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Ina Haendle
Claudia Röder

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Vaccination with Mage-3A1 Peptide-pulsed Mature, Monocyte-derived Dendritic Cells Expands Specific Cytotoxic T Cells and Induces Regression of Some Metastases in Advanced Stage IV Melanoma

By Beatrice Thurner,* Ina Haendle,* Claudia R öder,* Detlef Dieckmann,* Petra K eikavouss,i Helmut Jonuleit,§ Armin Bender,* Christian M aczek,* Doris Schreiner,* Peter von den Driesch,* Eva B. Bröcker,‡ Ralph M. Steinman,i Alexander Enk,§ Eckhart K ämpgen,‡ and Gerold Schuler*

From the *Department of Dermatology, University of Erlangen-Nuremberg, D-91052 Erlangen, Germany; the ‡Department of Dermatology, University of Würzburg, D-97080 Würzburg, Germany; the §Department of Dermatology, University of Mainz, D-55131 Mainz, Germany; and the Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021

Summary

Dendritic cells (DCs) are considered to be promising adjuvants for inducing immunity to cancer. We used mature, monocyte-derived DCs to elicit resistance to malignant melanoma. The DCs were pulsed with Mage-3A1 tumor peptide and a recall antigen, tetanus toxoid or tuberculin. 11 far advanced stage IV melanoma patients, who were progressive despite standard chemotherapy, received five DC vaccinations at 14-d intervals. The first three vaccinations were administered into the skin, $3 \times 10^6$ DCs each subcutaneously and intradermally, followed by two intravenous injections of $6 \times 10^6$ and $12 \times 10^6$ DCs, respectively. Only minor (less than or equal to grade II) side effects were observed. Immunity to the recall antigen was boosted. Significant expansions of Mage-3A1–specific CD8$^+$ cytotoxic T lymphocyte (CTL) precursors were induced in 8/11 patients. Curiously, these immune responses often declined after the intravenous vaccinations. Regressions of individual metastases (skin, lymph node, lung, and liver) were evident in 6/11 patients. Resolution of skin metastases in two of the patients was accompanied by erythema and CD8$^+$ T cell infiltration, whereas nonregressing lesions lacked CD8$^+$ T cells as well as Mage-3 mRNA expression. This study proves the principle that DC “vaccines” can frequently expand tumor-specific CTLs and elicit regressions even in advanced cancer and, in addition, provides evidence for an active CD8$^+$ CTL–tumor cell interaction in situ as well as escape by lack of tumor antigen expression.

Key words: dendritic cells • vaccination • active immunotherapy • melanoma • cytotoxic T lymphocytes

I t is now established that the immune system has cells, particularly CD8$^+$ CTLs, that can recognize tumor antigens and kill tumors (1, 2). Nevertheless, a major problem is that these T cells are either not induced or only weakly induced, i.e., the T cells are not evident in the systemic circulation. One possibility is that there is inadequate tumor antigen presentation by dendritic cells (DCs),1 “nature’s adjuvant” for eliciting T cell immunity (3). Another is that tumor-reactive T cells are tolerized by the tumors (1, 4). Melanoma provides a compelling setting in which to pursue a current goal of cancer immunotherapy, the generation of stronger tumor-specific T cell immunity, particularly with CTLs (4). The majority of tumor antigens identified so far are expressed by melanomas (2). Limited antimalanoma CTL responses have been detected (5), and infusions of IL-2 expanded killer cells can lead to rejection of melanoma (6).

Conventional adjuvants promote antibody rather than CTL responses. Therefore, several novel strategies are being explored to induce tumor-specific T cell immunity. DC vaccination is one of these (3). Immature DCs capture

1Abbreviations used in this paper: CNS, central nervous system; DCs, dendritic cells; DTH, delayed-type hypersensitivity; MCM, monocyte-conditioned medium; RT, reverse transcriptase; TT, tetanus toxoid.
antigens but lack full T cell-stimulatory activity (7). In the presence of appropriate stimuli, such as inflammatory cytokines, the DCs mature. DCs upregulate T cell adhesion and costimulatory molecules as well as select chemokine receptors that guide DC migration into lymphoid organs for priming of antigen-specific T cells. The use of DCs as adjuvants is supported by many animal experiments with primarily mature DCs (3, 8). These studies have shown that the injection of tumor antigen–loaded DCs reliably induces tumor-specific CTL responses, tumor resistance, and in some cases, regression of metastases (3, 8). In the few pilot trials reported so far for humans, immature DCs have been employed (9–11). Scattered tumor responses are reported, but evidence for the induction of tumor-specific CTLs by DC vaccination has not been shown.

We have developed a technique to generate large numbers of homogenous populations of mature and stable DCs from monocytes in the absence of nonhuman proteins (12, 13). We are now exploring the use of these DCs as vaccine adjuvants in humans. Here we present the proof of the principle by demonstrating that three intracutaneous injections of Mage-3A1–specific CTLs are active in vivo. DC-mediated immunization and were accompanied by some cases, underscoring the potent adjuvant properties of DCs. As regressions of metastases also occurred upon injection of tumor antigen–loaded DCs reliably induces mature DCs (3, 8). These studies have shown that the presence of appropriate stimuli, such as inflammatory cytokines but lack full T cell–stimulatory activity (7). In the absence of nonhuman proteins (12, 13). We are now exploring the use of these DCs as vaccine adjuvants in humans. Here we provide the proof of the principle by demonstrating that three intracutaneous injections of Mage-3A1 peptide–pulsed mature DCs reliably enhance Mage-3A1–specific CD8+ and recall CD4+ T cell immunity in heavily pretreated, progressive stage IV melanoma patients with large tumor loads. Expansions of Mage-3A1–specific CTL responses have not been previously detected after Mage-3A1 peptide vaccination in less advanced melanoma patients (14), underscoring the potent adjuvant properties of DCs. As regressions of metastases also occurred upon DC–mediated immunization and were accompanied by CD8+ T cell infiltration, we propose that the induced Mage-3A1–specific CTLs are active in vivo.

Materials and Methods

Patient Eligibility Criteria

Patients were eligible if they suffered from stage IV (i.e., distant metastases) cutaneous malignant melanoma (1988 American Joint Committee on Cancer/U nion Internationale Contre Cancer TNM staging system) that was not curable by resection and was progressive despite chemoinmunotherapy. Further inclusion criteria were an expected survival of 4 mo, Karnofsky index of 60%, age 18 yr, measurable disease, HLA-1A1 positivity, expression of Mage-3 gene shown by reverse transcriptase (RT)-PCR in at least one excised metastasis, and no systemic chemo-, radio-, or immunotherapy within 4 wk (6 wk in the case of nitrosourea drugs) preceding the first DC vaccination. A positive skin test to recall antigens was not required. Important exclusion criteria were active central nervous system (CNS) metastases, any significant psychiatric abnormality, severely impaired organ function (hematological, renal, liver), active autoimmune disease (except vitiligo), previous splenectomy or radiation therapy to the spleen, organ allografts, evidence for another active malignant neoplasm, pregnancy, lactation, or participation (or intent to participate) in any other clinical trial. Concomitant treatment (chemo- or immunotherapy, corticosteroids, investigational drugs, paramedical substances) was prohibited. Palliative radiation or surgical therapy of selected metastases and certain medications (acetaminophen, paracetamol, nonsteroidal anti-inflammatory drugs, opiates) to control symptoms were allowed.

Clinical Protocol and Study Design

The study was performed at the Departments of Dermatology in Erlangen, Würzburg, and Mainz, Germany according to standards of Good Clinical Practice for Trials on Medicinal Products in the European Community. The protocol was approved by the Protocol Review Committee of the Ludwig Institute for Cancer Research (New York, N.Y.) and performed under supervision of its Office of Clinical Trials management as study LUD #97-001. The protocol was also approved by the ethics committees of the involved study centers.

The study design is shown in Table II. All patients gave written informed consent before undergoing a screening evaluation to determine their eligibility. Extensive clinical and laboratory assessments were conducted at visits 1, 5, and 8 (Table II) and consisted of a complete physical examination, staging procedures, and standard laboratory values as well as special ones (pregnancy test, free testosterone in males, autoantibody profile, and antibodies to HIV-1/2, human T cell lymphotropic virus type I, hepatitis B virus, and hepatitis C virus). Patients were hospitalized and examined the day before each vaccination and were monitored for 48 h after the DC injections. Adverse events and changes in laboratory values were graded on a scale derived from the Common Toxicity Criteria of the N ational Cancer Institute, National Institutes of Health, Bethesda, M.D.

Production of the DC Vaccine

During prestudy screening, we tested a small amount of fresh blood to verify that appropriate numbers of mature DCs could be generated from the patient's monocytes (12). Sufficient DC numbers could be successfully generated in all patients, but in some patients the test generation revealed that TNF-α had to be added to assure full maturation. To avoid repetitive blood drawings, we performed a single leukapheresis during visit 2 to generate DCs as described (13). In short, PBMCs from the leukapheresis (≥10^10 nucleated cells) were isolated on Lymphoprep™ (Nycomed Pharma) and divided into three fractions. The first fraction of 10^9 PBMCs was cultured on bacteriological petri dishes (Cat. #1000; Falcon Labware) coated with human Ig (100 μg/ml; Sandoglobulin™; Sand- doz GmbH) in complete RPMI 1640 medium (BioWhittaker) supplemented with 2 μg/ml gentamicin (Refobacin 10; Merck), 2 mM glutamine (BioWhittaker), and 1% heat-inactivated human plasma for 24 h to generate monocyte-conditioned medium (MCM) for later use as the DC maturation stimulus. The second fraction of 3 × 10^8 PBMCs was used for the generation of DCs for vaccination 1 and delayed-type hypersensitivity (DTH) test 1. Adherent monocytes were cultured in 1,000 U/ml GM-CSF (10 × 10^7 U/mg; Leukomax™; Novartis) and 800 U/ml IL-4 (purity ≥98%; 4.1 × 10^7 U/mg in a bioassay using proliferation of human IL-4+ CTLL; CellGenix; expressed in Escherichia coli and produced under good laboratory practice conditions but verified for good manufacturing practice [GMP] safety and purity criteria by) for 6 d, and then MCM was added to mature the DCs. MCM was supplemented in patients 04, 06, 09, 11, and 12 with 10 ng/ml GMP–rhu TNF-α (purity ≥99%; 5 × 10^7 U/mg in a bioassay using murine L-M cells; a gift of Dr. G.R. Adolf, Boehringer Ingelheim Austria, Vienna, Austria) to assure full maturation of DCs. Mature DCs were harvested on day 7. The third fraction of PBMCs was frozen in aliquots and stored in the gas phase of liquid nitrogen to generate DCs for later vaccinations and DTH tests.

DCs for vaccinations were pulsed with the Mage-3A1 peptide (15) (EVDPIGHLY, synthesized at GMP quality by Clinalfa) as tumor antigen, and as a recall antigen and positive control, tetanus toxoid (TT) or tuberculin (if at visit 1 the DTH to TT in the
M. mult test. M. erieux was >10 mm; both purchased from the Bacterial 
Vaccine Department of the Statens Serum Institute, Copenhagen, 
Denmark). The recall antigen was added at 0.05 μg/ml for the last 24 h, and the Cag-3A1 peptide was added at 0.05 μM di-
rectly to the cultures for the last 8 h (if immunity to recall anti-
gens was strongly boosted, the dose of recall antigen was reduced 
to 1.0 to 0.1 μg/ml or was omitted for the intravenous DC in-
jections to avoid a cytokine release syndrome). On day 7, mature 
DCs were harvested, resuspended in complete medium, washed, 
pulsed once more with Mage-3A1 peptide (now at 30 μM) for 60 min at 37°C. DCs were finally washed and resuspended in PBS (GMP quality PBS; BioWhittaker) for injection. DCs to be 
used for Mage-3A1 DTH tests were pulsed with Mage-3A1 (but 
with no recall antigen); DCs that served as negative control in the 
DTH tests were not pulsed at all. An aliquot of the DCs to be 
used for vaccinations was analyzed as described (13) to assure that 
DTH tests were not pulsed at all. An aliquot of the DCs to be 
used for vaccinations was analyzed as described (13) to assure that 
dose for Mage-3A1. Recall antigen was added at 10 μg/ml directly to 
the PBMCs (1 or 5 × 10^6 PBMCs flat-bottomed 96-well plate). 
Assays were performed on fresh PBMCs. Spots were evaluated 
and counted using a standard computer-assisted video imaging analy-
sis system (Carl Zeiss Vision) as described (16).

Semiquantitative Assessment of CTL Precursors. The multiple 
microculture method developed by Romero et al. (17) was used to 
obtain a semiquantitative assessment of CTP precursors specific 
specific for Mage-3A1 peptide. Aliquots of frozen PBMCs were 
thawed and assayed together. CD8^+ T cells were isolated with 
biological microbeads (MACS™ separation columns; Miltenyi Bio-
tec) and seeded at 10^5/ml well in 96-well round-bottomed plates in 
RPMI 1640 with 10% heat-inactivated human serum. The CD8^+ 
PBMCs were pulsed with peptide Mage-3A1 or the influenza 
PB1 control peptide VSDGGPNLY (10 μg/ml; 30 min at room 
temperature), irradiated (30 Gy from a cesium source), and added 
as an APC population at 10^5/ml with IL-2 (10 IU/ml; final) and IL-7 (10 ng/ml final) in a total volume of 200 μl/well. On day 7, 100 μl fresh medium was substituted, and peptide 
 Mage-3A1 or PB1 (1 μg/ml final) and IL-2 (10 U/ml) was 
added. On day 12, each microwell was divided into three equal 
samples to test cytolytic activity in a standard 4-h 51Cr-release 
assay on peptide-pulsed (10 μg/ml with 1 h at 37°C) T2A1 cells, 
nonpulsed T2A1 cells, and K562 target cells, respectively. All 
the assays were performed with an 80-fold excess of nonlabeled 
K562 to block NK activity. Microwells were scored positive if 
lysis of T2A1 targets with peptide minus lysis without peptide was 
≥12% and this specific lysis was greater than or equal to 
twice the lysis of T2A1 targets without peptide plus six as de-
described (18). We aimed at testing 30 microwells of 10^6 CD8^+ 
T cells. Therefore, 1/30 positive wells equals at least one CTP in 
3 × 10^5 (i.e., 30 wells at 10^6 CTP per well) CD8^+ T cells (cor-
responding to ~3 × 10^6 PBMCs).

Immunization Schedule
A total of five vaccinations (three into the skin followed by 
two intravenously) with antigen-pulsed DCs were given at 14-d 
intervals (Table II). This design was chosen to explore the toxic-
ity and efficacy of various routes in this trial. For vaccinations 1–3, 
3 × 10^6 DCs were given subcutaneously at two sites (1.5 × 10^6 
DCs in 500 μl PBS per site) and 3 × 10^5 intradermally at 10 sites 
(3 × 10^5 DCs in 100 μl PBS per site). The injection sites were 
the ventromedial regions of the upper arms and the thighs close to 
the regional lymph nodes and were rotated clockwise. Limbs where 
draining lymph nodes had been removed and/or irradiated were 
excluded. For intravenous vaccinations 4 and 5, a total of 6 and 
12 × 10^6 antigen-pulsed DCs (resuspended in 25 or 50 ml 
PBS plus 1% autologous plasma) was administered over 5 and 10 
min, respectively. Premedication with an antipyretic (500 mg acet-
aminophen/paracetamol p.o.) and an antihistamine (2.68 mg cle-
mastinhydrogenfumarat i.v.) was given 30 min before intravenous 
DC vaccination.

Evaluation of Immune Status
Recall Antigen-specific Proliferation and Cytokine Production. 
PBMCs were cultured in triplicate at two dose levels (3 × 10^4 
and 1 × 10^5 PBMCs/well) plus or minus TT or tuberculin at 
0.1, 1, and 10 μg/ml and pulsed on day 5 with [3H]thymidine for 
differentiation at sites (10^5 Mage-3A1 peptide–
medium (R PM I 1640 and 5% heat-inactivated human serum) per 
well. For the detection of Mage-3A1-reactive T cells, the APCs 
were irradiated T2.A1 cells (provided by P. van der Bruggen, 
Ludwig Institute of Cancer Research, Brussels, Belgium) pulsed 
with MHC class I–restricted peptides (Mage-3A1 peptide and the 
HIV-1 p17-derived negative control peptide GSEELRSLY) added 
at 7.5 × 10^4/well (final volume 100 μl/well). After incubation 
for 20 h, cells were washed six times, incubated with biotiny-
lated second mAb to IFN-γ (7-B6-1; Mabtech) for 2 h, washed, 
and stained with Vectastain Elite kit (Vector Labs.). For detec-
tion of TT-reactive T cells, TT was added at 10 μg/ml directly to 
the PBMCs (1 or 5 × 10^6 PBMCs flat-bottomed 96-well plate). 
Assays were performed on fresh PBMCs. Spots were evaluated 
and counted using a standard computer-assisted video imaging analy-
sis system (Carl Zeiss Vision) as described (16).

Statistical Analysis
For analysis of the immune response, pre- and postimmuniza-
tion values were compared by paired t test after logarithmic trans-
formation of the data. Significance was set at P < 0.05.
Results

Patient Characteristics

All 13 patients were HLA-A1+, had proven MAGE-3 mRNA expression in at least one excised metastasis, and suffered from advanced stage IV melanoma, i.e., distant metastases that were progressive despite chemotherapy and, in some cases, chemoinmunotherapy (Table I). We offered DCs to all patients who fulfilled the inclusion and exclusion criteria, i.e., we did not select for subsets of patients. Two patients (numbers 01 and 03) succumbed to melanoma after two and three vaccinations, respectively. 11 patients received all five planned DC vaccinations in 14-d intervals (Table II) and were thus fully evaluable.

Quality of the Vaccine

All vaccine preparations were highly enriched in mature DCs. More than 95% of the cells were large and veiled in appearance, expressed a characteristic phenotype by flow cytometry (HLA-DR++ CD86++ CD40+ CD25− CD14−), and acted as strong stimulators of an MLR at DC/T cell ratios of ≤1:300 (13). Mst (mean 80%) expressed the CD83 mature DC marker (19). These features were stable upon removal of cytokines and culture for one to two more days (13). The DCs were pulsed with MAGE-3 A1 peptide as a tumor antigen and TT or tuberculin as a recall antigen. The latter were internal controls for immunization and possibly provided help for CTL responses (20).

Toxicity

No major (above grade II) toxicity or severe side effects were observed in any patient, including the two patients who were not fully evaluable. We noticed, however, local reactions (erythema, induration, pruritus) at the intracuta-

Table I. Patients’ Characteristics, Status before DC Vaccination, and Response to DC Vaccination

<table>
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<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>Onset stage IV</th>
<th>Previous therapy</th>
<th>Metastases at study entry</th>
<th>Clinical Response</th>
<th>Survival</th>
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<td>04 M48</td>
<td>1/98</td>
<td>PCI</td>
<td>1/15</td>
<td>CNS 2/12</td>
<td>complete regression of all but 1 lung metastasis, overall progression</td>
<td>10 + 9</td>
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<td>10/97</td>
<td>CI</td>
<td>3/19</td>
<td>CNS 2/12</td>
<td>complete regression of 1 lung + 4 s.c. metastases, overall progression</td>
<td>6 + 16</td>
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<tr>
<td>07 F48</td>
<td>6/97</td>
<td>C</td>
<td>1/7</td>
<td>CNS 2/12</td>
<td>complete regression of 1 lung + 2 s.c. metastases, overall progression</td>
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<td>08 M67</td>
<td>11/97</td>
<td>PC</td>
<td>2/54</td>
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<tr>
<td>09 F43</td>
<td>5/98</td>
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<td>1/28</td>
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<td>Partial regression of 1 lung metastasis, overall progression</td>
<td>4 + 11</td>
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<tr>
<td>12 M54</td>
<td>9/96</td>
<td>CI</td>
<td>7/80</td>
<td>CNS 2/12</td>
<td>partial regression of axillary LN metastases, overall progression</td>
<td>26 + 9</td>
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Patients without objective tumor regression

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<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>Onset stage IV</th>
<th>Previous therapy</th>
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<th>Clinical Response</th>
<th>Survival</th>
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<tr>
<td>13 M34</td>
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<td>12 + 5†</td>
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Treatment centers: three patients (04, 08, and 12) were treated in Wuerzburg, two in Mainz (patients 10 and 13), and the others in Erlangen. Pretreatment therapy: PCI, polychemoimmuno. Preceding excisions and radiotherapies are not listed. Metastases at study entry: the number and diameter of the largest metastases present at study entry are listed (number/diameter in millimeters). m, multiple (>3 metastases). Survival: (since onset of stage IV and as of 5 August 1999) is listed as months since onset stage IV until study entry + number of months since study entry. †Patient deceased.

*CNS metastases were regressing at study entry after gamma knife treatment.

*Determined by autopsy.

*Sudden death from bleeding into CNS metastasis on visit 8.

The regressions of lung metastases in patients 06 and 07 were documented at a staging 3 mo after visit 8. mediast., mediastinum; pancr., pancreas.
neous vaccination sites that increased with the number of vaccinations. In 9/11 patients, strong DTH reactions (induration \( >10 \text{ mm} \) in diameter) were noted to DCs carrying a recall antigen (Fig. 1). Elevation of body temperature (grade I and II fever) was observed in most (9/11) patients and was also related to pulsing DCs with recall antigen. The most striking example was patient 02, who progressively developed fever (up to \( 40^\circ C \)) upon successive vaccinations but did not show a rise in body temperature when TT was omitted for the final (fifth) vaccination. We observed slight lymph node enlargement, clinically in 63\% and by sonography in 83\% of patients, after the intracutaneous DC injections. Interestingly, these were delayed, being inapparent during the 2 d of monitoring after vaccinations but detected when patients were investigated again the day before the next vaccination (Table II).

Immunological Responses

Boosting of Recall Antigen-specific Immunity. PBMCs that had been frozen before vaccination and 14 d after vaccination were thawed and assayed together, as specified in the protocol (Table II). In most patients, a significant boost of antigen-specific immunity developed to TT (and tuberculin in patient 10) \((P < 0.004; \text{Fig. 2})\). Supernatants from the proliferative assays contained large amounts of IFN-\( \gamma \) (mean 1,679 pg/ml, range 846–4,325) but little IL-4 (IFN-\( \gamma \)/IL-4, 317:1). In five patients, we studied the kinetics of the immune response to TT by IFN-\( \gamma \)-ELISPOT analysis. We found an increase after the intracutaneous vaccinations \((P < 0.02)\) but a peculiar decrease after the intravenous vaccinations \((P < 0.008; \text{Fig. 3})\). Thus, comparing recall immunity before and after all five vaccinations (Fig. 2) as prespecified in the protocol (Table II) obviously underestimated the extent of boosting.

Table II. Study Design

<table>
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<tr>
<th>Activities</th>
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<th>Leukapheresis</th>
<th>Vacc. #1</th>
<th>Vacc. #2</th>
<th>Vacc. #3</th>
<th>Vacc. #4</th>
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<td>DTH to Mage-3.A1 peptide-loaded DC</td>
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<td>Recall-antigen proliferation</td>
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</table>

X, prespecified in the protocol as obligatory; x, optional.

Figure 1. Local reactions to DCs carrying Mage-3A1 peptide and TT at the intradermal and subcutaneous vaccination sites in patient 09 (24 h after vaccination 2; top panel) and 02 (48 h after vaccination 3; bottom panel). Erythema at the 10 intradermal (left) and 2 subcutaneous (right) vaccination sites was followed by induration \( >10 \text{ mm} \) in diameter (with secondary purpura in patient 02). These local reactions represent strong DTH reactions to DCs carrying TT, as such strong reactions did not occur in response to unpulsed DCs or DCs pulsed with Mage-3A1 peptide alone in DTH tests I–III (Table II; reactions not shown).
Aliquots of PBMCs, frozen before the first and after the third and fifth vaccinations, were thawed at the same time (Table II) and subjected to a semiquantitative recall assay for CTLp (reference 17; Fig. 4). Before vaccination, CTLp frequencies were low or undetectable. In 8/11 patients, we found a significant expansion of Mage-3A1-specific CTLp as indicated by the increase (mean, eightfold; $P = 0.008$) of positive microcultures in the multiple microculture procedure employed for the semiquantitative assessment of CTLp (17). Interestingly, in six patients, the CTLp frequencies were maximal after the three intracutaneous vaccinations ($P < 0.0013$) but then decreased after the two additional intravenous vaccinations in all but one of these patients ($P = 0.026$). Only in 1/11 patients did we observe an increase of CTLp to an irrelevant PB1 influenza peptide that served as a specificity control (not shown).

**Clinical Responses**

At the end of the trial, i.e., ~2 wk after the fifth vaccination (Table II), we observed temporary growth cessation of some individual metastases, but more intriguingly, in 6/11 patients, complete regression of individual metastases in skin, lymph nodes, lung, and liver (Table I and Fig. 5). Resolution of skin metastases was found in three patients (Table I, patients 06, 07, and 08) and in two of them (06 and 07), it was preceded by local pain, itching, and slight erythema. The six regressing skin lesions of patients 06 and 07 (Table I) were also excised and examined by immunohistology. Clusters of CD8$^+$ T cells were seen around and in the tumor, the latter often necrotic, suggesting an immune attack (Fig. 6).

In patients 06 and 08, the metastases excised at study entry (four and two, respectively) proved to be Mage-3 mRNA$^+$. However, all of the samples removed at the end (two and six, respectively) were Mage-3 mRNA$^-$, suggesting immunoselection for antigen-negative tumor cells. Remarkably, significant infiltration of CD8$^+$ T cells was not found in any of these lesions.

**Discussion**

In deciding on the source of DCs for this phase I trial, we selected mature, monocyte-derived DCs for the follow-
Monocyte-derived DCs currently represent the most homogenous and potent DC populations, with several defining criteria and quality controls (12, 13, 21). The method for generating production of these DCs is very reproducible and allows the cryopreservation of large numbers of cells at an identical stage of development (12, 13). Furthermore, these DCs can be produced in the absence of potentially hazardous FCS (12, 13, 21). FCS exposure also leads to large syngeneic T cell responses in culture, so their clinical use (11) might produce nonspecific immunostimulatory effects. Unlike other investigators (9–11), we chose to use mature rather than immature DCs for our first melanoma trial. The DCs that have been used with efficacy in animal experiments were primarily mature (3, 8). Mature DCs are much more potent in inducing CTL and Th1 responses in vitro (reference 22 and Jonuleit, H., A. Gieseke, A. Kandemir, L. Paragnik, J. Knop, and A.H. Enk, manuscript in preparation), and the DCs are also resistant to the immunosuppressive effects of IL-10 (23) that can be produced by tumors (24–26). Mature DCs also display an extended half-life of antigen-presenting MHC class I (26a) and class II molecules (27). Finally, mature DCs have a high migratory activity (21) and express CCR7 (28), a receptor for chemokines produced constitutively in lymphoid tissues (28). Mature DCs, as used in this cancer therapy trial, have recently also been shown to rapidly generate broad T cell immunity in healthy subjects (28).

Mature DCs were loaded with only one melanoma peptide, Mage-3A1, to avoid uncertainties regarding loading of DCs with multiple peptides (11) of varying affinity and off rate. Successful loading was verified with a Mage-3A1-specific CTL clone and ELISPOT analysis (not shown). The Mage-3A1 peptide (15) was selected for several reasons. It is essentially tumor specific (2) and expressed in tumors other than melanoma (2), and the Mage-3A1 epitope is likely a rejection antigen (14). Moreover, the Mage-3A1 CTLp frequency is exceedingly low in noncancer patients (reference 18; 0.4–3 per 10^7 CD8^+ T cells) as well as in cancer patients, even after peptide vaccination (14). Thus, any induction or boost of Mage-3A1 CD8^+ T cell responses would indicate a significant superiority in the adjuvant capacities of DCs.

DTH assays with peptide-pulsed DCs were carried out as described by Nestle et al. (11) to detect Mage-3A1 immunity (not shown). However, we did not detect unequivocal DTH. This was due to the frequently observed background to nonpulsed DCs (possibly due to cytokine production by DCs) and the noteworthy variability from test site to test site. As Mage-3A1-specific T cells are CD8^+.

Figure 4. Mage-3A1 CTLp frequency analysis as assessed by semiquantitative recall assay. The y-axis and the numbers above the bars indicate the percentage of positive wells found before vaccination 1, before vaccination 4 (14 d after vaccination 3), and after therapy (usually 14 d after vaccination 5).

Figure 5. Regression (arrows) of a globular (13 mm in diameter) lung metastasis in patient 07 that was then no longer detectable in serial 6-mm-thick computed tomography scans.
T cells and DTH assays typically detect primed CD4+ T cells, we suspect that DTH to MHC class I peptide-pulsed DCs may also for this reason prove not to be a sensitive or reliable way to monitor specific CD8+ T cell-mediated immunity.

In contrast, we found sizable expansions of Mage-3A1-specific CTL precursors in PBMCs from a majority (8/11) of patients (P < 0.008; Fig. 4). This is an important proof of the principle of DC-based immunization, and it is also significant from the point of view that tumors can induce tolerance or anergy. It is very promising that CTLp expansions can be induced in far advanced and heavily pretreated stage IV melanoma patients. However, active Mage-3A1-specific effectors were generally not observed in ELISPOT assays, except for in two patients with high frequencies (>5,000/10^7 CD8+ T cells). Perhaps active CD8+ effectors were rapidly sequestered in the numerous metastases, as suggested by the biopsy studies illustrated in Fig. 6. An alternative explanation is that looking for effectors in peripheral blood 14 d after a preceding vaccination might simply be too late.

Interestingly, in six patients, CTLp had increased to their highest levels after the three intracutaneous vaccinations (P < 0.0013) and then decreased (P < 0.026) with subsequent intravenous immunizations (Fig. 4). The decrease in CTLp might be due to emigration of activated Mage-3-reactive CTLs into tissues, tolerance induction, or clonal exhaustion via the intravenous route. We also observed decreased responses to recall antigens in the five patients that we studied before and after intravenous vaccination (Fig. 3). The effect of the intravenous route requires additional study, as it may be counterproductive. In contrast, our results clearly demonstrate that the intracutaneous route is effective, so that the less practical intranodal injection propagated by other investigators (11) does not seem essential. It will, however, be necessary to compare subcutaneous and intradermal routes to find out if one is superior.

We found regression of individual metastases in 6/11 patients when patients were staged 14 d after the fifth vaccination (Table I). This percentage of responses was unexpected in far advanced stage IV melanoma patients who were all progressive despite standard chemotherapy and even chemoinmunotherapy. In the study by Nestle et al. (11), chemotherapy was only given to 4/16 melanoma patients, and objective tumor responses were observed in 5/16. Therefore, we attribute the regressions to DC-mediated induction of Mage-3A1-specific CTLs. This interpretation is supported by the heavy infiltration with CD8+ T cells of regressing but not nonregressing (skin) metastases. The observation that all of the metastases in patients 06 and 08 that were excised at the end of the study were Mage-3 mRNA (whereas those removed at the onset were uniformly positive) suggests immune escape of and selection for Mage-3 antigen-negative tumors. Immune escape might also have been responsible for the lack of tumor response in those nonresponders that had mounted a Mage-3A1-specific CTL response.

After the end of the trial, surviving patients received further vaccinations with DCs and several tumor peptides (Mage-1, tyrosinase, and Mage-3) that were no longer part of the protocol. It is encouraging that 5/11 patients are still alive (Table I) 9–17 mo after study entry, as the expected median survival in patients progressive after chemo(immuno)therapy is only 4 mo (29, 30). One of the initial responders (patient 06) has recently experienced a complete response and has now been disease free for 2 mo. It is interesting that Marchand et al. (14) have also observed that regressions, once they have started, proceed slowly and may take months to complete.

In conclusion, the use of a defined DC vaccine combined with detailed immunomonitoring provides proof that vaccination with mature DCs expands tumor-specific T cells in advanced melanoma patients. In addition, we have found some evidence for the direct interaction between...
CD8+ CTLs and tumor cells as well as for escape of antigen-negative metastases. We are convinced that DC-mediated immunization can be intensified further to reveal the presence of expanded populations of effector cells. Efficacy might be increased at the level of the DC, e.g., by optimizing variables such as DC maturation state, route, dose, and schedule or by improving the short life span of DCs in vivo (31, 32); at the level of the T cell, e.g., by providing melanoma-specific CD8+ T cell help (33, 34) or IL-2 (35); and by treating patients earlier in their disease course.

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Address correspondence to Gerold Schuler, Dermatologische Klinik mit Poliklinik, Hartmannstr. 14, D-91052 Erlangen, Germany. Phone: 49-9131-85-6175; E-mail: schuler@derma.med.uni-erlangen.de

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