Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells

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Melanoma is the main cause of death in patients with skin cancer[1]. Cytotoxic T lymphocytes (CTLs) attack melanoma cells in an HLA-restricted and tumor antigen-specific manner. Several melanoma-associated tumor antigens have been identified[2]. These antigens are suitable candidates for a vaccination therapy of melanoma. Dendritic cells (DCs) are antigen-presenting cells (APCs) specialized for the induction of a primary T-cell response[3]. Mouse studies have demonstrated the potent capacity of DCs to induce antitumor immunity[4–11]. In the present clinical pilot study, DCs were generated in the presence of granulocyte/macrophage-colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) and were pulsed with tumor lysate or a cocktail of peptides known to be recognized by CTLs, depending on the patient's HLA haplotype. Keyhole limpet hemocyanin (KLH) was added as a CD4 helper antigen and immunological tracer molecule. Sixteen patients with advanced melanoma were immunized on an outpatient basis. Vaccination was well tolerated. No physical sign of autoimmunity was detected in any of the patients. DC vaccination induced delayed-type hypersensitivity (DTH) reactivity toward KLH in all patients, as well as a positive DTH reaction to peptide-pulsed DCs in 11 patients. Recruitment of peptide-specific CTLs to the DTH challenge site was also demonstrated. Therefore, antigen-specific immunity was induced during DC vaccination. Objective responses were evident in 5 out of 16 evaluated patients (two complete responses, three partial responses) with regression of metastases in various organs (skin, soft tissue, lung, pancreas) and one additional minor response. These data indicate that vaccination with autologous DCs generated from peripheral blood is a safe and promising approach in the treatment of metastatic melanoma. Further studies are necessary to demonstrate clinical effectiveness and impact on the survival of melanoma patients.

Current vaccination strategies do not induce a sufficient antigen-specific immune response for tumor eradication in patients with advanced melanoma. Two preliminary reports using intradermal delivery of peptides for vaccination observed some clinical remissions[12,13]. In one additional study, expansion of peptide-specific CTLs was achieved, but no clinical response was observed[14]. Presentation of tumor antigen by APCs is crucial for the induction of an antigen-specific CTL response[15]. Under physiological circumstances, tissue-resident but highly motile DCs take up antigen, move to an adjacent lymph node and activate effector T cells[3,15,16]. Therefore, DCs have been proposed to be the ideal candidate for the generation and amplification of a primary immune response in a vaccination setting, for example, for the induction of antitumor immunity[15,16]. In recent years, techniques have been developed to generate large numbers of functionally active dendritic cells by culturing bone marrow or peripheral blood cells in the presence of GM-CSF and/or other cytokines such as IL-4, tumor necrosis factor, stem cell factor, and FLT3 ligand[17–20].

Here we report for the first time on the vaccination of 16 melanoma patients with peptides or tumor lysates using DCs for antigen delivery. Our clinical and immunological monitoring data provide further insight into the powerful antigen-presenting capacity of DCs for induction of an antitumor-specific immune response. DCs were generated from peripheral blood by using GM-CSF and IL-4 over 7 days and were pulsed with a cocktail of peptides known to be recognized by CTLs, depending on the patient's HLA haplotype. In four patients, tumor lysate was used instead of peptide as source of tumor antigen. Multiple peptides were used to diminish the chances of immune escape in a given patient. Generation of potent CTL immune responses requires the presence of CD4 helper T cells and the presence of both the helper and the CTL determinant on the same APCs (ref. 21). Because of a lack of defined tumor helper antigens, we added KLH as a helper antigen to induce a potent KLH-specific memory T-cell response. KLH also has the advantage of being a neo-antigen and can therefore serve as an immunological tracer molecule. Fetal calf serum (FCS)-derived antigens may also contribute to T-helper cell responses. Primary immune responses are generated in professional lymphoid organs[22]. Injecting DCs in a peripheral tissue site (such as skin) or intravenously may lead to a substantial loss of DCs during migration into spleen or lymph node. We decided therefore to inject our DC preparation directly into a professional lymphoid organ. Direct delivery of antigen-loaded DCs (1×10^6 cells per injection) was done by weekly injections into an uninvolved inguinal lymph node over 1 month. Booster immunizations were repeated after 2 weeks and thereafter in monthly intervals. Thus DCs were reproducibly generated from peripheral blood of patients with advanced melanoma and injected into uninvolved inguinal lymph nodes.

As yet, only one study on DC vaccination has been published, describing vaccination of four lymphoma patients and a different isolation protocol[23]. Future studies will most likely use *in vitro* generated monocyte-derived DCs generated in the presence of IL-4 and GM-CSF (refs. 19, 20). A major end point of our study was therefore toxicity. Vaccination was well tolerated in all patients and was administered on an outpatient basis. Occasionally mild fever or swelling of the injected lymph node occurred, lasting 1–2 days. No

Table 1 Patients' characteristics, status before DC vaccination and response to DC vaccination.

Patient			Primary tumor			Status before vaccination		Response to DC vaccination		
No.	HLA type	Age, sex	Tumor type[a]	Tumor thickness	Clark level	Previous therapies[b]	Metastases before DC vaccination	DCs pulsed with	Clinical response[c]	DTH reactivity to DCs loaded with[d]
1	A2	60 f	SSM	?	II	brain radiation intra-art. embolization surgery	pancreas liver[e]	A2 peptides	PR (pancreas) 7 mo	Pep ↑ KLH ↑↑
2	A3	57 f	SSM	1.8 mm	IV	surgery (multiple) DTIC IL-2 + IFN IL-2 + IFN + carboplatin	skin (multiple)	tumor lysate	PD	Pep ϕ KLH (↑)
3	A11, A31	29 f	NM	?	IV	surgery (multiple) DTIC IL-2 + IFN IL-2 + IFN + carboplatin radiation	skin (multiple) soft tissue (multiple) lungs liver bone	tumor lysate	PD	Pep ϕ KLH ↑
4	A2	65 m	?	?	?	surgery (multiple) radiation DTIC	skin (multiple) lungs	tumor lysate	CR (skin, lung) 15 mo +	Pep ↑↑ Lysate ↑↑ KLH ↑↑↑
5	A2	48 f	SSM	2.0 mm	IV	surgery (multiple) hyperthermia DTIC + IFN DTIC + carboplatin radiation	LN (multiple) intestine liver kidney ovary	A2 peptides	PD	Pep ϕ KLH ↑↑
6	A2	42 m	NM	2.8 mm	III	surgery (multiple) hyperthermia radiation IL-2 + IFN	skin (multiple) LN (multiple)	A2 peptides	PD	Pep ϕ KLH ↑↑
7	A2	54 m	NM	1.58 mm	IV	surgery (multiple) IFN (i.t.)	skin (multiple)	tumor lysate	PR (skin) 3 mo	Pep (↑) Lysate ↑ KLH ↑↑↑
8	A1	62 m	NM	7.0 mm	IV	surgery	skin (multiple) soft tissue (multiple) bone	A1 peptides	PR (soft tissue, skin, bone) 12 mo	Pep ↑ KLH ↑
9	A2	69 f	ALM	2.5 mm	V	surgery limb perfusion (TNF) IL-2 (i.t.)	skin (multiple)	A2 peptides	CR (skin) 15 mo +	Pep ↑ Lysate ↑↑ KLH ↑↑
10	A1, A2	69 m	NM	4.5 mm	IV	surgery IFN (i.t.)	skin (multiple) soft tissue liver, bone	A1 + A2 peptides	PD	Pep ϕ KLH ↑
11	A1, A2	23 m	SSM	1.08 mm	III	brain surgery radiation (multiple) surgery	brain intestine	A1 + A2 peptides	PD	Pep (↑) KLH ↑↑
12	A1	40 f	?	?	?	surgery iscador	lung LN (multiple)	A1 peptides	PD	Pep (↑) KLH (↑)
13	A1	54 m	SSM	0.9 mm	III	surgery radiation IFN	bone soft tissue lung	A1 peptides	PD	Pep ↑ KLH ↑↑
14	A2	37 m	NM	3.8 mm	IV	surgery (multiple)	LN (multiple)	A2 peptides	PD	Pep (↑) KLH ↑
15	A1	59 m	SSM	2.6 mm	III	surgery	lung soft tissue	A1 peptides	PD	Pep (↑) KLH (↑)
16	A2	64 m	NM	3.15	III	surgery	lung	A2 peptides	MR 3 mo	Pep ↑↑↑ KLH ↑↑↑

Sites of response (in parentheses) and duration of clinical response are given.
[a]Tumor type: ALM, acral lentiginous melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma.
[b]Therapies: DTIC (dimethyltriazenyl) imidazole carboxamide or dacarbazine; IFN, interferon; IL, interleukin; i.t., intratumoral; TNF, tumor necrosis factor.
[c]Clinical response: CR, complete response; MR, mixed response; PD, progressive disease; PR, partial response.
[d]DTH reactivity to DCs loaded with peptides, KLH and tumor lysate: ϕ, erythema and induration after 48 h below 6 mm in diameter; (↑), between 6 and 10 mm; ↑, between 11 to 20 mm; ↑↑, between 20 and 30 mm; ↑↑↑, >30 mm.
[e]At the entry of patient 1 into the study, the hypodense lesion in segment VI of the liver had the morphological features of a liver cyst (DD: liver metastasis). In the follow up, the likelihood of that being a metastasis was further decreased, since PET was negative at that region and because the lesion remained stable for >1 year.
Other abbreviations: ?, unknown; DC, dendritic cell; DTH, delayed-type hypersensitivity; HLA, histocompatibility leukocyte antigen; KLH, keyhole limpet hemocyanin; LN, lymph node.

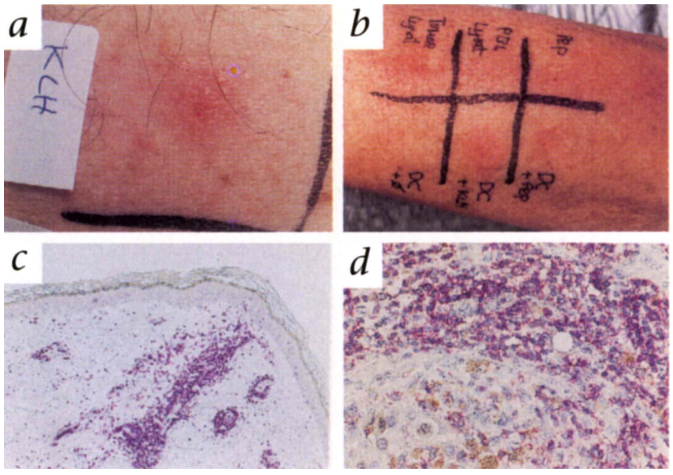

Fig. 1 DTH reactivity and histological response to DC-based vaccination. ***a***, Typical DTH reaction toward KLH, which occurred in all patients after vaccination. Note erythema and induration (here: 14 × 15 mm) at injection site. ***b***, DTH reactivity in patient 9 after 48 h against DCs pulsed with KLH (12 × 12 mm), as well as toward A2-peptide loaded DCs (6 × 5 mm). No reactions occurred after injection of peptide alone or lysates of peripheral blood lymphocytes. This patient, who was treated with A2-peptide loaded DCs, also developed DTH skin reactivity against autologous tumor cell lysate (15 × 15 mm). ***c***, Immunohistochemical characterization of the lymphocytic infiltrate at the DTH site injected with KLH shown in Fig. 1*a* (×100). Alkaline phosphatase–anti-alkaline phosphatase (APAAP)-staining with a CD45RO monoclonal antibody (mAb) was performed, which demonstrated a strong perivascular and epidermal infiltration of CD45RO-positive memory T cells. These cells were CD4-positive in serial sections (not shown). ***d***, CD45RO-stain by APAAP-technique (×400) of a metastasis in regression of patient 8. Note abundant peritumoral infiltration of CD45RO-positive cells. The majority of these cells were CD8-positive in serial stainings (not shown).

physical signs of autoimmune disease were observed in any of the patients. In three patients (nos. 8, 10 and 13), anti-TSH receptor antibodies became detectable after initiation of DC vaccination, however, without clinical effect on thyroid function. In patient 1, antinuclear antibodies (1:320) were observed after the sixth treatment, also without clinical relevance. In patient 11, an individual melanocytic nevus regressed and lost pigmentation under vaccination. Thus, DCs pulsed with antigen can be repeatedly injected into patients without significant toxicity.

Next we asked whether DC vaccination induces antigen-specific immunity. Analysis of tumor peptide-specific CTL activity *in vitro* is currently much debated, and no consensus has been reached about the optimal method for CTL detection. We used DTH reactions as a convenient method for detecting antigen-specific immunity. Vaccination of patients with DCs pulsed with a globular protein antigen (KLH), with peptides binding to the HLA-A1 or -A2 molecule, or with autologous tumor lysate was capable of inducing an immune response. Before vaccination there was no DTH reactivity to the antigens tested in any of the patients. First, we investigated DTH responses to KLH, which has to be taken up, processed and loaded on MHC class II molecules by DCs for effective presentation to helper T cells. After six cycles of vaccination, all patients demonstrated strong DTH reactivity toward the neo- and helper-antigen KLH (Table 1, Fig. 1*a*), indicating the efficient generation of KLH-specific memory T cells. Biopsies taken from DTH test sites revealed a dense infiltration of CD45RO lymphocytes into the KLH challenge site (Fig. 1*c*). These data were confirmed by KLH-specific *in vitro* proliferation tests (M.G. *et al.*, manuscript in preparation). As is well known, tumor peptides have to be presented in the context of MHC molecules to be recognized by effector T cells during DTH reactions. Labeling of tissue-resident APCs with peptide is difficult because peptides are quickly degraded. Therefore, *ex vivo* peptide-pulsed DCs were injected intradermally and used to detect tumor peptide-specific immune responses. Background of DTH reaction with DCs alone was occasionally observed; however, infiltrations remained below 6 mm in diameter (∅) in most patients, except patient 1 (∅ 9 mm), patient 13 (∅ 7 mm), patient 15 (∅ 20 × 15 mm) and patient 16 (∅ 30 mm). Peptide-pulsed DCs clearly induced stronger DTH reactions in all patients, except patient 1. In this patient, DTH reactivity against peptide alone was observed. Significant DTH reactivity (>10 mm in diameter) against DCs pulsed with melanoma-associated peptides (Fig. 1*b*) was observed in five patients, with four of these patients (1, 8, 9, 16) demonstrating a major clinical response to treatment (Table 1). Furthermore, two patients, who both expressed the HLA-A2 molecule (nos. 4 and 7), developed DTH reactivity toward the lysate, as well as DTH reactivity to DCs pulsed with tyrosinase, gp100 and Melan-A peptides after immunization with tumor lysate-pulsed DCs. In these two patients, skin reactivity was associated with clinical response, whereas two other patients (nos. 2 and 3) demonstrated no DTH reactivity to the tumor lysate and did not respond clinically. In patient 9, who also had a complete response, vaccination with A2 peptides pulsed with DCs led to DTH reactivity against peptides used for immunization, as well as *de novo* DTH reactivity toward autologous tumor lysate (Fig. 1*b*; Table 1). These data indicate an association between clinical response and DTH reactivity. To prove the relevance of DTH tests for detection of peptide-specific CTLs, we performed immunohistochemistry and *in vitro* tests for peptide-specific cytotoxicity. Staining of cryosections with anti-CD8 monoclonal antibody revealed large numbers of infiltrating $CD8^+$ T cells at the DTH challenge site (Fig. 2*a*). Infiltrating cells were expanded *in vitro* and tested for peptide-specific cytotoxicity. Specific cytotoxicity for Melan-A and

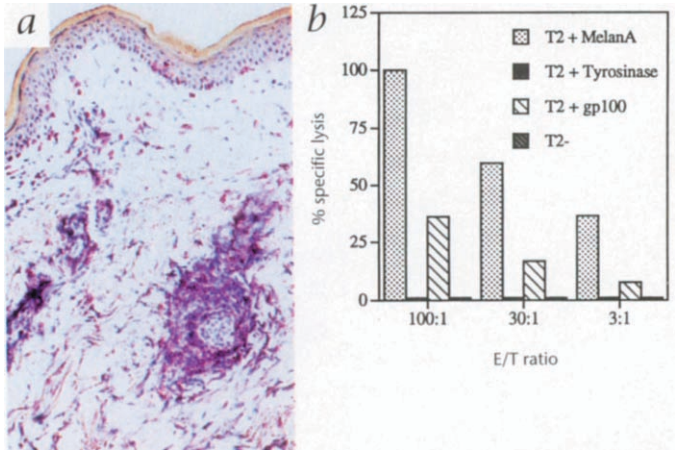

Fig. 2 Recruitment of peptide-specific CTLs into a DTH challenge site in patient 16. ***a***, Immunohistochemical characterization of infiltrating cells into an A2 peptide DTH challenge site (×100). APAAP staining with a CD8 mAb was performed, which demonstrated a strong perivascular infiltration of $CD8^+$ T cells. ***b***, Expansion and testing of A2 peptide-specific cytotoxicity of infiltrating T cells. A biopsy of the DTH site was taken after 48 h. Infiltrating T cells were expanded in the presence of irradiated peptide-pulsed PBLs and IL-2 as described in the Methods section. Cultured cells were tested at different E:T ratios for specific cytotoxicity against peptide-pulsed T2 cells at day 14 using the chromium-release assay. Note potent CTL activity against Melan-A- and gp100-pulsed target cells.

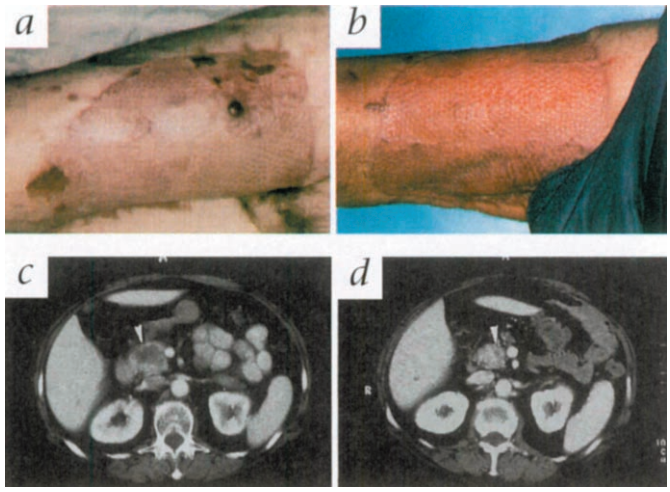

Fig. 3 Clinical responses to DC-based vaccination. Right leg (ventral thigh) of patient 4 with multiple erythematous skin metastases in an area of previous surgery before (***a***) and after (***b***) 6 cycles of vaccination with tumor lysate-pulsed DCs. The patient experienced a complete response with disappearance of all skin metastases, as well as lung metastases. CT scan of patient 1 with large pancreatic metastasis (white arrow) before (***c***) (dark tumor area: 30 × 20 mm) and after (***d***) (dark area: 10 × 8 mm) 6 cycles of vaccination with A2-peptides-pulsed DCs.

gp100 but not tyrosinase was observed in these cultures (Fig. 2*b*). These data were supported by expansion of Melan-A-specific CTLs in the peripheral blood (data not shown). In addition, immunohistochemical analysis of metastases in regression taken from responding patients was done. A representative metastasis is shown in Fig. 1*d*, which exhibits a predominant infiltration with CD45RO-positive memory lymphocytes.

Another aim of our study was to assess the clinical response to DC vaccination. Our selected group of patients treated had a Karnofsky index above 80 and had failed previous treatment regimens. Although tumor load was low, metastases could be found in multiple organs in most patients (Table 1). Two complete responses were observed in patients with predominantly cutaneous infiltrations (Table 1; patients 4 and 9; Fig. 3, *a* and *b*). However, reduction of tumor masses by more than 50% was also documented in other organ sites, such as pancreas and lung in four other patients (Table 1, Fig. 3, *c* and *d*). All clinical responses were detected between treatment week 6 to 10. Only one (of six patients) expressing the HLA-A1 haplotype and immunized with MAGE-1 and MAGE-3 peptides responded to DC vaccination (no. 8). Two out of six patients immunized with the HLA-A2 peptides exhibited a complete response (9) and a partial response (1). Four patients were immunized with DCs pulsed with autologous tumor lysates. Both patients expressing HLA-A2 responded to vaccination with regression of hundreds of metastatic skin nodules and two lung metastases (4) or a substantial regression of the cutaneous infiltrations (7), respectively. Both patients with a complete response are still free of tumor over the past 15 months, whereas the partial responses have a median duration of 6+ months with one partial response lasting 12 months (Table 1). We are well aware that the number of patients in our study for assessing clinical remissions is limited.

Vaccination of melanoma patients with peptide- or tumor lysate-pulsed DCs is a well-tolerated outpatient treatment and induces antigen-specific immunity, as well as clinical responses. A first report in humans described the intravenous application of autologous DCs pulsed with idiotypic proteins in four patients with follicular lymphoma with three patients responding to treatment[23]. In melanoma, there is limited experience with this treatment approach. When Mukherji and co-workers used "DC-like" cells pulsed with the MAGE-1 peptide, no clinical response was observed, but MAGE-1-specific CTLs were detected in two out of three patients[24]. We took advantage of the improved and better standardized conditions to culture DCs and designed a clinical pilot study for a DC-based vaccination approach in advanced melanoma. Vaccination of 16 patients with *in vitro* generated, autologous DCs pulsed with either melanoma-associated peptides or autologous tumor lysates combined with KLH was well tolerated with only few, mild adverse events. The approach to pulse DCs with peptides together with KLH as a highly immunogenic neo-antigen, allowed us to control for the induction of a specific immune response. Indeed, every patient demonstrated a positive immune response to KLH as evidenced by a KLH-specific DTH skin reactivity. Since it is well known that $CD8^+$ cytotoxic and $CD4^+$ helper T cells are involved in antitumor immunity, the introduction of KLH may be crucial, as it induces a strong T-helper cell response and the production of cytokines in the microenvironment of the lymph node necessary for the generation of an effective cytotoxic T-cell response. It is noteworthy that a positive DTH response was observed in two patients (nos. 4 and 8) more than 6 months after the last DC vaccination, indicating the induction of a long-lasting immune response (data not shown). Peptide-specific DTH reactivity used as a read-out system for the clinical monitoring was associated with a clinical response in our patients. Finally, even though only a small number of selected patients were treated, the clinical responses observed are encouraging and demand future studies for DC vaccination in other human cancers.

These data indicate that vaccination of advanced melanoma patients with peptide-pulsed, as well as tumor lysate-pulsed, DCs is well tolerated and is able to induce antitumor immunity *in vivo*, which is associated with measurable DTH reactivity and clinical responses.

Methods

Patient selection. The study protocol was approved by the Clinic Institutional Ethical Review Boards of the participating centers. According to the protocol, patients were required to have histologically proven metastatic melanoma, adequate hepatic and renal function (bilirubin, <3 mg%; serum creatinine, <1.5 mg%) and a life expectancy of at least 12 weeks. Furthermore, patients with any severe cardiac or psychiatric disease, or concurrent acute infection with hepatitis virus or HIV, were excluded. All participants gave informed consent before enrolling in the study. Treatment was carried out at the Departments of Dermatology, Virchow Clinic-Berlin and University of Zurich Medical School. Patient recruitment started in April 1996, and the study was closed in March 1997. Sixteen patients were enrolled and basic data are summarized in Table 1.

Treatment. One week before vaccination, medical history was taken, and the following baseline studies were performed: a physical examination; hematological testing for hemoglobin, hematocrit, β_2-microglobulin, neopterin, antinuclear antibodies, rheumatoid factor, anti-thyroid antibodies [anti-thyroxin-binding globulin, anti-thyroid peroxidase and anti-thyroid-stimulating hormone (TSH)-receptor antibodies]; C-reactive protein; IgG subclasses; leukocyte and platelet count; blood chemistry panel; and urine analysis. Blood was also taken for immunological testing. Chest X-ray and computer tomographic scans of brain, chest and abdomen were taken unless obtained within 8 weeks prior. Eligible patients received four vaccinations at weekly intervals. The fifth vaccination was administered during week 6, and immunization was subsequently continued at monthly intervals for up to 10 vaccinations, depending on clinical response. In most cases, the vaccine preparation was administered intralymphatically into an inguinal lymph node under ultrasound control or was injected in close proximity to the regional lymph nodes. Comprehensive immunological screening, hematological testing and a crude clinical assessment (physical examination, chest X-ray, ultrasound examination of the abdomen)

was performed during week 5 before the fourth vaccination. A complete clinical and immunological screening identical to the initial work-up was done during treatment week 10 and was repeated every two months for final evaluation.

Clinical response and toxicity criteria. Tumor sites were evaluated by physical examinations and scans at 6-week intervals. Standard definitions of major (complete or partial) objective responses were used. A minor response was defined as a 25% to 50% decrease of lesions lasting at least one month, or a more than 50% decrease of lesions lasting less than a month. Stable disease was defined as less than a 25% change in size with no new lesions developing for 6 weeks. Adverse effects were recorded using common WHO toxicity criteria.

Generation of DCs from peripheral blood. Leukocytes were prepared from peripheral blood (100 ml) according to previously published protocols[19,20,25] by using Ficoll-Hypaque density centrifugation. DCs were generated as described previously[19,20,25]. Briefly, PBMCs were resuspended in RPMI 1640 plus 10% FCS (Life Technologies, Eggenstein, Germany) and allowed to adhere to plastic dishes (Costar, Cambridge, MA). After 2 h at 37 °C, the nonadherent cells were removed, and the adherent cells were subsequently cultured for 7 days with GM-CSF (800 U/ml; kindly provided by U. Haus; Sandoz, Nürnberg, Germany) and IL-4 (500 U/ml; PharMingen, Hamburg, Germany). Phenotypic changes were monitored by light microscopy and flow cytometric analysis. Cultured DCs expressed high levels of HLA class I, HLA class II and costimulatory molecules (CD80, CD86) as described[25].

Pulsing of *in vitro* generated DCs. Routinely, 1×10^6 DCs were generated after 7 days of culture in GM-CSF/IL-4. DCs were pulsed either with HLA-A2-binding melanoma-associated peptides for tyrosinase (MLLAVLYCL), Melan-A /MART-1 (AAGIGILTV) and gp100 (KTWGQYWQV) or HLA-A1-binding peptides derived from MAGE-1 (EADPTGHSY) and MAGE-3 (EVDPIGHLY), depending on the HLA type of the patient at a concentration of 50 μg/ml for 2 h at room temperature (RT). In case of the lack of expression of HLA-A1 or HLA-A2 and/or the availability of autologous tumor tissue, DCs were pulsed with autologous tumor lysates (100 μg/ml) for 4 h at RT. In all cases, the DC preparation was loaded with keyhole limpet hemocyanin *Megathura crenulata* (KLH) protein (Calbiochem, Bad Soden, Germany, 50 μg/ml) for at least 2 h. Before injection, DCs were washed three times in sterile PBS and resuspended in a total volume of 0.5 ml PBS. Antigen-loaded DCs were administered immediately in an uninvolved inguinal lymph node.

Delayed-type hypersensitivity (DTH). Delayed-type hypersensitivity skin tests were performed with peptide- or/and tumor lysate-pulsed DCs, and nonpulsed DCs before vaccination, at week 6 and week 10. Thereafter, DTH tests were repeated at 2-month intervals. DCs without peptides, DCs pulsed with each peptide (50 μg/ml) or peptide alone, tumor lysate (10 μg) and KLH (5 μg) were injected intradermally into the forearm. In parallel, a commercially available recall-DTH test (Multi-Test Merieux, Leimen, Germany) was administered on the opposite forearm. A positive skin-test reaction was defined as >5-mm diameter erythema and induration 48 h after intradermal injection.

Preparation of tumor lysates. Tumor biopsies were immediately placed in PBS. Subsequently, adjacent nonmalignant tissue was removed by scalpel, and tumor cells were dispersed to create a single-cell suspension. Aliquots were taken for cell counting and vitality staining by trypan blue. Cells were lysed by 4 to 5 freeze cycles (on liquid nitrogen) and thaw cycles (room temperatures). Lysis was monitored by light microscopy. Larger particles were removed by centrifugation (10 min, 600 r.p.m.), supernatants were passed through a 0.2-μm filter, protein contents determined and aliquots stored at –80 °C until use.

Expansion and testing of CTLs infiltrating a DTH challenge site. A biopsy of a DTH site was taken after 48 h. Biopsies were minced and placed in culture (Iscove's modified Dulbecco's medium, Life Technologies) supplemented with 8% human serum) using 48-well plates with 1×10^6/well irradiated peptide-pulsed (50 μg/ml) PBLs in complete medium supplemented with 60 U/ml IL-2 (R&D, Abingdon, UK). Responder T cells were harvested at day 7, resuspended in a 24-well plate at 0.5×10^6/well and restimulated with 1×10^6 irradiated peptide-pulsed PBLs in complete medium supplemented with IL-2 (10 U/ml). Cultured cells were tested at different effector-to-target (E:T) ratios for specific cytotoxicity against T2 cells (1000 targets/well) pulsed with or without peptide (1 μg/ml) in a standard 4-h chromium-release assay at day 14.

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