Natural Human Plasmacytoid Dendritic Cells Induce Antigen-Specific T-Cell Responses in Melanoma Patients

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Abstract

Vaccination against cancer by using dendritic cells has for more than a decade been based on dendritic cells generated \textit{ex vivo} from monocytes or CD34\textsuperscript{+} progenitors. Here, we report on the first clinical study of therapeutic vaccination against cancer using naturally occurring plasmacytoid dendritic cells (pDC). Fifteen patients with metastatic melanoma received intranodal injections of pDCs activated and loaded with tumor antigen-associated peptides \textit{ex vivo}. \textit{In vivo} imaging showed that administered pDCs migrated and distributed over multiple lymph nodes. Several patients mounted antivaccine CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell responses. Despite the limited number of administered pDCs, an IFN signature was observed after each vaccination. These results indicate that vaccination with naturally occurring pDC is feasible with minimal toxicity and that in patients with metastatic melanoma, it induces favorable immune responses. \textit{Cancer Res}; 73(3); 1–13. ©2012 AACR.

Introduction

Dendritic cells constitute a family of antigen-presenting cells (APC) defined by their morphology, phenotype, and unique capacity to process exogenously encountered antigens and to present them to naive T cells. Following infection or inflammation, dendritic cells undergo a complex process of maturation and migrate to lymph nodes to present antigens and activate T cells. This decisive role in inducing immunity was the rationale for dendritic cell–based immunotherapy, in which dendritic cells loaded with tumor antigens were injected into patients with cancer to stimulate T cells to eradicate tumors (1–3). So far, most dendritic cell–based vaccination studies were conducted in patients with metastatic melanoma because of the immunogenicity of this tumor and the characterization of antigens such as gp100 and tyrosinase (4, 5). Although numerous vaccination studies showed the immunogenicity of tumor antigen-loaded dendritic cells, the number of objective clinical responses has been limited, hampering its implementation as a novel form of standard treatment (6). Because of the limited number of naturally circulating dendritic cells, virtually all vaccination studies have used dendritic cells differentiated \textit{ex vivo} from monocytes or CD34\textsuperscript{+} progenitors. Recently, we proposed that these “artificial” dendritic cells may be less effective than their natural counterparts that circulate in the blood (7). Two major subsets of natural circulating dendritic cells can be found: plasmacytoid dendritic cells (pDC) and myeloid dendritic cells.

pDCs are the major producers of type I IFN, which is of major importance in combating viral infections. Freshly isolated pDCs have been associated with tolerance or Th2 responses (8, 9), but several groups have now shown that activated human pDCs produced large amounts of type I IFNs and induced Th1 responses (8, 10). Human pDCs induced strong allogeneic T-cell responses (11) and primed CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells against viruses or tumor antigens (12, 13). Preclinical studies showed that antigen-loaded pDCs protected against \textit{Leishmania major} (14) and induced antitumor responses that inhibited tumor growth (15, 16). Recently, Takagi and colleagues showed that pDCs were crucial for the initiation of inflammation and T-cell immunity (17). Together, these findings prompted us to test the capacity of activated human pDCs to elicit antitumor immune responses in patients.

In this first-in-human study, we report on vaccinating patients who have metastatic melanoma, with autologous activated pDCs loaded with tumor-associated peptides. Despite their low abundance in peripheral blood, we succeeded in isolating sufficient numbers (0.3–3 million per vaccination) of highly purified cells to carry out this study. We used the inactivated thick-borne encephalitis virus vaccine as a natural Toll-like receptor (TLR) agonist to activate pDCs and used these...
antigens as an immunomonitoring tool. We show that these activated pDCs were safe to use and induced antigen-specific CD4+ and CD8+ T-cell responses in patients suffering from melanoma.

Materials and Methods

Patient characteristics and clinical protocol

Sixteen patients with distant metastatic melanoma (according to the 2001 American Joint Committee on Cancer staging system; ref. 18) were enrolled in this feasibility study. One patient did not complete the scheduled vaccinations and monitoring due to rapid progression of disease. Fifteen of the patients were considered evaluable. Eligibility criteria included measurable target lesions, HLA-A2.1 phenotype, histologically documented metastatic melanoma expressing gp100 (compulsory) and tyrosinase (noncompulsory), no active infection or immune suppressive conditions, serum lactate dehydrogenase (LDH) concentration within normal limits (<450 U/L) and World Health Organization (WHO) performance status 0 or 1. Primary endpoint was toxicity related to vaccination and immunologic response. The trial, CMO 2004/093, was approved by the local Institutional Review Board (Committee on Research involving Human Subjects Arnhem-Nijmegen) and in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. The primary objective of this study was to generate clinical grade mature pDC preparations and to determine a safe and effective dosage of the vaccine. When the appropriate dosage of the vaccine was determined, we evaluated the efficacy of the pDCs to initiate antitumor T-cell responses in patients with stage IV melanoma. Clinical trial registration number is NCT01690377.

CliniMACS pDC isolation and immunization schedule

Patients were vaccinated with autologous pDCs loaded with HLA-A2.1-binding tumor peptides derived from the melanoma-associated antigens gp100 and tyrosinase. The following HLA-A2.1-restricted peptides were used: gp100-derived peptides gp100154–162 (KTWGQYWQV) and gp100280–288 (YLEPGPVTA) and tyrosinase-derived peptide tyrosinase369–377 (YMNGTMSQV). The clinical study was designed as a feasibility study. The first patient received 0.3 x 10^6 pDCs per vaccination, patients 2 and 3 received 1.0 x 10^6 pDCs per vaccination, and patients 4 and 5 received 3 x 10^6 pDCs per injection. Considering the yield from pDC isolation, this is the maximum feasible dose, and was also given to patients 6 to 15. Three intranodal injections were given once every 2 weeks followed by a delayed-type hypersensitivity (DTH) challenge (Fig. 1). In the absence of disease progression, patients were eligible for a maximum of 2 maintenance cycles consisting of 3 biweekly vaccinations and a DTH challenge, each with a 6-month interval.

pDCs were directly isolated from apheresis products using the fully closed immunomagnetic CliniMACS isolation system (Miltenyi Biotec). GMP-grade magnetic bead-coupled BDCA4 antibodies were used, following the manufacturer’s guidelines. This procedure resulted in clinically applicable purified pDCs, which had an average purity of 75% and a yield between 13 x 10^6 and 33 x 10^6 (Supplementary Fig. S1).
Following apheresis and CliniMACS isolation, pDCs were cultured overnight at a concentration of 10^6 cells/mL in X-VIVO 15 (Cambrex) containing 2% pooled human serum (Sanquin), supplemented with 10 ng/mL recombinant human interleukin-3 (rHIL-3; Cellgenix). For the first vaccination, these pDCs were subsequently activated for 6 hours by addition of FSME-IMMUN (1:10 v/v; Baxter AG). During the last 3 hours of activation, pDCs were loaded with the melanoma-associated peptides gp100_{154}, gp100_{280}, and tyrosinase (19). For subsequent vaccinations and DTH skin tests, overnight IL-3–cultured pDCs were activated for 3 hours by addition of FSME-IMMUN (1:10 v/v); thereaf e, these 3-hour activated pDCs were frozen in X-VIVO 15 medium containing 10% dimethyl sulfoxide (DMSO). Upon subsequent vaccinations/DTH, 3-hour activated pDCs were thawed and activated for an additional 3 hours with FSME-IMMUN (1:10 v/v) and loaded with the melanoma-associated peptides. The peptide-loaded pDCs were administered intranodally in a clinically tumor-free lymph node region under ultrasound guidance (20). This procedure gave rise to mature pDCs meeting the following release criteria: more than 50% viability, IFN-α secretion, high expression of MHC class I, MHC class II, CD83, CD80, CD86, and CCR7.

**Phenotype**

The purity of pDCs after CliniMACS isolation and the phenotype of the pDCs were determined by flow cytometry. The following primary monoclonal antibodies (mAb) and the appropriate isotype controls were used: anti-CD45-FITC, anti-BDCA2-PE, and anti-CDC23-PEC (all Miltenyi Biotec); anti-HLA-ABC-PE (W6/32), anti-HLA-DR/DP-FITC (Q5/13), anti-CD80-PE, and CD86-PE (all BD Biosciences Pharmingen); anti-CD83 (Beckman Coulter); and anti-CCR7 (R&D Systems) followed by goat-anti-mouse phycoerythrin (PE).

**Cytokine detection**

Supernatants were collected from pDC cultures either directly after 6 hours of stimulation or after 6 hours of stimulation followed by a washing step and subsequent reconstitution of cells in fresh medium for an additional 12 hours, and IFN-α production was analyzed with murine monoclonal capture and horseradish peroxidase-conjugated anti-IFN-α antibodies (BenderMed Systems) using standard ELISA procedures.

To analyze the T-helper cell profile, supernatants were collected after 2 days of pDC-PBL coculture. Cytokine production (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), TNF-α, TNF-β, IFN-γ) in the supernatant was analyzed with a human Th1/Th2 Multiplex kit (BenderMed System) according to the manufacturer’s instructions.

**111In-oxinate labeling and scintigraphic imaging**

Six-hour Frühsummer-Meningoenzephalitis (FSME)-activated pDCs were labeled with 5 MBq 111In-oxinate (GE Healthcare) in 0.1 mol/L Tris–HCl (pH 7.0) for 15 minutes at room temperature as described previously (21, 22). Cells were washed 3 times with PBS/1% HSA, and the labeling efficiency was calculated as the percentage of the activity that remained associated with the cell pellet. 111In-labeled pDCs (3 × 10^6; 4 MBq) in 200 μL saline were injected under ultrasound guidance directly into a clinically tumor-free lymph node.

In vivo planar scintigraphic images (256 × 256 matrix, 174 and 247 keV 111In photopeaks with 15% energy window) of the injection depot and corresponding lymph node basin were acquired with a gamma camera (Siemens ECAM) equipped with medium energy collimators, 15 minutes, 24, and 48 hours after injection. Migration was quantified by region-of-interest analysis of the individual nodes visualized on the images and expressed as the fraction of 111In-labeled dendritic cell that had migrated from the injection depot to follow ing lymph nodes after 15 minutes, 24, and 48 hours.

**Immunomonitoring of patients**

Blood samples were obtained before the start of the vaccination regimen and after each individual vaccination. Each sample was tested for the presence of FSME-specific T cells by proliferation and 3H-thymidine incorporation, and for the presence of FSME-specific antibodies in the serum using an ELISA specific for Tick-borne encephalitis immunoglobulin G (IgG; Serion/Virio). Four days after the third vaccination, a DTH skin test was conducted as described previously (23, 24). Monocyte-derived dendritic cells (moDC) pulsed with gp100 or tyrosinase peptides (1 × 10^6) and pDCs pulsed with gp100 or tyrosinase peptides (0.2 × 10^6) were injected intradermally in the skin of the back of the patient at 4 different sites. The maximum diameter of induration was measured after 48 hours. Punch biopsies (6 mm) were obtained from all DTH sites. Half of the biopsy was cryopreserved and the other half was manually cut and cultured in RPMI-1640 (Gibco-BRL Life Technologies) containing 7% human serum and IL-2 (100 U/mL, Proleukin). Every 7 days, half of the medium was replaced by fresh medium containing human serum and IL-2. After 2 to 5 weeks of culturing, T cells were tested for specificity against gp100 and tyrosinase (19). DTH-derived cells were stained with tetramer-MHC complexes containing the gp100_{154–167}, gp100_{280–288}, or tyrosinase_{369–376} peptide (Sanquin) combined with CD8 staining as described previously (23). All samples were tested with HIV_{77–85}-HLA-A21-tetramers recognizing the irrelevant HIV-epitope (SLYNTVATL) for background staining.

**Mixed lymphocyte-peptide cultures**

Blood frequencies of antivaccine CD8^+ T cells were estimated using mixed lymphocyte-peptide cultures as described previously (25). Briefly, peripheral blood mononuclear cells (PBMC) isolated before and after 1 cycle of 3 pDC injections, were thawed and divided in 3 groups incubated for 1 hour at room temperature in Iscove’s medium (Life Technologies) with 1% human serum and 2 μmol/L of the peptides tyrosinase_{369}, wild-type gp100_{154} (KTVGQYQWQY), or wild-type gp100_{280} (YLEPGPVVTA). These pulsed cells were then washed, pooled, and distributed at 2 × 10^6 cells/0.2 mL in round-bottom microwells in Iscove’s with 10% human serum, l-arginine (116 mg/L), l-asparagin (36 mg/L), l-glutamine...
(216 mg/L), 1-methyl-L-tryptophan (100 μmol/L), IL-2 (20 U/mL), and IL-7 (10 ng/mL). On day 7, 50% of the medium was replaced by fresh medium containing IL-2 and peptides at 4 μmol/L. Tetramer labeling was conducted on day 14 as described previously (25). Anti-gp100154 T-cell clones were derived that represented either the spontaneous anti-gp100 T cells present before vaccination in patients 2, 5, 6, 10, and 11, or the pDC-induced anti-gp100 T cells present after vaccination in patients 1, 4, 8, and 12. Tetramer-positive CD8+ T cells were sorted at 1 cell per well and restimulated weekly with irradiated HLA-A2* EBV-transformed B cells pulsed with the gp100154 peptide at 2 μmol/L, and irradiated allogeneic PBMC as feeder cells, in medium supplemented with IL-2 and IL-7. Established T-cell clones were stained with the relevant tetramer and their functional avidity was evaluated by testing their production of IFN-γ after stimulation with HLA-A2* EBV-B cells pulsed with various concentrations (from 10 pmol/L up to 10 μmol/L) of the gp100154 peptide.

RNA isolation and qPCR

To determine mRNA expression of IFN and IFN-stimulated genes, blood was drawn from 1 patient during 1 cycle of 3 vaccinations before each vaccination and at 4 and 24 hours after each vaccination using PAXgene tubes. RNA isolation was conducted using PAXgene Blood RNA Kit according to the manufacturer’s instructions (Qiagen). RNA isolations from PBMCs from healthy volunteers were done using the ZR RNA Isolation Kit (Zymo Research) according to manufacturer’s instructions. RNA was treated with DNase I (amplification grade; Invitrogen) and reverse-transcribed into cDNA by using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). To exclude genomic DNA contamination, we included a “-RT” control in which the reverse transcriptase was replaced with RNase-free water. The -RT control was taken along in the quantitative PCR (qPCR) analysis and was used as a cutoff value. cDNA was stored at −20°C until further use. mRNA levels for the genes of interest were determined by qPCR with a Bio-Rad CFX apparatus (Bio-Rad) with SYBR Green (Applied Biosystems). Analysis was done using Biorad CFX-1.6 software, and expression levels were determined relative to Porphobilinogen deaminase (PBGD) expression. Primer sequences are available on request and were from the Primer Bank database (26).

Matched historical controls

Matched historical controls were identified from records of patients with metastatic melanoma from the Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands), the Netherlands Cancer Institute—Antoni van Leeuwenhoek Hospital (Amsterdam, the Netherlands), and University Hospital Essen (Essen, Germany) who had received first-line dacarbazine (DTIC) chemotherapy at 850 to 1,000 mg/m2 i.v. at 3 week intervals, between March 2000 and March 2010. All matched controls were HLA-A*02:01-positive and were required to have received at least 3 infusions, a therapy time frame that is consistent with 1 cycle of vaccinations. Historical controls were matched to study subjects, in ratio 1:3, primarily for M substage at baseline according to American Joint Committee on Cancer criteria, number of distant metastases, number of metastatic sites, localization of distant metastases, and baseline serum LDH. These criteria currently represent the most important prognostic factors for survival (27). In case of more than 3 matches for 1 study subject, demographic criteria (age, gender), and systemic salvage treatment after progression on DTIC were used to select the closest match.

Statistical analysis

Significant differences from controls were determined according to paired Student t test or by one-way ANOVA analysis followed by the Tukey post hoc test. Differences between the groups were evaluated using an unpaired t test. Differences between pre- and postvaccination were evaluated with a Wilcoxon signed-rank test. Kaplan–Meier probability estimates of progression-free survival (PFS) and overall survival (OS) were calculated, statistical differences between the survival of the groups were determined with a log-rank test. Statistical significance was defined as P < 0.05. SPSS19.0 was used for survival analyses.

Results

Vaccination of metastatic melanoma patients with activated pDCs is safe with minimal side effects

It is of utmost importance that pDCs administered to patients with cancer have an immunostimulatory phenotype (19). Previously, we have shown that the commonly used preventive FSME-vaccine induced both IFN-α secretion and a mature pDC phenotype (28). Thus, FSME could be used as a clinically applicable natural TLR agonist. In this first-in-human study, we vaccinated 15 patients with increasing pDC numbers, ranging from 0.3 to 3 million per vaccination, with 3 vaccinations at biweekly intervals (Fig. 1). Patient characteristics are shown in Table 1. The expression of CD80, CD83, CD86, MHC class I, and II upon FSME-induced activation indicated that the generated pDCs were highly mature and capable of providing costimulatory signals needed for optimal T-cell activation (Fig. 2A). Furthermore, pDC activation for 6 hours led to the secretion of high levels of type I IFN (Fig. 2B). In addition, we also washed 6-hour activated pDCs and reconstituted the cells in fresh medium. Thereafter, pDCs were maintained for an additional 12 hours to show that pDCs have a sustained ability to secrete IFN-α (Fig. 2B). Thus, all patients received purified and activated peptide-loaded pDCs that met the predefined release criteria with the ability to secrete type I IFN in vivo (6). Furthermore, the vaccines were well tolerated and no signs of severe toxicity (common toxicity criteria grade 3–4) were observed. Six vaccinated patients developed grade 1 flu-like symptoms and 1 patient reported grade 2 non-treatment–related pain resulting from progressive subcutaneous metastasis. In none of the vaccinated patients did we detect antibodies to the murine antibody used during the isolation procedure (data not shown). We conclude that
Table 1. Patient characteristics and clinical and immunologic responses after pDC vaccination

<table>
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<tr>
<th>Patient</th>
<th>Sex/age</th>
<th>Prior treatment</th>
<th>Stage at baseline</th>
<th>Site of metastases</th>
<th>Number of apheresis</th>
<th>Injected pDCs</th>
<th>Number of vaccinations</th>
<th>Anti FSME response</th>
<th>Anti tumor response</th>
<th>PFS</th>
<th>OS</th>
<th>Salvage treatment</th>
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<td>+</td>
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<td>++</td>
<td>++</td>
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<td>3</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>PD</td>
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Abbreviations: CT, chemotherapy; IT, immunotherapy; MR, mixed response; OS, overall survival; PD, progressive disease; PFS, progression-free survival; RT, radiotherapy; S, surgery; SD, stable disease.

aNo T-cell response; + Stimulation index >1-2; ++ SI ≥2:10.
bNo Ab or <15 U/mL; ± Ab titer < 28 U/mL; + Ab titer > 28 <100 U/mL; ++ Ab titer >100 U/mL.
cPFS in months.
dOS in months.
eAnti-CTLA4 antibody.
fAkt inhibitor.
gIfosfamide-pazopanib.
hMitogen-activated protein/extracellular signal–regulated kinase (MEK) inhibitor (AZD6244).
it is feasible and safe to administer activated and tumor-peptide–loaded pDCs to patients.

Migration of activated pDCs in vivo

For effective immunization, injected dendritic cells have to migrate into the T-cell areas of draining lymph nodes to present antigens to naïve T cells. Because of the limited amount of pDCs available, we opted for intranodal injection to maximize the number of pDCs in the lymph node. In 3 patients, we examined the migration in vivo of activated pDCs labeled with 111In (21, 29). Forty-eight hours after intranodal injection, a significant proportion of 111In-labeled pDCs remained at the injection site and distinct amounts were detected in distant lymph nodes (Fig. 2C). These results showed that the pDCs injected in a single lymph node distributed into downstream nodes.

Vaccination with activated pDC induce an IFN signature in vivo

To verify whether activated pDCs secreted significant quantities of type I IFNs in vivo, we investigated in 1 patient the IFN signatures in great detail (multiple time points/3 vaccinations). We measured in blood mononuclear cells the expression of type I IFNs genes and of 5 IFN-induced genes in blood mononuclear cells 1 hour before and 4 and 24 hours after vaccination, and compared these expression levels with those of healthy individuals. Transcription of both IFN-α and IFN-β genes was clearly induced 4 hours after vaccination and decreased 20 hours later (Fig. 3A), indicating a temporal systemic induction of type I IFNs. As expected, we observed increases in the expression of the IFN-induced genes RIG-I, PKR, OAS-1, OAS-2, and IRF-7 after 4 hours and further increases after 24 hours (Fig. 3B). These results show that after each vaccination even small numbers of injected pDCs induced a systemic type I IFN signature.

Plasmacytoid dendritic cell vaccination leads to CD4+ T-cell proliferation and antibody production

We next analyzed the ability of activated pDCs to prime T-cell responses to the FSME antigens, which served as an internal control for the tumor-associated antigenic peptides. After 3 pDC vaccinations, FSME-stimulated T-cell proliferation increased in 9 of 14 tested patients (Fig. 4A and Table 1). Cytokine production experiments carried out in 6 patients showed higher levels of IFN-γ, IL-10, and IL-5 after vaccinations than before (Fig. 4B and data not shown).

Furthermore, we followed the levels of serum anti-FSME antibodies after each vaccination. Twelve of 15 tested patients significantly increased their anti-FSME IgG titers starting...
after the second vaccination (Fig. 4C and Table 1). One patient (pt 14) already had anti-FSME antibodies before vaccination.

In only 1 of 15 patients neither T cells nor antibodies to FSME were detected. Thus, the majority of patients developed de novo proliferative and humoral FSME-specific immune responses upon vaccination, indicative of a functional immune system that can be primed by activated and antigen-loaded pDCs.

Activated pDCs induce tumor antigen-specific CD8+ T-cell responses in vivo

We used tetramers to detect the presence of tumor antigen-specific CD8+ T cells in blood, and in biopsies taken from skin DTH reactions as described previously (23). In blood, the proportions of total CD8+ T cells did not change following vaccination (Fig. 5A), and ex vivo tetramer staining was negative both before and after vaccination (data not shown). We resorted to an in vitro restimulation of blood mononuclear cells in limiting dilution conditions over 2 weeks with the 3 antigenic peptides, before screening all microcultures for the presence of CD8+ tetramer+ cells. This procedure allows to estimate the frequencies of blood CD8+ T cells that recognize a given antigen and proliferate in vitro in response to this antigen. As shown in Fig. 5B, 7 of 15 patients showed a significant increase (≥5-fold) of the frequency of gp100154-specific CD8+ T cells. To evaluate the affinity of these antivaccine T cells, we derived anti-gp100154 T-cell clones from pre- and postvaccination PBMC and measured IFN-γ production after a stimulation with various concentrations of peptide. The concentration of peptide that stimulates a half-maximal cytokine production reflects the functional avidity of the T cells. As shown in Fig. 5C, 2 of 8 postvaccination clones had a higher (>10-fold) functional avidity than that of all the other pre- or post vaccination clones, suggesting the efficacy of pDCs at priming high-affinity T cells.

In the skin, we observed in 10 of 15 patients positive DTH responses with indurations of up to 22 mm at the sites of intradermal injection of 0.2 × 10^6 peptide-loaded activated pDCs (Supplementary Table S1). Furthermore, we detected

Figure 3. Vaccination with activated pDCs induces an IFN signature. A, the graphs show the IFN-α and IFN-β gene expression levels in the blood relative to PBGD expression at indicated times after each vaccination. B, the graphs show the mRNA expression of indicated IFN-stimulated genes determined as in A. Healthy, steady-state expression levels of indicated genes in 8 healthy individuals. n.a., not analyzed; n.d., not detected (†, P < 0.05; ‡, P < 0.01; ‡‡, P < 0.001).
anti-gp100\textsubscript{154} CD\textsuperscript{8}{+} T cells in 1 DTH biopsy obtained after 1 cycle of vaccination (pt 1) and in 1 obtained after 2 cycles (pt 13; Fig. 5D). T cells obtained from biopsies of DTH reactions to unloaded pDCs did not proliferate showing the specificity of this response. We conclude that vaccination with small numbers of peptide-loaded and activated pDCs can induce tumor-specific CD\textsuperscript{8}{+} T-cell responses in patients with metastatic melanoma.

**Clinical outcome of late-stage melanoma patients vaccinated with plasmacytoid dendritic cell**

Although the patient number does not allow drawing significant conclusions about the clinical outcome, we did observe some interesting trends. Two patients (pt 13 and 14) showed durable stable disease and were eligible for 2 additional cycles consisting of 3 pDC vaccinations. Patient 14 developed a mixed response with regression of lung metastases and progression of a nodal metastasis. Patient 10 had a documented progression but achieved complete remission after surgery and subsequently received maintenance treatment. To have an indication whether pDC vaccination has an influence on clinical outcome, we compared the clinical outcome of 15 pDC-vaccinated patients with the outcome of carefully selected matched control patients (n = 72) who received standard DTIC (dacarbazine) chemotherapy as a first-line treatment (Supplementary Table S2). The median PFS in the pDC group was 4.0 versus 2.1 months in the control group (not significant; Fig. 6A). Although the initial endpoint of this study was safety and feasibility, the median OS showed a remarkable improvement as compared with matched control patients: 22.0 [95% confidence interval (CI), 1.8–42.2] versus 7.6 months (95% CI, 5.8–9.4), P = 0.001. Furthermore, 7 of 15 patients were alive, even 2 years after the start of pDC vaccination, compared with 6 of 72 patients treated with standard chemotherapy (Fig. 6B).

**Discussion**

After almost 15 years of dendritic cell–based immunotherapy, the clinical efficacy of the ex vivo generated monocyte or CD34\textsuperscript{+} progenitor derived-dendritic cells is disappointing. Therefore, properly activated naturally occurring dendritic...
cells might represent the next generation of anticancer cellular therapy to induce tumor-specific immunologic responses and improve clinical efficacy. In this first clinical study with pDCs in patients with metastatic melanoma, with activated pDCs injected into lymph nodes, we observed: (i) distribution of injected pDCs over multiple lymph nodes, (ii) enhanced systemic secretion of type I IFNs, (iii) de novo T- and B-cell responses to control antigen, and (iv) induction of tumor antigen-specific CD8^+ T cells, including some with high functional avidity.
pDCs are known to sense viral or self-nucleic acids and to become activated to produce type I IFN at sites of inflammation. These IFNs initiate protective immunity through maturation of resident myeloid dendritic cells and subsequent activation of infiltrating T cells and natural killer (NK) cells. In contrast, nonactivated pDCs promote T regulatory cell-mediated immunosuppression (30), and the presence of pDCs in tumors has been correlated with poor clinical outcome (31, 32). We hypothesized that properly activated pDCs, because of their IFN production, might stimulate myeloid dendritic cells and enhance their ability to cross-prime CD8$^+$ T cells, thereby inducing more efficient antitumor T-cell responses when compared with in vitro generated dendritic cells. This notion is supported by 2 independent studies showing in mice that type I IFNs were critical for the induction of antitumor immune responses (33, 34). Therefore, to warrant clinical efficacy, pDCs should secrete type I IFNs at the time of administration. Because previous studies showed that pDCs produced IFN-α within 12 hours after activation and then became refractory to further stimulation (35–37), isolated pDCs were maintained overnight in IL-3 and subsequently activated for 6 hours with FSME. Our approach resulted in clinically applicable pDCs with a stimulatory phenotype and secretion of type I IFNs in vivo. Accordingly, we now show that vaccination with such pDCs indeed induced an IFN signature based on the upregulated IFN-induced genes in the blood after vaccination, validating our approach.

For an optimal immune response, the injected pDCs should migrate to neighboring lymph nodes and then to T-cell areas. The observed migratory behavior and distribution over multiple lymph nodes of the injected pDCs might be linked to the expression of CCR7 (Supplementary Fig. S2), the homing receptor for the chemokines CCL19 and CCL21 that are expressed by high endothelial venules and stromal cells in the T-cell areas (30, 38, 39). The pDC migratory behavior is in agreement with previous studies, where although the majority of injected dendritic cells reside at the injection depot (22, 29, 40, 41), a substantial fraction of the pDCs migrated to subsequent lymph nodes. It is important to consider that the microenvironment influences cell migration (42). Therefore, a high number of pDCs at the injection site might affect the microenvironment. For example, pDC-derived IFNs could drive expression of chemokines resulting in the recruitment of immune cells, thereby circumventing the need for active migration of the pDCs themselves. Unfortunately, the clinical protocol did not allow lymph node resections, to further substantiate this important aspect.

We used FSME as natural TLR agonist to activate pDCs. As FSME comprises a total inactivated virus, we reasoned that during activation pDCs will process FSME-derived antigens and present antigenic peptides on human leukocyte antigen (HLA) molecules. Indeed, we observed cellular as well as humoral responses against FSME in almost all patients. Therefore, commonly used vaccines are potentially interesting, to serve both as a TLR agonist and as a control antigen. In addition, it cannot be excluded that anti-FSME Th1 responses contribute to the expansion of CD8$^+$ tumor specific T cells, or whether synthetic CpG oligonucleotides also will be sufficient for clinical benefit.

We have previously shown that the presence of anti-vaccine CD8$^+$ T cells at sites of vaccine-induced skin DTH reactions was correlated with favorable clinical outcome (23, 43). Several clinical studies in patients with cancer have reported antivaccine T-cell responses in the blood but only

Figure 6. pDC vaccination improves OS. Clinical outcome to pDC vaccination was compared with a group of carefully matched historical control patients who received dacarbazine as first-line treatment. PFS for pDC vaccination was 4.0 versus 2.1 months in the control group. Median OS data showed a remarkable improvement compared with matched control patients; 22.0 versus 7.6 months. Furthermore, the OS of pDC-vaccinated patients was significantly improved compared with matched controls. Statistical significance between the survival of the groups was determined by a log-rank test, $P = 0.001.$
in a minority of patients or after prolonged in vitro restimulation with antigen (44–47). Here, we observed the specific induction of tumor-specific CD8⁺ T cells, which indicates that even small numbers of pDCs can evoke antigen-specific immune responses. To verify if this tumor-specific T-cell response contributed to long-term tumor control, we compared the clinical outcome of our patients with that of carefully selected controls treated with standard DTIC chemotherapy. Although it must be emphasized that this study was not designed to evaluate clinical outcome, several interesting observations were made. First, PFS in vaccinated patients was similar to that of the matched control patients, matching PFS reported in literature (48–50). Remarkably, the 1- and 2-year survival rates were 60% and 45% after pDC vaccination compared with 30% and less than 10% of the matched control patients, respectively. To exclude that this discrepancy in survival might partially be influenced by subsequent salvage treatments, we carefully matched the total number of different treatments and the types of salvage treatments for both control group and the vaccinated patients (Supplementary Table S1). Second, we detected tumor-specific CD8⁺ T cells in 4 of 6 patients who showed a clinical response (stable disease or mixed response) and had a long OS (defined as more than 24 months). Therefore, we hypothesize that the improved OS is related to the priming or restimulation of antigen-specific CD8⁺ T cells, which would be in line with our previous findings (23). However, a uniform framework as a reference for immunomonitoring is mandatory to appreciate immune responses in clinical trials. To date, no monitoring assay that evaluates a single T-cell function exists that relates to clinical outcome. Most information about CTL frequencies is derived from cancer germ-line genes such as MAE (51). These frequencies are comparable with the here-observed frequencies for differentiation antigen-specific T cells. In our previous study, in which we compared wild-type peptides and modified peptides on moDCs, increased T-cell frequencies were observed in responding patients, slightly higher than in this study (0.01%; ref. 52). Third, given the low numbers of CTLs that we detected, as compared with numbers reported in trials with monocyte- or CD34⁺ progenitor-derived dendritic cells, it is tempting to speculate that other immune cells from the innate arm play an important role in assisting CTLs. Therefore, it would be extremely interesting to study the immune cell repertoire within resided lymph nodes in more detail. Comparable patterns, no effect on PFS but significantly extended OS, were recently described for other immunotherapies, especially anti-CTLA4 blockade (49). These findings support the notion that immunotherapy may act indirectly, rather than having a direct cytotoxic antitumor effect.

The interesting observation about the OS, albeit retrospectively, urge for a randomized phase II clinical trial to confirm the potential of natural pDCs as an anticancer vaccine.

Disclosure of Potential Conflicts of Interest
G. Winkels is employed as a Senior Project Manager in Miltenyi Biotec GmbH. No potential conflicts of interest were disclosed by the other authors.

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