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*Featured Article*

# Maturation of Dendritic Cells Is a Prerequisite for Inducing Immune Responses in Advanced Melanoma Patients

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## Abstract

*Purpose:* **We have investigated the capacity of immature and mature monocyte-derived DCs pulsed with melanoma-associated peptides (gp100 and tyrosinase) to induce a primary cytotoxic T-lymphocyte response *in vivo*.**

*Experimental Design:* **Advanced HLA-A2.1$^+$ melanoma patients were vaccinated with peptide- and keyhole limpet hemocyanin (KLH)-pulsed DCs, either immature (9 patients) or matured by monocyte-conditioned medium/tumor necrosis factor α/prostaglandin $E_2$ (10 patients).**

*Results:* **All patients vaccinated with mature DCs showed a pronounced proliferative T-cell and humoral response against KLH. By contrast, KLH responses were absent in most of the patients vaccinated with immature DCs. Delayed-type hypersensitivity (DTH) reactions against antigen-pulsed DCs were only observed in patients vaccinated with mature DCs and not in patients vaccinated with immature DCs. MHC-peptide tetramer staining of DTH-derived T cells revealed the presence of specific T cells recognizing the melanoma-associated peptides in 1 patient. In a second patient, DTH-derived T cells showed specific lysis of tumor cells expressing the antigens used for DC pulsing. Only patients vaccinated with mature DCs showed objective clinical responses. Interestingly, both patients with long-term progression-free survival (22 and >40 months) were both vaccinated with mature DCs and demonstrated antigen-specific T-cell reactivity of DTH-derived T cells.**

*Conclusions:* **We conclude that mature DC are superior to immature DC in the induction of immunological responses in melanoma patients, which may translate into improved clinical results.**

## Introduction

DCs[2] constitute a family of antigen-presenting cells defined by their morphology and their unique capacity to initiate a primary immune response (1). It has been shown that DCs pulsed with MHC class I peptides are potent inducers of a CTL response *in vitro* (2). Furthermore, the presence of DCs in tumor tissue has been correlated with a favorable clinical prognosis (3, 4). Therefore, DCs are thought to play a pivotal role in the induction of T-cell-mediated antitumor responses *in vivo* (1, 5, 6).

The availability of class I-restricted peptides derived from tumor-associated antigens such as gp100, tyrosinase, MAGE, and NY-ESO-1, the ability to grow large numbers of DCs from monocytes, as well of antigen-pulsed DCs to induce specific CTL reactivity *in vitro* led to the use of peptide-pulsed DCs in antitumor vaccination trials (2, 7, 8). Recent studies have demonstrated the safety and the ability of antigen-loaded DCs to induce an immune response in humans (9–12). Evidence is accumulating that the type of DCs and the route of administration play a critical role in determining the quality and quantity of the immune response (13, 14). We recently showed that migration of DCs is highly dependent on their maturation status (15). To further optimize clinical efficacy, DC vaccination strategies need more investigation.

In this study, we compare the efficacy of immature and mature DCs in inducing an immune response in advanced stage IV melanoma patients. Melanoma is well suited to explore vaccination strategies because it is one of the more immunogenic tumors in which melanoma-associated antigens such as gp100 and tyrosinase and specific T-cell responses toward these antigens have been identified (16, 17). DCs used for vaccination were pulsed with HLA-A2.1-binding peptides derived from gp100 and tyrosinase and KLH (9). The latter was used to provide T-cell help and to verify the immunogenicity of the two

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[2] The abbreviations used are: DC, dendritic cell; KLH, keyhole limpet hemocyanin; LDH, lactate dehydrogenase; i.d. intradermal; PE, phycoerythrin; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; MCM, monocyte-conditioned medium; IL, interleukin; GM-CSF, granulocyte monocyte-colony stimulating factor; HS, human serum; TNF, tumor necrosis factor; ELISPOT, enzyme-linked immunospot; Th1, T-helper 1; $PGE_2$, prostaglandin $E_2$; DTH, delayed type hypersensitivity.

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DC populations, as well as competence of the patients' immune system to mount an immune response.

The results of this study implicate that maturation of DCs is a crucial step for the induction of T- and B-cell responses in melanoma patients and a correlation with a favorable clinical outcome is suggested.

## Materials and Methods

**Patient Criteria.** Inclusion criteria were: histological evidence of metastatic melanoma; progressive disease; measurable disease parameters; focal or diffuse expression of gp100 and/or tyrosinase in at least one metastasis as determined by immunohistochemistry; HLA-A2.1 phenotype; WHO performance status 0 or 1; and written informed consent. Patients were staged according to the 2001 American Joint Committee on Cancer staging system: patients with distant nonvisceral metastases were categorized as stage M1a; patients with metastasis to the lung were categorized as stage M1b; and patients with metastases to any other visceral site or with an elevated serum LDH were categorized as stage M1c (18). Patients with clinical signs of brain metastases, serious concomitant disease, or a history of second malignancy were excluded. Prior treatment was allowed, provided a treatment-free period of at least 4 months was observed and all related toxicity had resolved. Approval from the local regulatory committee was obtained.

**Clinical Protocol and Immunization Schedule.** In eligible patients, a leukapheresis was performed from which DCs were generated. The protocol consisted of two parts. In the first part, antigen-pulsed DCs were administered three times at biweekly intervals, either i.v. and s.c. or i.v. and i.d. In the second part, patients without tumor progression received 3 monthly vaccinations with peptides alone (100 μg) and KLH (2 μg), either s.c. or i.d. as in the first part of the study. Before each vaccination, 80 ml of blood were collected for immunological monitoring.

A clinical response was defined as stable disease for >4 months or any partial or complete response. Stable disease and partial response were defined according to WHO criteria (19). Toxicity was assessed according to National Cancer Institute Common Toxicity Criteria. Progression-free survival was calculated from the day of the first vaccination. Patients were evaluated for response after completing the first and second part of the protocol and every 3 months thereafter.

Patients who remained free of disease progression were eligible for two maintenance cycles at 6 months interval, each consisting of three biweekly intranodal vaccinations in a clinically tumor-free, usually inguinal lymph node region under ultrasound guidance with mature DCs, pulsed with modified gp100- and tyrosinase peptides and KLH (20, 21).

**Antibodies and Immunostaining.** To characterize and compare the phenotype of the DC populations, flow cytometry was performed using either FITC-conjugated or PE-conjugated mAbs. The following FITC-conjugated mAbs were used: anti-HLA class I (W6/32) and anti-HLA DR/DP (Q5/13); and PE-conjugated mAbs anti-CD80 (Becton Dickinson, Mountain View, CA), anti-CD14, anti-CD83 (both Beckman Coulter, Mijdrecht, the Netherlands), and anti-CD86 (BD PharMingen, San Diego, CA).

For immunohistochemistry, the following mAbs were used: HMB-45 (Dako, Glostrup, Denmark) against gp100 and T311 (Novocastra, Newcastle, United Kingdom) against tyrosinase (22).

**DCs: Preparation, Characterization, and Route of Administration.** DCs were generated as described previously (23). Briefly, PBMCs were isolated by Percoll (1.073) density gradient centrifugation (30 min, 4°C, 2100 rpm; Pharmacia) after leukapheresis. PBMCs were washed and $1 \times 10^{10}$ cells were used for the generation of MCM (24). From the remaining PBMCs, monocytes were isolated by either counterflow centrifugation or by adherence to plastic. Monocytes isolated by elutriation were cultured at $15 \times 10^6$/75-$cm^2$ tissue culture flasks (Costar, Badhoevedorp, the Netherlands) in 20 ml of IL-4 (500 units/ml; Schering-Plough International, Kenilworth, NJ), GM-CSF (800 units/ml, Schering-Plough International), and 2% HS (Bloodbank; Rivierenland, Nijmegen, the Netherlands) containing X-VIVO 15 medium (BioWhittaker, Walkersville, MD). On day 6, half of the medium was replaced by autologous MCM supplemented with IL-4 and GM-CSF, and cells were harvested on day 9. Thereafter, immature DCs were cultured from adherent PBMCs in 10 ml of X-VIVO 15 medium, of which half was replaced on day one with X-VIVO 15 medium supplemented with IL-4 and GM-CSF, and cells were harvested on day 6 (23, 25). The first 4 patients were vaccinated i.v. and s.c. with these phenotypically immature DC (Fig. 1; Ref. 23), but analysis of the skin injection site revealed that many DCs remained in the s.c. fat (data not shown). On the basis of this finding and our data from murine experiments, we switched from s.c. to i.d. together with i.v. vaccinations (26). The injected immature DCs were characterized by the presence of MHC class I and class II, a moderate expression level of CD86 and a low expression of CD14, CD80, and CD83 (Fig. 1).

Mature DCs were cultured as immature DCs from adherent PBMCs with addition of autologous MCM enriched with 10 μg/ml $PGE_2$ (Pharmacia & Upjohn, Puurs, Belgium) and 10 ng/ml TNF-α (kindly provided by Dr. Adolf Bender, Vienna, Austria) on day 7 (30%, v/v; Ref. 27). This procedure gave rise to mature DCs on day 9 as demonstrated by high expression levels of MHC class I and II, CD80, CD83, and CD86, and absence of CD14 (Fig. 1; Ref. 23). Both immature as well mature DC preparations were endotoxin free.

**Cryopreservation of PBMCs and DCs.** All DCs were cultured directly after leukapheresis and frozen as immature or mature DCs for multiple vaccinations. DCs and PBMCs were frozen using a cryo 1°C freezing container (Nalgene, Rochester, NY), which was put in −80°C for 24 h, in freezing medium consisting of 50% XVIVO-15 (5% HS), 40% HS albumin, and 10% DMSO (final concentration; Sigma). Cells were frozen in 1 ml/vial containing a maximum of $40 \times 10^6$ cells (23).

Cells were thawed in a 37°C water bath, after which the cells were washed once in cold medium and once in medium of room temperature before additional use. We previously showed that the recovery of viable immature DCs (69 ± 15%) did not differ from that of mature DC (65 ± 10%; Ref. 23).

**Peptide Pulsing.** DCs were pulsed with wild-type peptides, gp100:154-167, gp100:280-288, or modified peptides (gp100:154-167 Q→A, gp100: 280-288 A→V) and wild-type tyrosinase 369–376 (16, 20, 21, 28, 29). Pulsing was done

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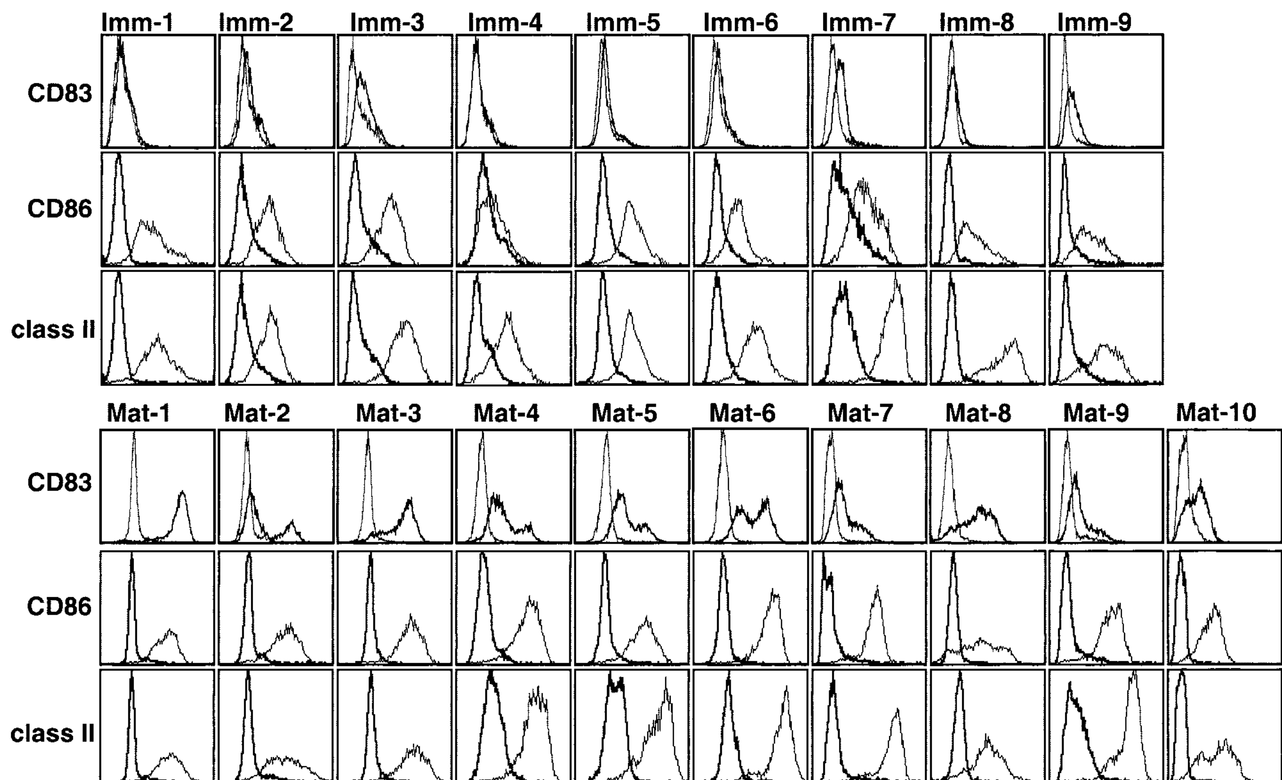


*Fig. 1* CD83, CD86, and MHC class II expression on DCs used for all vaccinations (first vaccination fresh DCs, thereafter frozen DCs). Imm designates vaccination with immature DCs ($CD83^{neg\text{-}low}$) and Mat with mature DCs ($CD83^{high}$). CD83, CD86, and MHC class II expression (*dark overlays*) were measured by flow cytometry, and *gray lines* represent the isotype-matched control.

directly after harvesting or after thawing. On the day of vaccination, we added peptides (50 μg/ml) for 90 min and kept DC at 37°C/5% $CO_2$. Thereafter, fresh peptides (25 μg/ml) were added, and DCs were kept at room temperature for 60 min. After peptide loading, DCs were washed once in 0.9% sodium chloride and resuspended in 0.2 ml or 1 ml for i.d/s.c. or i.v. injections, respectively.

**DTH.** One to 2 weeks after the three DC vaccinations, a DTH skin test was performed. Briefly, unpulsed DCs, DCs pulsed with peptides, DCs pulsed with KLH, and DCs pulsed with peptides plus KLH ($2 \times 10^5$ DCs each) were injected i.d. in the skin of the back of the patients at four different sites. The diameter (in millimeters) of induration was measured after 48 h, each time by the same investigator (M. J. P. G.), and an induration of >2 mm was considered positive.

From positive DTH sites, punch biopsies (6 mm) were obtained under local anesthesia. Biopsies were cut in half, one part for immunohistochemistry, and the other part was cut in small pieces and cultured in RPMI/7%HS supplemented with IL-2 (100 units/ml). Every 7 days, half of the medium was replaced by fresh IL-2-containing RPMI/7%HS. After 2–4 weeks of culturing, T cells were tested for antigen recognition in a cytotoxicity assay or tested for tetramer binding.

**Humoral Response to KLH.** Antibodies against KLH were measured in the serum of vaccinated patients by ELISAs as described by Holtl *et al.* (30). Briefly, 96-well plates were coated overnight at 4°C with the protein KLH (25 μg/ml) in PBS (0.1 ml/well). After washing the plates, different concentrations of patient serum (range 1 in 100 to 1 in 500,000) were added for 1 h at room temperature. After extensive washing, specific Abs (total IgG, IgG1, IgG2, and IgG4) labeled with horseradish peroxidase were allowed to bind for 1 h at room temperature. Peroxidase activity was revealed using 3,3′ 5,5-tetramethyl-benzidine as substrate and measured in a microtiter plate reader at 450 nm. A positive signal at a ≥1 in 400 dilution of the patients' serum was considered positive.

**Proliferative Response and Cytokine Production to KLH.** Cellular responses against KLH were measured in a proliferation assay. Briefly, $1 \times 10^5$ PBMCs, isolated from blood samples taken before each vaccination, were plated per well of a 96-well tissue culture microplate either in the presence of KLH or without. After 16 h of culture, supernatants (50 μl) were taken, and IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were measured by a cytometric bead array (Th1/Th2 Cytokine CBA 1; BD PharMingen) according to the manufacturer's instructions. After 4 days of culture, 1 μCi/well of tritiated thymidine was added. Incorporation of tritiated thymidine was measured in a β-counter. A proliferation index >2 was considered positive.

**MHC Tetramer Staining.** Tetrameric-MHC complexes were kindly provided by Drs. Rosalie Luiten and Hergen Spits from the Netherlands Cancer Institute (Amsterdam, the Netherlands; Ref. 31). Each tetramer was validated by staining against

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a CTL line specific for HLA-A2 in association with the peptide of interest.

PBMCs ($1 \times 10^5$ cells in 10 μl) were incubated with PE-labeled tetrameric-MHC complexes for 1 h at room temperature. After washing, the samples were analyzed by flow cytometry.

**Cytotoxicity Assay.** Cytotoxic activity of DTH-infiltrated lymphocytes was measured using the chromium release assay as described previously (32). Briefly, target cells HLA-A2.1-positive BLM either transfected with control antigen G250 or with the antigens of study gp100 or tyrosinase were incubated with 100 μCi $Na_2[^{51}Cr]O_4$ (Amersham, Bucks, United Kingdom) for 45 min at 37°C. After extensive washing, chromium-labeled BLMs ($10^3$) were mixed with unlabeled K562 cells (ratio 1:10) and added to lymphocytes ($10^5$ cells) in triplicate wells of a round-bottomed microtiter plate (total volume 150 μl). After a 4-h incubation, 100 μl of the supernatant were harvested, and its radioactivity content was measured. The specific percentage of cytotoxicity was defined by: [(experimental release − spontaneous release) ÷ (maximum release − spontaneous release)] × 100%.

**IFN-γ ELISPOT Assay.** Production of IFN-γ in response to the peptides used for DC pulsing was determined by the ELISPOT assay as described previously (33). In short, $CD8^+$ T cells ($10^5$) and T2 cells ($7.5 \times 10^4$/well) with the peptides (final concentration 50 μg/ml) were incubated for 20 h at 37°C on multiscreen HA plates (Millipore, Bedford, MA), which were coated with antihuman IFN-γ. Captured cytokine was detected by biotinylated mAb antihuman IFN-γ, avidin-peroxidase complex, and peroxidase staining. Spot numbers were automatically determined with the use of computer-assisted video image analysis.

## Results

**Patients Characteristics.** In this study, a total of 19 stage IV melanoma patients were vaccinated, 9 with immature DCs, and 10 with mature DCs. Patient characteristics are shown in Table 1. Both groups were comparable considering age, sex, and previous therapy. A higher number of patients vaccinated with mature DCs had a performance status 0, but in this group, there were also more patients with a more advanced stage of disease (Table 1).

**Toxicity and Side Effects.** No severe toxicity (common toxicity criteria grade III–IV) occurred. In patients vaccinated with immature DCs, mild fatigue, anorexia, and nausea but no fever was observed. However, fever (≥38°C) developed in 2 of 10 patients vaccinated with mature DCs after the second vaccination and in 7 of 10 after the third vaccination. Furthermore, erythema was observed at the injection site of the second or third vaccination, only after vaccination with mature DCs.

**Proliferation and Cytokine Production of PBMCs upon Stimulation with KLH.** To determine the ability of immature *versus* mature DCs to mount an immune response against KLH, PBMCs collected and frozen after each DC vaccination were thawed and analyzed for the presence of KLH-reactive T cells in a proliferation assay. Patients receiving mature DCs ($n = 10$) showed a strong proliferative response against KLH that could already be detected after a single DC vaccination (Fig. 2). The observation that this response was not additionally augmented after the second and third DC vaccination or after administration of KLH protein i.d. suggests that a single DC vaccination was sufficient to obtain maximal proliferative responses against KLH. This may also explain why we did not observe a clear correlation between the increase in proliferation and the number of injected DCs (Table 2).

*Table 1* Patient characteristics

| Patient | Sex/age | Previous therapy | Performance status (WHO) | Stage |
|---|---|---|---|---|
| Imm-1 | M 33 | RT, HT[a] | 0 | M1a |
| Imm-2 | M 55 | RLND, RT | 1 | M1c |
| Imm-3 | F 56 | RLND | 1 | M1b |
| Imm-4 | M 52 | RLND, DTIC | 1 | M1c |
| Imm-5 | F 78 | | 1 | M1b |
| Imm-6 | M 48 | RLND, IFN | 0 | M1c |
| Imm-7 | M 41 | RLND, 2xMT | 0 | M1c |
| Imm-8 | M 62 | RLND, MT | 0 | M1c |
| Imm-9 | M 46 | RLND | 0 | M1a |
| Mat-1 | M 22 | | 0 | M1c |
| Mat-2 | M 59 | RLND, MT, IFN | 0 | M1c |
| Mat-3 | F 33 | RLND | 0 | M1c |
| Mat-4 | F 35 | RLND | 0 | M1c |
| Mat-5 | M 63 | RLND | 1 | M1c |
| Mat-6 | M 57 | RLND, IFN | 0 | M1b |
| Mat-7 | M 73 | RT | 0 | M1b |
| Mat-8 | M 54 | RLND, IFN | 0 | M1c |
| Mat-9 | F 50 | RLND, 2xMT, IFN | 0 | M1c |
| Mat-10 | M 66 | | 0 | M1a |

[a] RT, radiotherapy; HT, hyperthermia; MS, metastasectomy (in all patients of nonvisceral metastases); IFN, IFN-α; DTIC, dacarbazine; RLND, regional lymph node dissection.

By contrast, we observed no significant increase in KLH-specific proliferation of PBMCs derived from patients vaccinated once with immature DC ($n = 9$; Fig. 2). Proliferative responses measured after subsequent immature DC vaccinations, either i.v./s.c. or i.v./i.d. remained much less pronounced when compared with PBMCs from patients vaccinated with equal numbers of mature DCs (Table 2).

To determine the type of the induced immune response, PBMCs obtained after three vaccinations with either immature or mature DCs were stimulated with KLH for 16 h, and cytokine production was measured. No cytokine production was observed by PBMCs after three vaccinations with immature DCs (Fig. 2). However, PBMCs from patients vaccinated with mature DCs produced high amounts of IL-2 (60 pg/ml), TNF-α (212 pg/ml), and IFN-γ (398 pg/ml) but no IL-4 in response to KLH (Fig. 2). From these results, we conclude that mature DCs induce a Th1 response.

**Humoral Response against KLH in Serum.** Humoral responses against KLH (total IgG) were detected in serum of most patients (9 of 10) vaccinated with mature DCs given i.v. and i.d. (Table 2). After a single vaccination with KLH-pulsed DCs, IgG antibodies reactive with KLH were readily detectable by ELISA (Fig. 3). After subsequent vaccinations with mature DCs, the IgG antibody titers increased and remained elevated after vaccinations with the protein KLH at days 56, 84, and 112 (Fig. 3).

In contrast, in serum of all of the patients vaccinated with immature KLH-pulsed DCs, IgG antibodies reactive with KLH remained absent (Fig. 3, Table 2) even after three DC vaccina-

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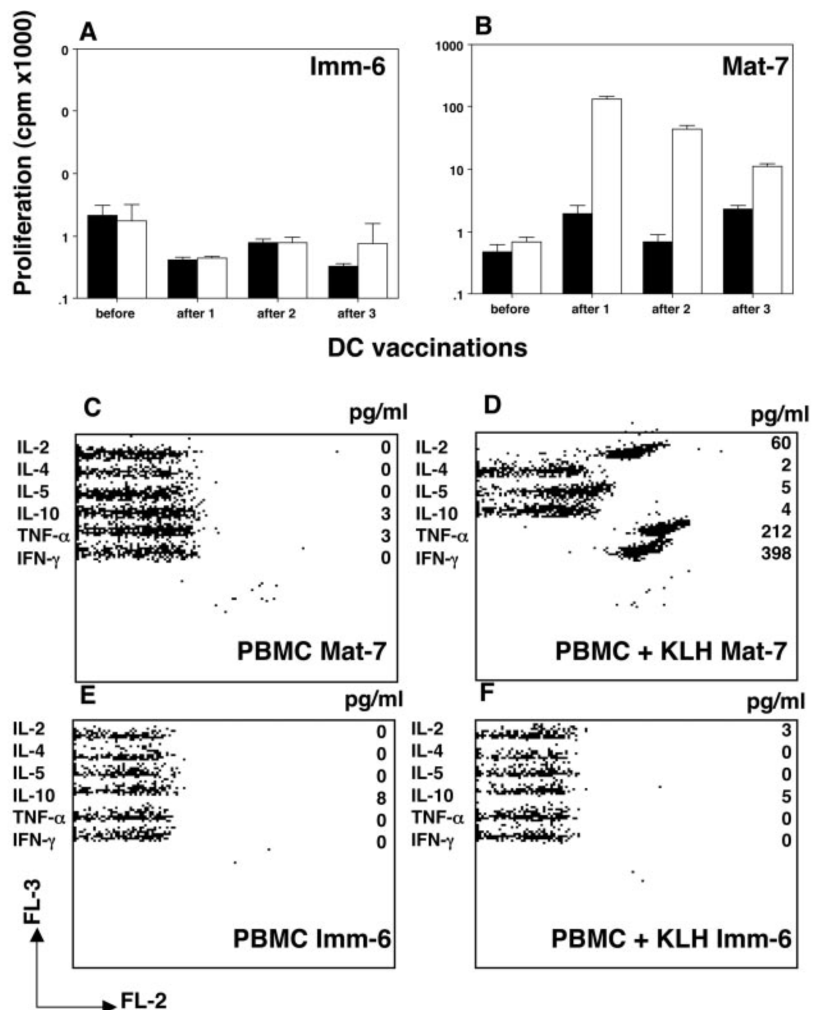


*Fig. 2* KLH-specific proliferation of PBMCs before and after DC vaccination. Patients were vaccinated either with immature DCs (*A*) or mature DCs (*B*). One representative patient for each DC subset is shown (■, PBMC without KLH; □, PBMCs with KLH). Results of the proliferation to KLH of all patients are shown in Table 2. Cytokine production by PBMCs of patients vaccinated three times either with mature (Mat-7; *C* and *D*) or immature DCs (Imm-6; *E* and *F*); unstimulated PBMCs (*C* and *E*) and PBMCs stimulated with KLH (*D* and *F*).

tions. To exclude the possibility that these patients were refractory to KLH, 2 patients received a subsequent single vaccination with KLH protein. High titers of IgG against KLH could be obtained, demonstrating that their immune system was not affected (Fig. 3).

Sera containing KLH antibodies after vaccination with mature DCs were further analyzed for subtypes of IgG (IgG1, IgG2, and IgG4). We did not observe anti-KLH antibodies of the IgG4 subclass in any of the patients. In 4 patients, we observed equal amounts of IgG1 and IgG2, whereas in 5 patients, the levels of IgG2 were significantly higher compared with the levels of IgG1, indicating the presence of IFN-γ and hence a Th1 response (Fig. 3; Refs. 34, 35). Taken together, both antibody and proliferative responses against KLH demonstrate the superiority of mature DCs in inducing Th1 responses.

**DTH Reactions.** No DTH skin reactions against immature DCs either unpulsed or pulsed with KLH and/or peptides were observed in patients vaccinated three times with immature DCs (Table 2). In contrast, in all patients vaccinated (i.v./i.d.) with mature DCs, positive DTH reactions with indurations up to 12 mm were observed against mature DCs pulsed with KLH and/or peptides. However, also in 8 of 9 patients against unpulsed mature DCs (Table 2). Moreover, in 1 patient vaccinated with mature DCs, a DTH with unpulsed immature DCs induced induration as well (data not shown). These data imply that vaccination with mature but not with immature DCs provoke a DTH response.

**Detection of Tumor Peptide-Specific T Cells in Peripheral Blood and DTH.** To determine the presence of antigen-specific T cells in peripheral blood, ELISPOT measuring IFN-γ-production by CD8+ T cells in response to the vaccinated tumor-derived peptides and tetramer staining were used.

In some of the analyzed samples (in 1 of 4 tested patients vaccinated with immature DCs and 1 of 5 patients vaccinated with mature DCs), a small increase in reactivity against the vaccinated peptides was observed after vaccination, either by ELISPOT or by tetramer staining (data not shown). Furthermore, this increase was not consistent after subsequent vaccinations. From these experiments, we concluded that in peripheral blood no significant elevation of peptide-reactive T cells could be found.

Short-term T-cell cultures (2–4 weeks) from biopsies of positive DTH reactions of 7 patients vaccinated with mature

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*Table 2* Immune and clinical responses after vaccination with immature and mature DCs

| Patients | Number of injected DC ($10^6$ of cells) | | | DTH[a] | Anti-KLH response | | Response[d] | PFS (mos) |
|---|---|---|---|---|---|---|---|---|
| | Vacc. 1 | Vacc. 2 | Vacc. 3 | | T cell[b] | Ab[c] | | |
| Immature DC | | | | | | | | |
| Imm-1 | 9/3 | 5/3 | 8/4 | − | + | − | PD | 1 |
| Imm-2 | 20/5 | – | – | n.d. | − | − | PD | 0.5 |
| Imm-3 | 14/4 | 12/3 | 21/5 | − | + | − | PD | 3 |
| Imm-4 | 25/6 | 7/2 | – | n.d. | − | − | n.e. | 1.5 |
| Imm-5 | 8/6 | 7/5 | 2/5 | − | − | − | PD | 3 |
| Imm-6 | 30/8 | 27/5 | 30/5 | − | − | − | PD | 4 |
| Immature DC | (i.v./i.d.) | (i.v./i.d.) | (i.v./i.d.) | | | | | |
| Imm-7 | 18/5 | 14/3 | 9/9 | − | − | − | PD | 2 |
| Imm-8 | 33/10 | 16/3 | 13/13 | − | + | − | PD | 1 |
| Imm-9 | 45/15 | 25/10 | 52/15 | − | + | − | PD | 4 |
| Mature DC | (i.v./i.d.) | (i.v./i.d.) | (i.v./i.d.) | | | | | |
| Mat-1 | 31/5 | 30/8 | 55/7 | + | ++ | + | SD | 7.5 |
| Mat-2 | 45/15 | 34/6 | 50/13 | n.d. | + | − | n.e. | 2 |
| Mat-3 | 30/7 | 30/8 | 30/10 | ++ | +++ | + | PR[f] | >40[e] |
| Mat-4 | 50/15 | 17/5 | 30/15 | +++ | +++ | − | SD | 4.5[e] |
| Mat-5 | 33/15 | 19/12 | 27/15 | ++ | +++ | + | PD | 2 |
| Mat-6 | 70/25 | 50/17 | 60/30 | +++ | +++ | + | PD | 1.5 |
| Mat-7 | 12/10 | 12/12 | 10/10 | + | +++ | + | PD | 1.5 |
| Mat-8 | 15/7 | 26/9 | 13/13 | + | + | + | PD | 1.5 |
| Mat-9 | 30/14 | 18/8 | 20/10 | + | +++ | + | MxR | 6 |
| Mat-10 | 36/12 | 15/8 | 24/12 | + | +++ | + | SD | 22[e] |

[a] DTH was considered positive as at least two of the four DTH sites had a positive induration (− no induration; + induration ≥2<5; ++ induration ≥5<10; +++ induration ≥10).
[b] + standard index (SI) >2<10; ++ SI≥10<20; +++ SI≥20.
[c] − No Ab or < 1:400; + Ab titer ≥ 1:400.
[d] PD, progressive disease; SD, stable disease; MxR, mixed response; n.e., not evaluated; n.d., not done; PR, partial response.
[e] Patients received maintenance treatment with DC vaccinations.
[f] Complete response after excision of regressing tumor.

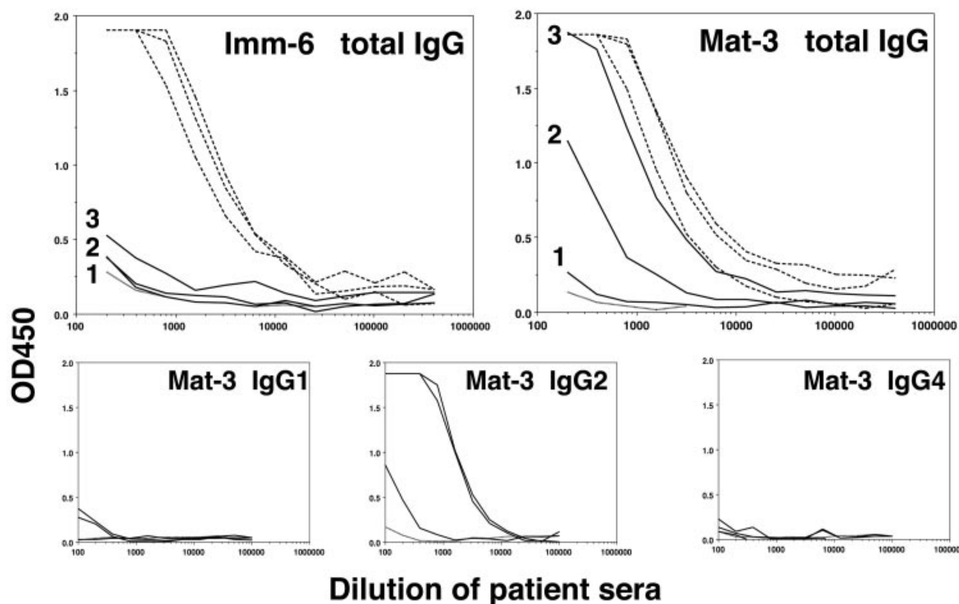


*Fig. 3* Representative humoral responses against KLH in the serum of patients vaccinated either with immature DCs (Imm-6) or mature DCs (Mat-3). Serum was obtained before (····), after each subsequent DC vaccination (— — —, number indicates number of DC vaccinations), and after vaccination with KLH protein (———). Total IgG antibodies with specificity for KLH were detected by ELISA in KLH-coated microtiter plates. Isotype-specific secondary antibodies were used to detect IgG1, IgG2, and IgG4 (patient Mat-3). Results of total IgG against KLH of all patients is shown in Table 2.

DCs provided the opportunity to test T-cell reactivity of the DTH-infiltrated T cells without restimulation *in vitro*. No T-cell outgrowth was observed from biopsies of DTH reactions against unpulsed DCs. From 4 of 7 patients, sufficient T cells were available after short time culture (*i.e.,* 2–4 weeks) to perform cytotoxicity assays. T cells derived from a biopsy of patient Mat-3 demonstrated specific lysis of gp100- and tyrosinase-BLM transfectants specifically (Fig. 4). In 5 of 7 patients, tetramer staining against the vaccinated peptides was performed. Because cultures from these biopsies consisted predominantly

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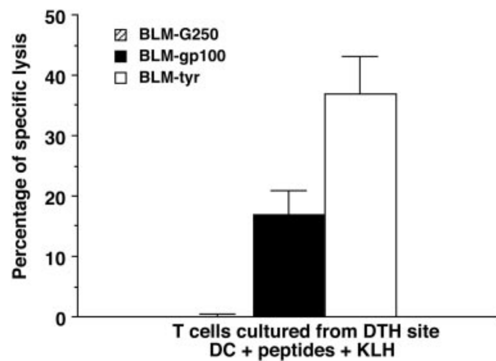


*Fig. 4* In a chromium release assay the lysis of HLA-A2.1-positive BLM cells, transfected either with G250 (negative control antigen), gp100, or tyrosinase by T cells (patient Mat-3) derived from a biopsy of a positive DTH site [DCs loaded with KLH and peptides (gp100:154, 280 and tyrosinase)] is shown.

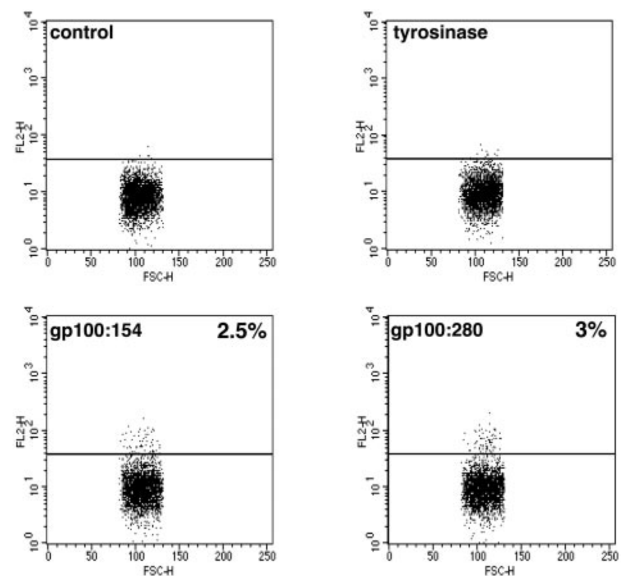


*Fig. 5* Tetramer analysis by flow cytometry of T cells derived from a biopsy of a positive DTH reaction of patient Mat-10. Depicted is the forward scatter on the *x*-axis (double staining with CD8 FITC might interfere with the tetramer staining and predominantly T cells grow out of these biopsies) and on the *y*-axis tetramer PE staining. Tetramer staining 1 log above the negative population was considered positive.

of T cells, we performed single tetramer staining. DTH-infiltrated T cells of patient Mat-10 were positive for tetramer gp100:154 (2.5%) and gp100:280 (3%) and negative for tyrosinase (Fig. 5).

**Clinical Outcome and Correlation with Immune Responses.** Of the immature DC group, 7 patients completed the DC vaccinations and 2 patients completed both parts of the protocol (Imm-1 and Imm-6). One of the 9 patients vaccinated with immature DCs was not evaluable for clinical response because of deteriorating performance status. All patients had either disease progression or stable disease with <4 month duration as best response (Table 2). The median overall survival was 7 months (range, 1.5–19 months).

Of the mature DC group, all patients completed the DC vaccinations, and 5 patients completed both parts of the protocol (Mat-1, Mat-3, Mat-4, Mat-9, and Mat-10). Three patients were eligible for maintenance therapy (Mat-3, Mat-4, and Mat-10), 2 of them received two additional DC vaccination cycles with a 6-month interval (Mat-3 and Mat-10).

Of the 10 patients vaccinated with mature DCs, 1 patient was not evaluable for clinical response because of deteriorating performance status, and 4 patients had disease progression. Stable disease was observed in 3 patients with a duration of 4.5, 7.5, and 22 months, respectively (Table 2). One patient (Mat-9) had a mixed response: four cutaneous metastases decreased in size (30–50%); however, one cutaneous metastasis increased with 35%. The median overall survival was 11 months (range, 2 to >40 months).

In 1 patient (Mat-3) with stage M1c melanoma vaccinated with mature DC, a partial response was observed in a distant lymph node metastasis with concomitant normalization of serum LDH. This metastatic site was subsequently resected. Upon pathological examination the resected lesion showed areas of necrosis and an abundant infiltrate of CD8+ T cells in the tumor, which was still gp100 positive, but demonstrated a decreased expression of tyrosinase as compared with a cutaneous melanoma lesion that was resected before DC vaccination (data not shown). This patient is now in complete remission for >3 years. In our study, the clinical outcome did not correlate with the pretreatment expression of gp100 or tyrosinase, neither with the percentage of positive cells, nor with the estimated staining intensity on the melanoma metastases (data not shown).

Although the number of patients in this study is limited and does not allow statistical analysis, it is intriguing that the best clinical outcome was observed in the 2 patients in whom tumor-specific T-cell responses of DTH-infiltrating lymphocytes was demonstrated. Patient Mat-3 and Mat-10 had a progression-free survival of >40 and 22 months, respectively. For comparison, in the patients in whom no specific T-cell reactivity could be observed, the median progression-free survival was 2 months (range, 1.5–7.5 months).

## Discussion

Our results in melanoma patients demonstrate a clear preference for the use of peptide-pulsed mature DCs as opposed to immature DCs in clinical vaccination studies. Monocyte-derived DCs matured with a mixture of proinflammatory cytokines and $PGE_2$ *in vitro* were superior in the induction of both humoral responses and proliferative responses against KLH *in vivo*. Analysis of KLH-specific T cells showed the production of the Th1 cytokine IFN-γ but not of the Th2 cytokine IL-4, and anti-KLH antibodies were of the IgG2 isotype, which is also indicative for a Th1 response (34, 35). Despite the previously observed lack of IL-12 production and subsequent Th1 development of $PGE_2$-matured DCs *in vitro* (36), we conclude vaccination with these fully matured DC polarize T cells toward IFN-γ production in patients.

The absence of T- and B-cell responses to KLH after vaccination with KLH-pulsed immature DCs might be partly explained by our recent observation that immature DCs are not

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able to efficiently migrate to the T-cell areas of lymph nodes (15). Because lymph nodes are the primary site of interaction among B cells, T cells, and DCs, DCs should migrate into the T-cell areas of secondary lymph nodes. Migration and subsequent induction of an immune response of the mature DC might be partly attributed to the use of $PGE_2$ in our maturation mixture. Recently, $PGE_2$ was described as a key factor in regulating the migratory capacity of DCs (Refs. 37, 38 and our own unpublished results). In addition to the lack of migration of immature DCs, the inability of these DCs to induce an immune response might also be attributable to the low production of DC-CK1 (CCL18), a chemokine that preferentially attracts both naïve T and B cells (39, 40). We previously demonstrated that DC-CK1 production by immature DCs is significantly lower when compared with mature DCs (23, 39–42). Our observation that injection of antigen-pulsed immature DCs does not lead to significant immune responses confirms and extends findings of Jonuleit *et al.* (43), who showed that immature DCs failed to induce peptide-specific cytotoxic T cells. Furthermore, the inability of immature DCs to give rise to an immune response also indicates that monocyte-derived DCs do not mature after injection *in vivo*. Thus, for active immunization in cancer patients, it appears to be critical that DCs are matured *in vitro* before injection.

Recently, it became clear that immature DCs are not simply ignored by the immune system but that antigen-pulsed immature DCs may blunt the capacity of the corresponding antigen-specific T cells to mount lytic activity *in vitro* (44). In addition, it has been shown that repetitive stimulation with immature DCs may lead to tolerance by inducing IL-10-producing regulatory T cells (45). In mice, DCs can, under steady-state conditions, induce T-cell tolerance and thereby maintain peripheral tolerance to self-antigens (46). Recent findings in humans also show circulating resident T-cell populations with potent regulatory properties in the peripheral blood (47). Whether regulatory T cells were induced by our vaccination with immature DCs remains to be investigated.

The superiority of mature DCs to induce an immune response was underscored by the observation that positive DTH reactions were only observed when the DCs were matured *in vitro*. In addition to DTH reactions to peptide/KLH-pulsed mature DCs, DTH reactions were also observed in response to nonpulsed mature DCs. Similar findings have been reported by others and can most likely be attributed to the production of large amounts of chemokines and cytokines by the mature DCs themselves (11, 23, 42). Of note, cultures from biopsies of positive DTH reactions to unpulsed DCs did not give rise to T cells.

Although significant differences were observed between vaccination with mature and immature DCs with respect to immunological response against KLH, the number of patients in our study is too small for a definite conclusion on the difference in clinical outcome between these vaccinations. Nevertheless, our results indicate improved clinical efficacy of peptide-pulsed mature DCs compared with immature DCs. Only in patients vaccinated with mature DCs could an objective clinical response be observed. A partial response was observed in a patient (Mat-3) with a distant lymph node metastasis with a concomitant normalization of serum LDH. After resection of the responding lesion, she currently is in ongoing remission for >40 months. Another patient (Mat-10) achieved disease stabilization of 22 months in irresectable bulky retroperitoneal lymph node metastases upon vaccination with mature DCs.

Prediction of clinical efficacy by immunological markers remains a challenge. Several clinical vaccination studies in cancer patients have reported T-cell responses in the peripheral blood but usually in a minority of patients or only after prolonged restimulation with antigen in culture (48–52). In our study, we could not detect significant antitumor T-cell reactivity in peripheral blood cells without restimulation *in vitro*. Therefore, we investigated the DTH reactivity as a tool to monitor the efficacy of our vaccine and its clinical outcome. Our results show that DTH-infiltrated T lymphocytes after short-term culture without antigenic restimulation show peptide-specific reactivity as determined by MHC-tetramer-positive T cells and cytotoxic reactivity to the vaccinated tumor antigens. Intriguingly, antigen-specific T-cell responses were detected in the 2 patients with the best clinical outcome (Mat-3 and Mat-10). In these patients, gp100-specific T cells were detected by MHC-tetramer staining in cultured T cells from the biopsy of a DTH reaction (Mat-10) and MHC-tetramer-positive cells by immunofluorescent staining on frozen biopsy sections (Mat-3).[3] Moreover, after a short time culture *in vitro*, these T cells were able to kill tumor cells expressing the antigens used for vaccination. The presence of antigen-specific T cells in the DTH of long-term responding patients suggests that the evaluation of T-cell reactivity in positive DTH sites may be a powerful tool in the monitoring of clinical T-cell-directed vaccination studies in cancer patients.

In conclusion, we here demonstrate that maturation of DCs before injection is pivotal to induce antigen-specific immune responses in melanoma patients. Moreover, clinical responses were only observed in patients vaccinated with mature DCs. Remarkable, T cells isolated from positive DTH sites of the patients with the best clinical outcome were specific for the antigens used for vaccination. This suggests that T cells isolated from positive DTH sites might be very helpful in monitoring vaccination therapy, but this method remains to be confirmed in larger clinical studies.

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