden may hamper the induction of effective immune responses, creating a suppressive tumor microenvironment by the secretion of suppressive cytokines and attraction of regulatory T cells. Robust immunologic responses on dendritic cell vaccination are induced more frequently in patients with no evidence of disease (72%) (manuscript in preparation) compared with patients with macroscopic tumor burden (32%). On the basis of the association of tumor-specific T cells and improved clinical outcome, this suggests that dendritic cell-based vaccination may have a more pronounced role in an adjuvant setting and should be initiated at an early stage after tumor resection. Patients with primary uveal melanoma usually have no detectable metastatic disease at the time of diagnosis, and most patients have a lengthy disease-free interval before metastasis become evident. Therefore, after treatment of the primary tumor, in the presence of only minimal residual disease and with little immune suppression, there is sufficient time to develop an effective immune response with adjuvant dendritic cell vaccination. Furthermore, patients with a high risk for relapse could be selected based on monosomy 3 status. The presence of monosomy 3 in the primary tumor is accepted widely as the most simple and reliable prognostic parameter, identified in approximately 50% of patients with primary uveal melanoma. Long-term studies have shown a 3-year survival rate of 40% if monosomy 3 is present, whereas tumors with normal chromosome 3 status rarely give rise to metastatic disease and have a 90% 3-year survival rate. To date, no adjuvant therapy has shown survival benefit in uveal melanoma, and because immunologic
responses are seen more frequently in patients before clinically detectable metastasis develop, dendritic cell vaccination may be a good candidate. We currently are investigating this strategy in a randomized study. In conclusion, we show that dendritic cell vaccination is feasible and safe in metastatic uveal melanoma. Our data suggest the potential of dendritic cell-based immunotherapy to enhance the host’s antitumor immunity and that it may be associated with longer than average overall survival times in metastatic uveal melanoma.

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REFERENCES


SUPPLEMENTAL METHODS. DENDRITIC CELL VACCINATION AND MONITORING OF IMMUNOLOGIC RESPONSES

- **DENDRITIC CELL VACCINE**: Monocytes were enriched from leukapheresis products by counterflow centrifugation using an Elutra-cell separator (Gambro BCT, Inc, Lakewood, Colorado, USA) and single-use, functionally sealed disposable Elutra sets, according to the manufacturer. Monocytes were cultured in the presence of interleukin (IL)-4 (500 U/mL) granulocyte-macrophage colony-stimulating factor (800 U/mL; both Cellgenix, Freiburg, Germany) and control antigen keyhole limpet hemocyanin (10 μg/mL; Calbiochem, Darmstadt, Germany). Dendritic cells were matured with autologous monocyte-conditioned medium (30%, v/v) supplemented with prostaglandin E2 (10 μg/mL; Pharmacia & Upjohn, Puurs, Belgium) and 10 ng/mL tumor necrosis factor-α (Cellgenix, Freiburg, Germany) for 48 hours as described previously.1 Plasmacytoid dendritic cells and myeloid dendritic cells were isolated directly from leukapheresis products using the fully closed immunomagnetic CliniMACS isolation system (Miltenyi Biotec, Bergisch-Gladbach, Germany). Good manufacturing practice-grade magnetic bead-coupled BDCA4 (plasmacytoid dendritic cells) or BDCA1 (myeloid dendritic cells) antibodies were used, following the manufacturer's guidelines. Plasmacytoid and myeloid dendritic cells were cultured overnight at a concentration of 106 cells/mL in X-VIVO-15 (Cambrex, Verviers, Belgium) containing 2% pooled human serum (Sanquin, Nijmegen, The Netherlands), supplemented with 10 ng/mL recombinant human IL-3 (plasmacytoid dendritic cells) or 800 U/mL granulocyte-macrophage colony-stimulating factor (both Cellgenix) and 10 μg/mL control antigen (Calbiochem; myeloid dendritic cells). The plasmacytoid dendritic cells were activated subsequently for 6 hours by addition of FSME-IMMUN (1:10 v/v; Baxter AG).2

Dendritic cells were pulsed with the human leukocyte antigen (HLA) class I gp100-derived peptides gp100:154–162, gp100:280–288, and the tyrosinase-derived peptide tyrosinase:369–377. In one protocol, dendritic cells from HLA-DRB*01:04-positive patients also were pulsed with HLA-DRB*01:04-binding peptides of both gp100 and tyrosinase (gp100:44–59 and tyro:448–462 analog).3 In protocols IV, V, and VIII, mature dendritic cells were electroporated with mRNA encoding gp100 or tyrosinase as described previously,4 and cells were resuspended in 0.1 mL for injection.

All administered dendritic cell vaccines met the release criteria described previously:5 mature phenotype with low expression of CD14, high expression of major histocompatibility complex (MHC) class I, MHC class II, CD83, and CD86 and expression of gp100 and tyrosinase for mRNA-electroporated cells.

- **FLOW CYTOMETRY**: Flow cytometry was used to characterize the phenotype of the ex vivo-generated dendritic cells and immune cell subpopulations in the peripheral blood. Flow cytometry measures multiple cell surface proteins simultaneously after staining the cells with fluorescently labeled antibodies specific for a certain antigen. The following monoclonal antibodies or appropriate isotype controls were used: anti–HLA ABC (W6/32), anti–HLA DR/DP (Q5/13), anti–HLA DR, anti–CD80 (all BD Biosciences, San Jose, CA, USA), anti–CD14, anti–CD83 (both Beckman Coulter), anti–CD86 (BD Pharmingen, San Jose, CA, USA), and anti–CCR7 (kind gift of Martin Lipp, Max Planck Institute, Berlin, Germany). For intracellular staining, NKI/beteb (immunoglobulin [Ig] G2b; purified antibody) against gp100 and T311 (IgG2a; Cell Marque Corp, Rocklin, CA, USA) against tyrosinase were used. Flow cytometry was carried out with FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences, San Jose, CA, USA).

- **CONTROL ANTIGEN-SPECIFIC PROLIFERATION**: CD4+ T-cell responses against the control antigen were measured using a 3H-thymidine incorporation proliferation assay with peripheral blood mononuclear cells of the patients before and after vaccination. Briefly, peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll-Paque density centrifugation, stimulated with control antigen (4 μg/2 × 10⁵ peripheral blood mononuclear cells) in X-VIVO with 2% human serum. After 3 days, cells were pulsed with 3H-thymidine for 8 hours, and incorporation was measured with a β-counter. Experiments were carried out in triplicate; nonspecific proliferation on stimulation with ovalbumin was used as control.

- **CONTROL ANTIGEN-SPECIFIC ANTIBODY PRODUCTION**: Antibodies against control antigen were measured in the serum of vaccinated patients using enzyme-linked immunosorbent assay (available at: http://www.klhanalysis.com). Briefly, microtiter plates were coated with control antigen, and different concentrations of patient serum were allowed to bind. After washing, patient antibodies were detected with mouse anti-human IgG, IgA, or IgM antibodies labeled with horseradish peroxidase (Invitrogen, San Diego, California, USA); 3,30–5,5-tetramethyl benzidine was used as a substrate. An isotype-specific calibration curve for the control antigen response was included in each plate; the detection limit was determined at more than 20 mg/L.
• ANALYSES OF SKIN TEST INFILTRATING LYMPHOCYTES FOR TUMOR RECOGNITION: To assess the immune response against tumor peptides generated in vaccinated patients, delayed-type hypersensitivity challenges were performed with mature dendritic cells loaded with gp100, tyrosinase, or both. We have shown that the presence of skin test-infiltrated, tumor-specific T cells correlated with clinical outcome.6,7 Skin tests were performed within 1 to 2 weeks after each vaccination cycle. Briefly, dendritic cells pulsed with gp100, tyrosinase, or both epitopes or electroporated with mRNA encoding either gp100, tyrosinase, or both were injected intradermally in the skin of the back of the patient at different sites. After 48 hours, the maximum diameter of induration was measured by palpation, and punch biopsy samples (6 mm) were obtained. Half of the biopsy was cryopreserved by snap freezing and the other part was cut manually and cultured for 2 to 4 weeks in RPMI-1640 containing 7% human serum and IL-2 (100 U/mL). Every 7 days, half of the medium was replaced by fresh medium containing human serum and IL-2. After 2 to 4 weeks of culturing, skin test-infiltrating lymphocytes of HLA-A*02:01-positive patients were stained with tetrameric-MHC complexes as described previously.3 Each tetramer was validated by staining against a cytotoxic lymphocyte cell line specific for HLA-A*02:01 in association with the peptide of interest. The ability of skin test-infiltrating lymphocytes to recognize vaccine-specific antigens and produce cytokines was determined by the production of cytokines and cytokotic activity of skin test infiltrating lymphocytes in response to T2 pulsed with the indicated peptides or BLM (a melanoma cell line expressing HLA-A*02:01 but no endogenous expression of gp100 and tyrosinase), transfected with control antigen G250, with gp100 or tyrosinase, or with an allogeneic HLA-A*02:01-positive, gp100-positive, and tyrosinase-positive tumor cell line (MEL624).6 In 2 HLA-A*02:01-negative patients, antigen recognition was determined using autologous EBV-transformed B cells as described previously.8

• MIXED LYMPHOCYTE–PEPTIDE CULTURES: Blood frequencies of antivaccine CD8þ T cells were estimated using mixed lymphocyte–peptide cultures in protocol V1 as described previously.2,9 Briefly, peripheral blood mononuclear cells isolated before and after 1 cycle of plasmacytoid dendritic cell vaccinations were thawed and divided in 3 groups incubated for 1 hour at room temperature in Iscove’s medium (Life Technologies, Carlsbad, California, USA) with 1% human serum and 2 μM of the peptides tyrosinase:369–377 (YMDGTMSQV), wild-type gp100:154–168 (KTWGQYWQV), or wild-type gp100:280–288 (YLEPVPVQVTA). These pulsed cells then were washed, pooled, and distributed at 2 × 10⁵ cells/0.2 mL in round-bottom microwells in Iscove’s with 10% human serum, L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L), 1-methyl-L-tryptophan (100 μM), IL-2 (20 U/mL), and IL-7 (10 ng/mL). On day 7, 50% of the medium was replaced by fresh medium containing IL-2 and peptides at 4 μM. Tetramer labeling was performed on day 14 as described previously.9 Anti-gp100:154–168 T-cell clones were derived that represented either the spontaneous anti-gp100 T cells present before vaccination or the plasmacytoid dendritic cell-induced anti-gp100 T cells present after vaccination. Tetramer-positive CD8þ T cells were sorted at 1 cell/well and restimulated weekly with irradiated HLA-A*02 þ EBV-transformed B cells pulsed with the gp100:154–168 peptide at 2 μM, and irradiated allogeneic peripheral blood mononuclear cells as feeder cells, in medium supplemented with IL-2 and IL-7.

• TETRAMER STAINING: To determine the presence of tumor-associated antigen-specific T cells, skin test-infiltrating lymphocyte cultures and peripheral blood mononuclear cells were reanalyzed and stained with tetrameric-MHC complexes containing the MHC class I epitopes gp100:154–168, gp100:280–288, or tyrosinase:369–377 (Sanquin) or MHC class II epitopes gp100:44–59 and tyrosinase:448–462 (provided by W.W. Kwok, Benaroya Research Institute, Seattle, Washington, USA), as described previously.3 In addition, in 2 patients, peripheral blood mononuclear cells were restimulated for 8 days with DR4-binding gp100 or tyrosinase peptides and were stained with tetrameric MHC complexes containing MHC-II epitopes gp100:44–59 and tyrosinase:448–462. Tetrameric MHC complexes recognizing the human immunodeficiency virus were used as correction for background binding. Tetramer positivity was defined as at least 2-fold increase in the double-positive population.

REFERENCES


### SUPPLEMENTAL TABLE

<table>
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<tr>
<th>Protocol</th>
<th>No. of Patients</th>
<th>Type of DC</th>
<th>Method of Antigen Loading</th>
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Class I mod = HLA class I-restricted modified gp100-derived peptides 154–162 Q→A and 280–288 A→V and HLA class I-restricted tyrosinase-derived peptide 369–377; Class I wt = HLA class I-restricted wt gp100-derived peptides 154–162 and 280–288 and HLA class I-restricted tyrosinase-derived peptide 369–377; Class II = HLA class II-restricted gp100-derived peptide 44–59 and tyrosinase-derived peptide 448–462 analog; DC = dendritic cell; i.d. = intradermal; i.n. = intranodal; i.v. = intravenous; mod = modified; moDC = monocyte-derived dendritic cell; mRNA = messenger RNA (encoding full-length gp100 and tyrosinase protein); myDC = myeloid dendritic cell; pDC = plasmacytoid dendritic cell; wt = wild type.

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### REFERENCES


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Biosketch

I. Jolanda M. de Vries is Professor at the Department of Tumor Immunology. Her work has been mainly focused on the modulation of dendritic cells for effective dendritic cell-based cancer immunotherapeutic purposes. Her primary scientific interest continues along the line of dendritic cell-immunotherapy and in particular the migratory and immunomodulatory behaviour of natural dendritic cell subsets. Throughout the past period she has developed a variety of immunomonitoring tools and established a multimodal imaging toolbox for human studies.