Effective clinical responses in metastatic melanoma patients after vaccination with primary myeloid dendritic cells

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Running title: Myeloid DCs induce effective responses in melanoma patients

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Translational relevance

Vaccination with dendritic cells (DCs) loaded with tumor-peptides is feasible, safe and can induce tumor-specific immune responses in advanced cancer patients. Thus far, DC-based immunotherapy of cancer was primarily based on in vitro-generated monocyte-derived DCs that require extensive in vitro manipulation, which may affect the survival of these cells in vivo, their capacity to migrate to the lymph nodes, as well as their immunogenic potential.

Here, we vaccinated metastatic melanoma patients with primary myeloid DCs, directly isolated from patient's blood and activated by only brief ex vivo culture. We found that vaccination is well tolerated and can induce de novo immune responses and objective clinical responses, which were associated with the presence of multifunctional tumor antigen-specific T cells in both blood and tissues. The rapid vaccine production procedure is highly standardized and will therefore expedite larger randomized multicentre trials.

Abstract

PURPOSE

Thus far, dendritic cell (DC)-based immunotherapy of cancer was primarily based on in vitro-generated monocyte-derived DCs, which require extensive in vitro manipulation. Here, we report on a clinical study exploiting primary CD1c+ myeloid DCs, naturally circulating in the blood.

EXPERIMENTAL DESIGN

Fourteen stage IV melanoma patients, without previous systemic treatment for metastatic disease, received autologous CD1c+ myeloid DCs, activated by only brief (16h) ex vivo culture and loaded with tumor associated antigens of tyrosinase and gp100.

RESULTS

Our results show that therapeutic vaccination against melanoma with small amounts ($3\text{-}10\text{x}10^6$) of myeloid DCs is feasible and without substantial toxicity. Four out of fourteen patients showed long-term progression-free survival (12-35 months), which directly correlated with the development of multifunctional CD8+ T cell responses in three of these patients. In particular, high CD107a expression, indicative for cytolytic activity, and IFNγ as well as tumor necrosis factor (TNF)α and CCL4 production was observed. Apparently, these T cell responses are essential to induce tumor regression and promote long-term survival by stalling tumor growth.

CONCLUSION

We show that vaccination of metastatic melanoma patients with primary myeloid DCs is feasible and safe and results in induction of effective anti-tumor immune responses that coincide with improved progression-free survival.

Introduction

Dendritic cells (DCs) are central players in immune responses. As professional antigen-presenting cells, DCs sample the tissue microenvironment and phagocytose both pathogen-derived products and dying host cells, including tumor cells(1). DCs have the unique capacity to attract and activate naive (tumor) antigen-specific CD4+ and CD8+ T cells. DC-based immunotherapy exploits this property of DCs: Tumor antigen-loaded DCs are injected into cancer patients to stimulate T cells and initiate tumor eradication(2,3). In clinical trials this approach resulted in effective immunological responses that coincided with favorable clinical outcomes(4,5). However, the number of objective clinical responses is limited, hampering its implementation as standard treatment.

In the majority of clinical DC vaccination studies performed so far, DCs differentiated in vitro from monocytes or CD34+ progenitors have been used, which may not be the optimal DC source for immunotherapy(6). The extensive culture period (8-9 days) and compounds required to differentiate these cells into DCs may negatively affect their immunological potential and their capacity to migrate to the T cell areas of the lymph nodes(7,8). Therefore, naturally circulating primary DCs may be a potent alternative for monocyte-derived DCs (moDCs), irrespective of the fact that they are relatively scarce (ranging from $1x10^7$ to $1x10^8$ DC in a single apheresis). A major advantage of exploiting primary DCs instead of moDCs is the brief ex vivo exposure to activate the cells and load them with tumor antigens, usually less than 24 hours. This might better preserve their functional capacities and prevent exhaustion.

Two main populations of naturally circulating primary DCs can be distinguished in human peripheral blood: myeloid (m)DCs and plasmacytoid (p)DCs. These subsets differ in function, localization, and phenotype. MDCs mainly migrate to, or reside in, the marginal zone of the lymph nodes (a primary entry point for blood-borne antigens) and are specialized in immunity against bacteria and fungi(9,10,11). PDCs on the other hand, mainly localize to the T cell areas of lymph nodes and seem specialized for viral antigen recognition(9). Depending on the pathogens they encounter, both DC subsets have the capacity to initiate suitable T cell responses. Anti-tumor responses induced by both mDCs and pDCs have been reported in animal models(12,13).

Recently, we completed the first vaccination trial exploiting ex vivo-activated and tumor antigen-loaded autologous primary pDCs in metastatic melanoma patients. Although the trial was

designed as a safety and feasibility study, we obtained promising clinical results; 7 out of 15 metastatic melanoma patients were still alive two years after initiation of treatment(14). Both activated pDCs, which produce high amounts of interferon (IFN)α, and activated mDCs have the capacity to induce T helper 1 cells, cytotoxic T cells, natural killer cells and natural killer T cells, leading to a potent cellular immune response(15,16,17). Moreover, primary mDCs isolated from healthy donors and cancer patients are able to prime tumor-specific CD8+ T cells in vitro(18). Human mDCs can be further subdivided into two populations, based on their differential surface expression of CD1c (BDCA1) and CD141 (BDCA3)(10,19). Here, we studied the feasibility and safety of vaccination of metastatic melanoma patients with ex vivo-activated and tumor antigen-loaded autologous CD1c+ mDCs and investigated whether primary mDCs are capable of inducing anti-tumor responses. In peripheral blood and cultures of skin test biopsies of vaccinated patients, we monitored the presence and functionality of tumor antigen-specific T cells. The results show that mDC vaccines induce cytotoxic T cells in vivo that express multiple functional markers simultaneously. The presence of these multifunctional T cells coincided with improved progression-free survival, demonstrating the potential of primary mDCs for anti-cancer immunotherapy.

Patients and Methods

Patient characteristics and clinical protocol

The study, CMO2004/093, was approved by the local Institutional Review Board (Committee on Research involving Human Subjects Arnhem-Nijmegen) and in concordance with the Declaration of Helsinki. Clinical trial registration number is NCT01690377. Written informed consent was obtained from all patients. Fifteen metastatic melanoma patients (stage IV or irresectable stage IIIc according to the 2009 American Joint Committee on Cancer staging system(20)) were enrolled in this phase I/II feasibility study (Table 1). One patient was excluded from analysis due to a protocol violation, the patient did not meet an inclusion criterion (VI-B-15; HLA-A*0201 negative). Therefore, fourteen patients were considered evaluable for immunological and clinical response. However, one patient did not complete the scheduled vaccinations due to rapid progression of disease (VI-B-04) so a delayed-type hypersensitivity (DTH) skin test was not available for immunomonitoring. Eligibility criteria included a measurable target lesion, HLA-A*0201 phenotype, histologically documented melanoma expressing gp100 (compulsory) and tyrosinase (non-compulsory), no serious active infection or immune suppressive conditions, serum LDH concentration within normal limits and WHO performance status 0 or 1. All vaccinations were administered between November 2010 and October 2013. The primary objective of this study was to generate clinical grade mature mDC preparations and to determine a safe and immunologically effective dosage of the vaccine. When the appropriate dosage of the vaccine was determined, we evaluated the efficacy of the mDCs to initiate anti-tumor T cell responses in patients with metastatic melanoma.

CliniMACS mDC isolation and immunization schedule

Patients were vaccinated with autologous mDCs loaded with HLA-A*0201-binding tumor peptides derived from the melanoma-associated antigens gp100 and tyrosinase. The first patient received a maximum of 3×10^6 mDCs per vaccination, patients 2 and 3 received maximally 5×10^6 mDCs per vaccination, and patients 4 and 5 received maximally 10×10^6 mDCs per injection. Considering the yield from mDC isolation, this is the maximum feasible dose, which was also given to patients 6 to 15. Three subsequent intranodal injections were given once every two weeks followed by a DTH challenge. In the absence of disease progression, patients were eligible for a maximum of two

maintenance cycles consisting of three biweekly vaccinations and a DTH challenge, each with a 6-month interval.

MDCs were directly isolated from apheresis products using the fully closed immunomagnetic CliniMACS isolation system (Miltenyi Biotec, Bergisch Gladbach, Germany) (Supplementary Figure 1). GMP-grade magnetic bead-coupled antibodies were used, following the manufacturer's guidelines. First, B cells were depleted using magnetic bead-coupled CD19 antibodies, followed by positive selection of CD1c+ mDCs with biotin-coated CD1c antibodies and magnetic bead-coupled anti-biotin antibodies. This procedure resulted in clinically applicable purified mDCs, which had an average purity of 93% and a yield between 27×10^6 and 96×10^6 cells (Figure 1A, B). Following apheresis and CliniMACS isolation, mDCs were cultured overnight (16 hours) at a concentration of 10^6 cells/ml in X-VIVO-15 (Lonza, Verviers, Belgium) containing 2% pooled human serum (HS) (Sanquin, Nijmegen, the Netherlands), supplemented with 800 U/ml recombinant human GM-CSF (Cellgenix, Freiburg, Germany) and 1 mg/ml keyhole limpet hemocyanin (KLH; Immucothel, Biosyn Arzneimittel GmbH, Fellbach, Germany) for immunomonitoring. This procedure gave rise to mature mDCs meeting the following release criteria: more than 50% viability, high expression of MHC class I, MHC class II, CD83 and CD86[2]. Part of these mDCs were directly loaded with the melanoma-associated HLA-A*0201-restricted peptides gp100$_{154-162}$ (KTWGQYWQV), gp100$_{280-288}$ (YLEPGPVTA) and tyrosinase$_{369-377}$ (YMNGTMSQV) for the first vaccination, which was given directly after peptide loading[21]. The remainder of the mDCs were frozen with 10% DMSO for subsequent vaccinations or DTH. Upon subsequent vaccinations/DTH, frozen mDCs were thawed and loaded with the melanoma-associated peptides. The peptide-loaded mDCs were administered intranodally in a clinically tumor-free lymph node under ultrasound guidance.

Flow cytometry

The purity of mDCs after CliniMACS isolation and the phenotype of the mDCs were determined by flow cytometry. The following primary monoclonal antibodies (mAbs) and the appropriate isotype controls were used: anti-CD1b/c-FITC (Diaclone, Besançon Cedex, France), anti-CD20-PE, anti-CD45-PerCP, anti-CD14-APC, anti-HLA-DQ-PE (all BioLegend, San Diego, CA, USA), anti-HLA-ABC-PE, anti-HLA-DR-PE, anti-CD80-PE, anti-CD83-PE, and CD86-PE (all BD Pharmingen, San Diego, CA, USA). Flow cytometry was performed with a FACS Calibur (BD Biosciences, San Jose, CA, USA).

Immunomonitoring

Peripheral blood mononuclear cells (PBMCs) and serum samples were obtained before the start of the vaccination regimen and after each individual vaccination. Samples were tested for the presence of KLH-specific T cells by proliferation and ^{3}H-thymidine incorporation, and the presence of KLH-specific antibodies in serum using ELISA[22].

Within one to two weeks after the third vaccination, a DTH skin test was performed as described previously[4,5]. Briefly, moDCs (1×10^6; cultured as previously described[21]) and mDCs (0.5×10^6), pulsed with gp100 or tyrosinase peptides, 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 and cultured as previously described[4,5]. After a culture period of 2 to 5 weeks, skin-infiltrating lymphocytes (SKILs) were tested for specificity against gp100 and tyrosinase.

PBMCs and SKILs were stained with tetrameric-MHC complexes containing the $gp100_{154-162}$, $gp100_{280-288}$ or $tyrosinase_{369-377}$ peptide (Sanquin) combined with CD8 staining as described previously[5]. All samples were tested with HIV_{77-85}-HLA-A*0201-tetramers recognizing the irrelevant HIV-peptide (SLYNTVATL) for background staining.

Antigen recognition was determined by the production of cytokines by SKILs in response to T2 cells pulsed with the indicated peptides or BLM cells transfected with control antigen G250, with gp100 or with tyrosinase, or the allogeneic HLA-A*0201positive, gp100-positive and tyrosinase-positive Mel624 tumor cell line. Cytokine production was measured in the supernatants after 16 hours of coculture by a cytometric bead array (eBioscience, San Diego, CA, USA).

Analysis of multifunctional T cells in peripheral blood

Analysis was performed as previously described[23]. Cryopreserved PBMCs or peripheral blood lymphocytes (PBLs) were thawed and suspended in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 10% HS (Sigma Aldrich, St. Louis, MO, USA) and antibiotic-antimycotic (Gibco). T cells were stimulated using CD28/49c-coated beads (BD Bioscience), either alone or in combination with 10 µg/mL peptide $gp100_{154-162}$, $gp100_{280-288}$ or $tyrosinase_{369-376}$. Brefeldin A (10 µg/mL, Sigma Aldrich) and Monensine (2 µM, eBioscience) were added and cells were incubated for 5

to 6 hours at 37°C and 10% CO_2. After incubation, T cells were stained with Fixable Viability Dye eFluor780 (eBioscience) and surface markers APC-Cy7- or PE-Cy7-labeled anti-CD4 (BD Pharmingen; eBioscience) and PE-CF594- or BV510-conjugated anti-CD8 (BD Horizon, San Jose, CA, USA). Subsequently, the cells were fixed using 4% paraformaldehyde (Merck, Darmstadt, Germany), permeabilized using 0.5% Saponin (Riedel-de Haën, Buchs, Switzerland) and stained with PerCP-Cy5.5- or BV421-conjugated anti-IFNγ (BD Pharmingen), PE-labeled anti-IL-2 (eBioscience), Alexa700- or APC-labeled anti-CD107a (BD Pharmingen), FITC-labeled anti-CCL4 (R&D systems, Minneapolis, MN, USA) and PE-Cy7- or PerCP-Cy5.5-labled anti-TNFα (eBioscience). Fluorescence was acquired using a CyAn ADP Analyzer (Beckman Coulter), a FACSAria or a FACSVerse (both Becton Dickinson, San Jose, CA, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). For SEB- and peptide-stimulated samples at least 10,000 and 25,000 viable CD8+ T cells were recorded. For each patient and time point 1 to 4 samples treated only with co-stimulatory beads were analyzed and at least 18,000 viable CD8+ T cells were recorded for each replicate sample. To analyze the functional diversity of CD8+ T cells, combination gates were created using the built-in Boolean gating algorithm of FlowJo. To account for unspecific activation, samples were background subtracted using the average values of 1 to 3 corresponding replicate samples stimulated only with co-stimulatory beads. To account for the different magnitude of total antigen-specific CD8+ T cell responses, the frequencies of cytokine combinations were normalized and given as the percentage of total tumor antigen-specific response. To reduce noise in the dataset, only samples with a minimum of 0.2% responding CD8+ T cells after background subtraction were considered positive based on an escalating variance model. Analysis and presentation of distributions was performed using SPICE version 5.3, downloaded from http://exon.niaid.nih.gov, and PRISM for windows version 5.03 (GraphPad Software Inc, La Jolla, CA, USA)(24).

Analysis of surgically resected tumors

Resected tumors were partially embedded in paraffin for immunohistochemistry and part of the resected tumors was used to make cell suspensions as previously described(25). Briefly, tumor tissue was cut into small fragments in Hanks Balanced Salt Solution medium (Gibco) with 50 µg/mL collagenase type 1A, 10 µg/mL DNase, and 1 µg/mL trypsin inhibitor (Sigma Chemical Co., Balcatta, WA, USA). The fragments were incubated for 30 min at 37°C. The resulting cell suspension was put

on a modified Ficoll gradient, and tumor infiltrating lymphocytes were harvested from the interphase. Flow cytometric analysis was performed with a FACS Calibur (BD Biosciences) using directly labeled mAbs against CD4, CD8, CD25, CD127 (all BD Pharmingen), FoxP3 (eBioscience), and tetrameric-MHC complexes containing the $gp100_{154-162}$, $gp100_{280-288}$ or $tyrosinase_{369-377}$ peptide.

Immunohistochemical staining of tumor tissue was done on paraffin-embedded tissue sections using mAbs against gp100, CD8 (both Dako, Glostrup, Denmark), tyrosinase (Monosan, Uden, the Netherlands), CD4 (Thermo Scientific, Waltham, MA, USA), and FoxP3 (eBioscience). Antigen retrieval was done by microwave boiling in 10 mmol/L sodium citrate buffer (pH 6.0) for 10 min. After rinsing with PBS, slides were pretreated with 20% normal horse serum for 10 min to reduce nonspecific staining. All sera and antibodies were dissolved in PBS with 1% bovine serum albumin. Subsequently, slides were incubated with the primary antibody at 4°C for 16 to 20 hours. The avidin-biotin complex (Vector, Burlingame, CA, USA) method was used for visualization with 3-amino-9-ethyl-carbazole or 3,3′-diaminobenzide hydrochloride solution. Slides were counterstained with hematoxylin solution or nuclear fast red, dehydrated, and mounted in Permount (Fisher Chemicals, Landsmeer, the Netherlands).

Statistical analysis

Kaplan-Meier probability estimates of progression-free survival and overall survival were calculated, statistical differences between the survival of the groups were determined with a log rank test. Progression-free survival and overall survival were defined as time from apheresis to the onset of progression of disease or to death of any cause, respectively. Patients without progression of disease or still alive at analysis, respectively, were censored at the time of last follow-up. The hazard ratio was calculated by the Cox proportional hazards model. Statistical significance was defined as $p<0.05$. SPSS20.0 was used for survival analyses.

Results

Patient & vaccine characteristics

In this study, we vaccinated metastatic melanoma patients with autologous, tumor peptide-loaded primary CD1c+ mDCs that were freshly isolated from peripheral blood and cultured overnight in the presence of GM-CSF (Supplementary Figure 1). Freshly isolated mDC had an average purity of 93% (Figure 1A, B). The phenotype of the ex vivo-activated DCs was determined by flow cytometry; all produced vaccines expressed high levels of MHC class I and II, CD83 and CD86 and met the predefined release criteria (Figure 1C, D). Expression of CD80 and CCR7 was lower and highly variable. The mean viability of injected DCs was 86% (range 53-100%) of the first (fresh) vaccination and 87% (range 55-98%) of thawed mDC vaccines (vaccinations 2 and 3; Figure 1E). Patients received on average $6×10^6$ DCs per vaccination, depending on the yield of mDCs after harvesting, with a maximum of $10×10^6$ DCs per vaccination. The yield, combined with lower CCR7 expression, provided the rationale to administer mDC vaccines intranodally.

A total of 14 HLA-A*0201-positive melanoma patients were vaccinated with three vaccinations at bi-weekly intervals. Patient characteristics are summarized in Table 1. One patient (VI-B-04) did not complete the scheduled vaccinations and immune monitoring due to rapid progression of disease and was excluded from further analysis of immunological responses (Supplementary Figure 2). Thirteen of the patients were considered evaluable for immunologic response. Five patients received a second cycle of three vaccinations and 3 of these patients completed the full 3 cycles. The vaccines were very well tolerated and no signs of severe toxicity were observed. The most common side effects associated with DC vaccination consist of transient flu-like symptoms, including fever, and erythema at the site of injection. However, none of the vaccinated patients experienced injection site reactions, and only 4 patients developed grade 1, and one patient grade 2 flu-like symptoms (Table 2). Besides one patient with grade 1 pain at the injection site and 4 patients with grade 1 fatigue, no other vaccine-related side effects were observed. From this we conclude that it is feasible and safe to administer activated and tumor peptide-loaded mDCs to melanoma patients.

MDC vaccination induces anti-KLH responses in metastatic melanoma patients

For immunomonitoring purposes and to provide CD4+ T cell help, all DCs have been loaded with the control antigen KLH. PBMCs, isolated after each vaccination, showed increased proliferation upon stimulation with KLH after the first cycle of vaccinations in 11 out of 13 patients (Supplementary Figure 3A, Table 2), indicating that the vaccine effectively induced de novo immune responses in these patients. We also followed the levels of serum anti-KLH antibody after each cycle of vaccinations. In 4 out of 13 patients, anti-KLH IgG could be detected, albeit just above detection limits in two of these patients (Supplementary Figure 3B, Table 2). In two patients anti-KLH IgG was already detectable before vaccination, but these patients showed increased levels after subsequent vaccinations. Patient VI-B-01 showed a clear IgG response after the second cycle of mDC vaccinations, which coincided with the presence of anti-KLH IgA. Anti-KLH IgM was not detected.

MDCs induce multifunctional tumor antigen-specific T cell responses in vivo

Cytotoxic T cells are crucial for immune-mediated tumor eradication. Previously we showed that the presence of tumor antigen-specific T cells in DTH skin tests positively correlates with clinical outcome in metastatic melanoma patients after moDC vaccination(4,5). We analysed the presence of tumor antigen-specific SKILs in biopsies taken from DTH skin tests after each cycle of vaccinations. In 4 of 13 patients tested, tetramer-positive CD8+ T cells were detected in SKIL-cultures (Table 2, Figure 2A, 2D). In 3 of these patients (VI-B-01, -08, and -13), the antigen-specific CD8+ T cells were fully functional and produced high levels of IFNγ upon antigen-specific stimulation with HLA-A*0201-binding gp100 or tyrosinase peptides (peptide recognition). SKILs of patient VI-B01 recognized $gp100_{154-162}$, $gp100_{280-288}$ and $tyrosinase_{369-277}$, whereas SKILs of patient VI-B-08 recognized $gp100_{154-162}$ and SKILs of patient VI-B-13 recognized both gp100 epitopes (Table 2, Figure 2B, 2E). SKILs of all three patients also produced high levels of IFNγ upon coculture with tumor cells expressing gp100 or tyrosinase protein (protein recognition). Interestingly, all 3 patients showed progression-free survival of more than 15 months and two of them showed objective clinical responses (see below).

Tetramer analysis of PBMCs after each cycle of 3 mDC vaccinations revealed tumor peptide-specific CD8+ T cells in peripheral blood of 4 out of 12 patients tested (VI-B-01, -08, -11, and -13) (Table 2, Figure 3A). Recent studies showed that T cell functionality is an important indicator for an effective immune response. In HIV patients the presence of T cells that simultaneously express more than three effector functions correlates with long-term disease control(26,27) and also in DC-

vaccinated melanoma patients these cells preferentially appear in patients with extended overall survival(23). We assessed the functionality of tumor-specific CD8+ T cells by measuring the simultaneous expression of CD107a (as a surrogate marker for cytolytic activity; expressed on the cell surface upon release of perforin and granzymes), the proinflammatory cytokines IFNγ, TNFα, and IL-2, and the chemokine CCL4 by tumor antigen-specific T cells after in vitro-stimulation (Figure 3B). Functional T cells, i.e. cells expressing at least one of the functional markers upon antigen-stimulation, could be identified in PBMC of 3 of the 4 patients that exhibited tetramer-positive CD8+ T cells in the blood, completely overlapping the three patients with IFNγ-producing SKILs (VI-B-01, -08, -13; Table 2, Figure 3C). We could not detect functional tumor antigen-specific T cells in blood of patients VI-B-03, VI-B-10, or VI-B-11, despite these patients were long survivors (VI-B-03, VI-B-10) or showed tetramer-positive CD8+ T cells in PBMCs (VI-B-11). Patient VI-B-08 already harboured high frequencies of tyrosinase-specific CD8+ T cells in the peripheral blood even before vaccination, which further increased after DC vaccination. However, tyrosinase-specific T cells were not detected in the SKILs of this same patient, indicative of the possible incapacity of these cells to home into the skin.

Functional T cell responses were dominated by CD107a expression, followed by secretion of IFNγ, CCL4, and TNFα (Figure 3D). Production of IL-2 was not observed. The functionality of tumor antigen-specific CD8+ T cells differed markedly between and within tested individuals and reached from high fractions of T cells with 3 or 4 functions (VI-B-01, -08) to mainly monofunctional responses (VI-B-01, -13). Patient VI-B-08 displayed high fractions of T cells with 3 functions already before vaccination, which increased after one cycle of DC vaccinations (Figure 3E). Furthermore, patient VI-B-01 displayed considerable fractions of tumor antigen-specific T cells with 3 functions after vaccination. T cells exhibiting 4 or more functions were only found in less than 4% of tumor antigen-specific T cells. From these data we conclude that vaccination with small numbers of primary CD1c+ mDCs can induce multifunctional tumor-specific CD8+ T cell responses in metastatic melanoma patients.

Clinical responses

Five patients showed at least stable disease upon the first vaccination cycle (7, 12, 15, 18, and 35+ months duration, respectively) and were therefore eligible for an additional vaccination cycle consisting of another three mDC vaccinations (Table 1 and 2). In two of these patients, disease

progression occurred after the second vaccination cycle. The three remaining patients also received a third cycle of three mDC vaccinations, of which one M1a, one M1b and one M1c patient. All patients were evaluated for clinical response at 3-month intervals with CT scans. Two patients showed objective tumor responses, which coincided with the presence of functional T cells in peripheral blood and DTH skin tests (Figure 2, 3). CT scans of patient VI-B-13 showed evident regression of pulmonary and mediastinal nodal metastases already after one cycle of DC vaccinations, which converted into a complete response after the second cycle of DC vaccinations (Figure 2C). The patient is in ongoing complete remission and good clinical condition 35 months after initiation of vaccinations with mDCs.

Patient VI-B-08 showed a mixed response; a subcutaneous lesion was strongly reduced in size after the first cycle of DC vaccinations and reduced even further after the second cycle (Figure 2F), while two other lesions remained stable. However, a subcutaneous metastasis on the scalp increased in size during the same time period. This lesion was resected during the second cycle of vaccinations and analyzed by flow cytometry and immunohistochemistry. Tumor antigens gp100 (90% of the tumor cells strongly positive) and tyrosinase (70% of the tumor cells weakly positive; not shown), and MHC class I (not shown) were still expressed by the tumor cells and CD8+ T cells were present both peritumorally and intratumorally (Figure 2G). Intriguingly, a massive infiltration of FoxP3+ T cells was observed (34% of CD4+ T cells; Figure 2G, 2H), indicative of regulatory T cells that might have locally inhibited an effective anti-tumor response. Since no other lesions showed progression at this stage the patient received a third cycle of vaccinations. Unfortunately, shortly after completion of the third cycle the patient progressed. Two further lesions were resected, which also expressed gp100, tyrosinase and MHC class I and were infiltrated by both CD8+ T cells and, to a lesser extent, CD4+ and FoxP3+ cells (data not shown). Subsequent treatments with both ipilimumab 3mg/kg (3 cycles) and vemurafenib (3 months) did not improve the clinical status and the patient died of disease 29 months after initiation of mDC vaccinations.

Despite the small sample size, our data strongly suggest a correlation between the immunological responses and progression-free survival (Figure 4A). The median progression-free survival was 17.6 months for patients with functional T cells in blood and IFNγ-producing SKILs (n=3), compared to 2.3 months for patients with no functional T cells (p=0.019, hazard ratio 0.15, 95%CI 0.04-0.57). The median overall survival of patients with functional T cells was 29.0 months, whereas in

the absence of functional T cells, the median overall survival was 10.9 months (Figure 4B; p=0.103, hazard ratio 0.43, 95%CI 0.12-1.54). The median overall survival of all patients was 13.3 months.

Discussion

Three conclusions can be drawn from this study: (i) Vaccination with naturally circulating primary CD1c+ mDCs can induce de novo immune responses and objective clinical responses, even in advanced metastatic melanoma patients. The treatment is well tolerated and warrants follow-up by a prospective randomized trial. (ii) Prolonged progression-free survival was associated with the presence of multifunctional tumor antigen-specific T cells in the blood and the DTH (SKILs). (iii) Primary mDC vaccines can, even at numbers as low as $3-10 \times 10^6$ mDCs per vaccination, induce de novo immune responses. This highly standardized and rapid vaccine production procedure (within a period of 48 hours) will certainly expedite larger randomized multicentre trials.

By overnight culture in the presence of GM-CSF, we obtained mDC that highly expressed MHC class I, MHC class II, CD83 and CD86. CD80 and CCR7 were expressed at lower and highly variable levels, which may be suboptimal. DC activation with appropriate adjuvants, such as TLR ligands, will induce increased levels of costimulatory molecules and production of proinflammatory cytokines, which is favorable for potent anti-tumor responses. Unfortunately, limited availability of GMP-compliant produced products impeded the use of TLR-ligands for mDC-maturation in this study. We recently optimized DC-maturation with clinical grade protamine-mRNA complexes that induce TLR7/8-mediated upregulation of maturation markers and production proinflammatory cytokines in mDCs as well as pDCs (28), which will be used in our future studies.

Five out of 14 vaccinated patients, including patients with widespread disease, showed long-term overall survival (22-40 months). In three of these patients, this coincided with the presence of tumor antigen-specific T cells and prolonged progression-free survival, while in the two patients without tumor antigen-specific T cells progression-free survival was short. Recent studies in HIV-infected patients showed that the functional quality, rather than the magnitude of the T cell response, is an important indicator for effective immune responses. In those HIV patients, the presence of T cells that simultaneously express more than three effector functions correlated with long-term disease control(26,27). Similarly, to obtain more insight in the functional capabilities of T cells from mDC-vaccinated melanoma patients, we examined their capacity to exert multiple functions at the same time. Besides cytotoxicity, necessary for induction of tumor cell apoptosis (analyzed by the expression of degranulation marker CD107a), also secretion of proinflammatory cytokines and chemokines such as IFNγ, TNFα, IL-2 and CCL4 proved important for long-term tumor control by increasing immune

16

recognition and inducing permanent senescence in cancer cells and by activation and recruitment of other immune cells, such as DCs and NK cells(29,30,31,23). Although the phenotype of induced peripheral blood CD8+ T cells was dominated by CD107a expression, also IFNγ, CCL4, and TNFα were secreted. Various levels of T cell functionality could be observed; two patients displayed high fractions of T cells expressing 3 or more functions (VI-B-01 and VI-B-08). Regression of the tumor was seen in two patients harboring functional T cells in their peripheral blood and DTH skin test (VI-B-08 and VI-B-13). Apparently, robust T cell responses are essential for tumor cell killing. These results highlight the ability of primary mDCs to induce comprehensive T cell responses in vivo.

Interestingly, patient VI-B-08 already displayed high frequencies of multifunctional tyrosinase-specific CD8+ T cells in the peripheral blood even before vaccination. MDC vaccination increased the frequency as well as the functionality of these T cells. However, as tyrosinase-specific T cells were not detected in the SKILs of this same patient, these cells may not have the capacity to home into the skin and tumor. By contrast, IFNγ-secreting gp100-specific cytotoxic T lymphocytes were detected in SKILs of this patient. Possibly, these gp100-specific T cells were responsible for regression of the responding lesion. Unfortunately, we could not obtain tumor material of this regressing lesion to confirm this hypothesis.

Five out of 14 patients (36%) had tetramer positive T cells in peripheral blood or SKILs. In three of these five patients (21%) functional T cells were detected. This response rate is similar to that of moDC-vaccinated stage IV melanoma patients in our previous studies(4). The high tumor burden in stage IV melanoma patients may hamper the induction of effective immune responses and cause the lack of response in the remaining patients, which is underscored by our observation that response rates in stage III melanoma patients are higher(32,33). Therefore, we hypothesize that DC vaccination may be more potent in the adjuvant setting.

Until 2013, the recommended standard therapy for metastatic melanoma patients was DTIC, which did not considerably affect overall survival(34). Recently, anti-CTLA4 antibodies (ipilimumab) (35,36) and anti-PD-1 antibodies (nivolumab and pembrolizumab)(37,38) proved to be effective forms of immunotherapy for patients with metastatic melanoma and replaced DTIC as standard treatment. Although the number of patients studied here is too small for direct comparison and to allow any conclusions regarding significant clinical efficacy, it is notable that the overall survival of mDC-vaccinated patients appeared to be comparable with that of similar patients treated with ipilimumab

reported in literature(35,36). However, four patients included in our study received ipilimumab upon disease progression, of which three patients were among the five patients with long-term overall survival. Nevertheless, none of these patients showed an objective response or long-term stable disease upon ipilimumab treatment; progression occurred between 5 and 8 months. Only one patient (VI-B-08) with a functional tumor-specific T cell response after mDC vaccination received ipilimumab; she showed progressive disease 7 months after start of ipilimumab treatment. Still, subsequent treatment has confounded the overall survival data. However, since the correlation of a functional T cell response with progression-free survival, where no such confounding has taken place, is significant, we believe this is due to mDC vaccination. We did not obtain sufficient data to speculate on the combination of mDC vaccination and checkpoint inhibitors, but as mDC vaccines specifically activate tumor antigen-specific T cells and immune-checkpoint inhibition activates T cells in a non-antigen-specific manner, one can hypothesize that a combination of the two could be beneficial, as CTLA4- or PD-1 blockade could improve the functionality and limit exhaustion of mDC-induced tumor antigen-specific T cells. This is supported by data suggesting a correlation between prior DC vaccination and objective responses upon subsequent treatment with ipilimumab(39). These results urge for a randomized phase II trial to explore the clinical efficacy of mDCs as an anti-cancer vaccine, possibly in combination with immune checkpoint inhibitors.

In our previous study using naturally circulating pDCs, vaccinated melanoma patients showed a beneficial effect on overall survival, irrespective of the observation that the induction of tumor antigen-specific T cells was not as prominent as in previous moDC-vaccination trials or in the present study(14). This might suggest that either the antigen-specific T cells that were induced by pDCs are very potent or more functional in terms of migration, cytokine secretion or survival, or that other mechanisms, such as activation of NK cells or other innate immune cells by the massive pDC-derived type I interferon secretion, are involved in pDC-related clinical outcome. MDCs and pDCs may utilize different mechanism and could synergize in the induction of clinical responses in metastatic melanoma patients. We therefore hypothesize that co-administration of naturally circulating mDCs and pDCs, and thus activation of multiple, possibly complementary, anti-tumor mechanisms, may generate more potent and longer-lasting anti-tumor responses in cancer patients compared to administration of individual naturally circulating DC subsets(17,40). Indeed, in a murine tumor model, simultaneous injection of mDCs and pDCs proved superior in generating anti-tumor responses when compared to

immunization with either DC subset alone(41). Future DC vaccination studies in cancer patients should elucidate whether this also holds true for human DC subsets. In vitro studies have already demonstrated that human mDCs and pDCs synergize via bi-directional cross-talk(42,11). Addition of CD141+ myeloid DCs may even further improve the potency of DC vaccines, as CD141+ DCs are highly efficient in antigen cross-presentation to CD8+ T cells and are able to secrete IFNγ and IL-12 upon activation, which allows the effective induction of T helper 1 and cytotoxic T lymphocyte responses(43,44,45,46,47,48). However, the limited presence of DCs in blood and lack of GMP-grade isolation reagents currently impedes the use of CD141^{+} DCs for DC-based immunotherapy.

In conclusion, we showed that vaccination of metastatic melanoma patients with primary mDC is feasible and safe and results in induction of effective anti-tumor immune responses that coincide with improved progression-free survival. The virtual lack of side effects of primary mDC vaccines, their swift and highly controlled production, and their potency in inducing multifunctional antigen-specific T cell responses, warrants further exploration of these natural circulating DC subsets in larger randomized trials.

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Table 1. Patient characteristics

Patient	Sex	Age (yrs)	Stage on entry	Site of disease	BRAF status	Prior treatment	LDH at apheresis (U/l)	gp100[a]	tyrosinase[a]	Nr of vaccines
VI-B-01	M	58	M1a	Skin[c]	mut	RLND	371[d]	+++	-	9
VI-B-02	M	71	M1c	LN, lung, skin, muscle	unknown	S	404[d]	++/+++	++/+++	3
VI-B-03	F	48	M1a	LN	mut	SN	460[d,e]	++/+++	++/+++	6
VI-B-04	M	31	M1c	LN, liver, spleen	mut	RLND	991[d,f]	+++	+++	2
VI-B-05	F	42	M1c	Liver	mut	RLND; adj IFN (EORTC18991); S	309[d]	++/+++	-	3
VI-B-06	M	52	M1c	Lung, liver, bone, soft tissue	wt	S	434[d]	+	+	3
VI-B-07	M	48	M1c	LN, liver	mut	Adj placebo/ipilimumab (EORTC18071)	428[d]	++/+++	+	3
VI-B-08	F	54	M1c	Skin, muscle, intestine	mut	S	196[g]	+++	+	9
VI-B-09	M	69	M1a	LN, skin	mut	S	224[g]	++/+++	+	6
VI-B-10	F	66	M1c	Lung, skin, mesentery	mut	S	109[g]	++	+	3
VI-B-11	M	48	M1c	Lung, mesentery, intestine	wt	S	228[g]	positive, not further specified	unknown	3
VI-B-12	M	46	M1b	LN, lung, skin	mut	Adj placebo/MAGE (GSK 2132231A), adj RT, S	163[g]	+++	++	3
VI-B-13	M	46	M1b	LN, lung	mut	S	186[g]	++	++	9
VI-B-14	M	73	IIIc[b]	LN, skin	mut	-	215[g]	++/+++	+++	3

Abbreviations: LN, lymph nodes; mut, BRAF mutation present; wt, BRAF wild type; RLND, radical lymph node dissection; SN, sentinel node procedure; S, surgery of regional or distant metastasis (other than RLND); adj, adjuvant; IFN, pegylated interferon-α2b; RT, radiotherapy; LDH, lactate dehydrogenase.

[a] gp100 and tyrosinase expression on the primary tumor was analyzed by immunohistochemistry. Intensity of positive cells were scored centrally and semiquantitatively by a pathologist. Intensity was scored as low (+), intermediate (++), or high (+++).

[b] Irresectable stage IIIc melanoma.

[c] The term 'skin' both includes subcutaneous and/or cutaneous metastasis.

[d] Upper limit of normal = 450 U/l.

[e] The LDH fluctuated between normal and elevated values. The elevated LDH was not considered due to tumor load.

[f] The LDH was normal (379 u/l) at screening. The elevated value at apheresis was due to rapid progressive disease.

[g] Upper limit of normal = 250 U/l.

Table 2. Immunological and clinical responses

Patient	Flu-like symptoms (CTC grade)	Anti-KLH response[a]		Tumor Ag-specific CD8+ T cells				PFS (months)	OS (months)	Best response	Salvage treatment
		T cell	Antibody	blood[b]		DTH[c]	Number of epitopes recognized[d]				
				Pre-vaccination	Post-vaccination						
VI-B-01	0	+	+	-	+(+)	+++	3	18	22	SD	S, T
VI-B-02	1	+	-	n.t.	-	-		<4	7	PD	C, RT
VI-B-03	1	+	-	-(-)	-(-)	-		7	40	SD	T, I
VI-B-04	0	n.a.	n.a.	n.t.	n.a.	n.a.		<4	3	PD	RT, C
VI-B-05	0	+	+	n.t.	-	+		<4	9	PD	I
VI-B-06	0	+	-	n.t.	-	-		4	13	SD	RT
VI-B-07	0	+	+	n.t.	-	-		<4	11	PD	T
VI-B-08	1	+	-	+(+)	+(+)	+++	2	15	29	MR	I, RT, T, S
VI-B-09	0	+	-	n.t.	-	-		12	15	SD	T, RT
VI-B-10	0	-	+	-(-)	-(-)	-		<4	38	PD	T, I
VI-B-11	2	-	-	+(-)	+(-)	-		<4	6	PD	-
VI-B-12	0	+	-	n.t.	n.t.	-		<4	11	PD	RT, T
VI-B-13	0	+	-	-	+(+)	+++	2	35+	35+	CR	-
VI-B-14	1	+	-	n.t.	-	-		<4	13	PD	C, RT

Abbreviations: n.a., not applicable; n.t., not tested; DTH, delayed-type hypersensitivity skin test; PFS, progression-free survival; OS, overall survival; SD, stable disease; PD, progressive disease; MR, mixed response; CR, complete remission; S, surgery; T, targeted therapy (BRAF inhibitor); C, chemotherapy; RT, radiotherapy; I, immunotherapy (anti-CTLA4 antibody).

[a] KLH-specific cellular proliferation and antibodies after vaccination with myeloid dendritic cells. Responses were scored as the best response after all cycles of DC vaccinations.

[b] Presence of tetramer-positive CD8+ T cells in peripheral blood is marked as +. In brackets, the presence of functional CD8+ T cells is marked as +. Functionality was defined as CD8+ T cells expressing at least one of the functionality markers CD107a, IFNγ, CCL4, TNFα or IL-2 above threshold level (0.2% after background subtraction) after in vitro peptide stimulation.

[c] Presence of tetramer-positive T cells in DTH is marked as +, presence of functional tetramer-positive T cells in DTH is marked as +++. Functionality was defined as the production of IFNγ after stimulation with T2 cells loaded with HLA-A*0201-binding gp100 or tyrosinase peptides (peptide recognition), BLM transfected with gp100 or tyrosinase protein (protein recognition) or the gp100- and tyrosinase-expressing tumor cell line Mel624 (tumor recognition). Responses were scored as the best immunologic response after all cycles of DC vaccinations.

[d] Total number of tumor antigen epitopes ($gp100_{154-162}$, $gp100_{280-288}$, $tyrosinase_{369-377}$) recognized by functional T cells from peripheral blood or DTH skin test in all cycles of DC vaccinations.

Figure legends

Figure 1. MDC vaccine characteristics.

(A) Purity (%) and yield (number of cells) in 22 CliniMACS-based CD1c+ mDC isolations on patients' apheresis material. Each dot represents the result of one mDC isolation. Lines represent the mean of all isolations. *mDCs of patient VI-B-06 had lower purity with unknown cause. (B) Representative example of flow cytometric analysis of mDC purity after CliniMACS isolation. (C) Expression of HLA-ABC, HLA-DQ, HLA-DR, CD80, CD83, CD86 and CCR7 on mDCs after overnight culture in the presence of GM-CSF was analyzed by flow cytometry. Data are shown as percentage of positive DCs used for the first vaccination cycle. (D) Representative flow cytometry plots of phenotype analysis. Filled histograms indicates marker expression, black line is the isotype control. (E) Viability of freshly administered mDCs (vaccination 1) and thawed mDCs (vaccination 2 and 3) of the first vaccination cycle of each patient. Each dot represents an mDC vaccine. The line represents the mean of all vaccines.

Figure 2. Immunological and clinical responses of patients VI-B-13 and VI-B-08.

(A-C) The presence and functionality of tumor antigen-specific T cells were tested in lymphocytes cultured from skin test biopsies (SKILs) of patient VI-B-13. (A) Example of tetramer analysis by flow cytometry of SKILs cultured from a biopsy of a positive post-treatment DTH reactions of patient VI-B-13 after the first cycle of three DC vaccinations. SKILs were stained with tetramers encompassing the HLA-A*0201-specific gp100$_{154-162}$, gp100$_{280-288}$, tyrosinase$_{369-377}$ peptide or an irrelevant peptide (HIV) and with anti-CD8. The percentage in the upper right quadrant represents the percentage of CD8+ tetramer+ cells. (B) IFNγ production by the same T cells after stimulation with T2 cells loaded with gp100$_{154-162}$, gp100$_{280-288}$ or tyrosinase$_{369-377}$ peptide (peptide recognition), BLM cells expressing gp100 or tyrosinase protein (protein recognition), or Mel624 cells expressing both gp100 and tyrosinase (tumor recognition). (C) CT scans of the chest of patient VI-B-13 showed clear reduction in size of a pulmonary metastasis after one cycle of mDC vaccinations (middle) and complete remission after three cycles of mDC vaccinations (right).

(D-H) The presence and functionality of tumor antigen-specific T cells were tested in lymphocytes cultured from skin test biopsies of (SKILs) patient VI-B-08. (D) Example of tetramer analysis by flow

cytometry of SKILs cultured from a biopsy of a positive post-treatment DTH reaction of patient VI-B-08 after the first cycle of three DC vaccinations. SKILs were stained with tetramers as in Figure 2A. (E) IFNγ production by the same T cells after stimulation with target cells as in Figure 2B. (F) CT scan of patient VI-B-08 before (left), after 1 cycle of mDC vaccinations (middle) and after 2 cycles of mDC vaccinations (right), showing an evident subcutaneous lesion dorsally of the left os ilium, which clearly reduces after the first cycle of DC vaccinations and further reduces after the second cycle of DC vaccinations. (G) Immunohistochemistry of progressive tumor showing that the melanoma-associated antigen gp100 is still expressed (left, gp100 in red). Both CD8+ T cells (middle, CD8 in brown) as well as FoxP3+ cells (right, FoxP3 in brown) are present in the tumor. (H) Flow cytometric analysis of tumor infiltrating lymphocytes isolated from a progressive tumor showing CD4+FoxP3+ lymphocytes.

Figure 3. Tumor antigen-specific T cell responses in peripheral blood.

(A) Examples of tetramer analysis of peripheral blood mononuclear cells (PBMCs) of patients VI-B-01, -03, -08, -10, -11 and -13. PBMCs were stained with tetramers encompassing the HLA-A*0201 specific peptides gp100$_{154-162}$, gp100$_{280-288}$, tyrosinase$_{369-377}$ or an irrelevant peptide (HIV) and with anti-CD8. The percentage in the upper right quadrant represents the percentage of CD8+ tetramer+ cells. Best results of all vaccination cycles per patient are shown. (B) To analyze the functionality of tumor antigen-specific CD8+ T cell responses, PBMCs or peripheral blood lymphocytess were stimulated with tumor peptides (gp100$_{154-162}$, gp100$_{280-288}$, tyrosinase$_{369-377}$) for 5-6 h and subsequently analyzed for expression of CD107a, IFNγ, CCL4, TNFα and IL-2 using flow cytometry. (C) Shown is the frequency of total tumor antigen-specific CD8+ T cells. Horizontal red line indicates cut-off value of 0.2% responding CD8+ T cells. (D+E) Displayed for each patient is the CD8+ T cell response with the highest fraction of T cells expressing 3 or more functions (indicated by ⇩ in C). (D) The relative contribution of each functional marker was measured. (E) Fractions of tumor antigen-specific CD8+ T cells with 1, 2, 3, 4, or 5 functions.
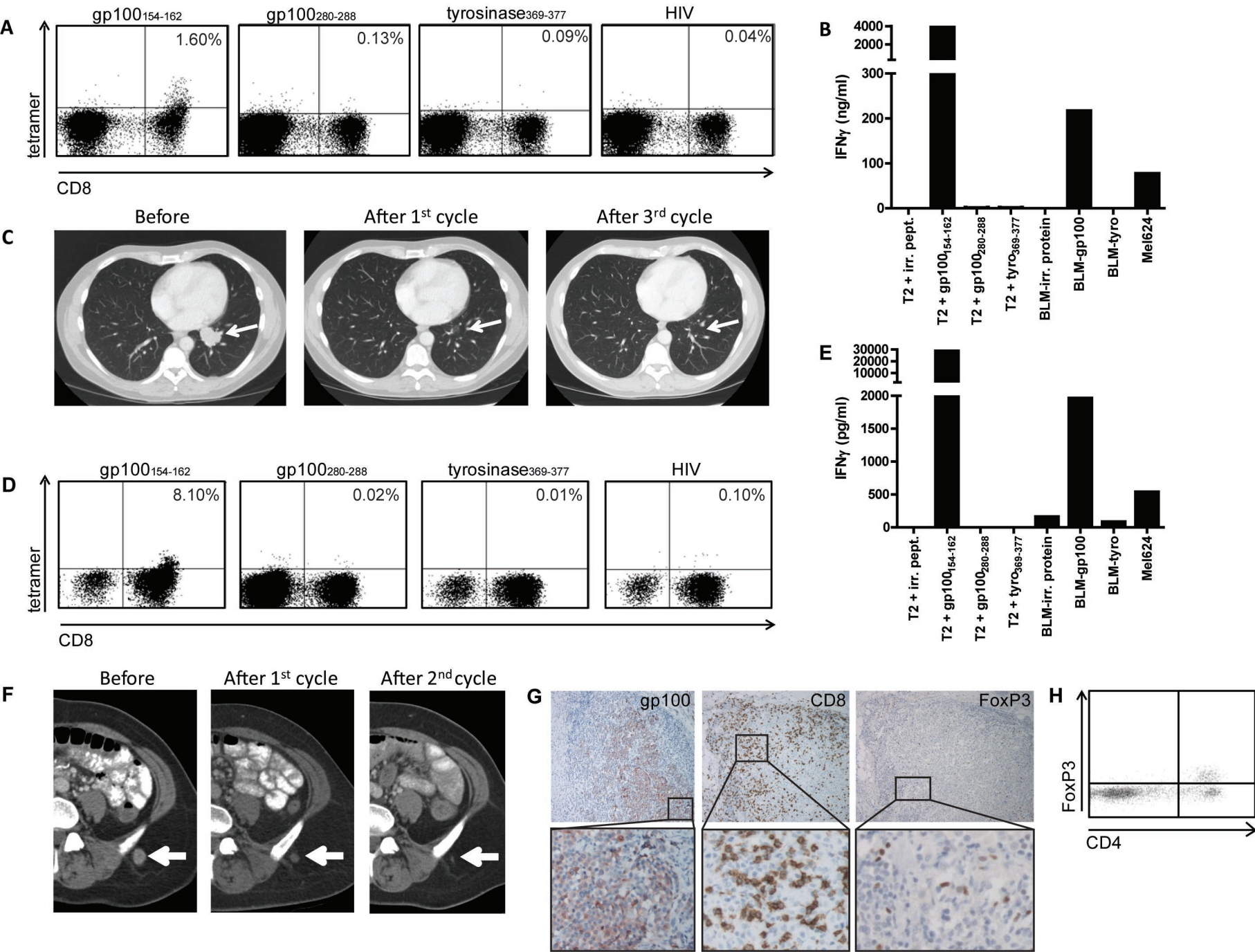
Figure 4. *Correlation between the presence of functional specific T cells and clinical outcome.*
Kaplan–Meier analyses of progression-free survival (A) and overall survival (B) according to the presence of functional tumor antigen-specific T cells in peripheral blood or skin-infiltrating
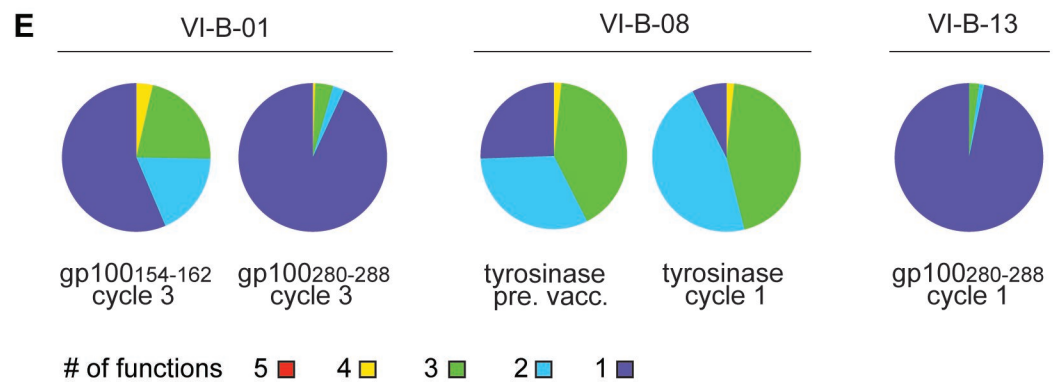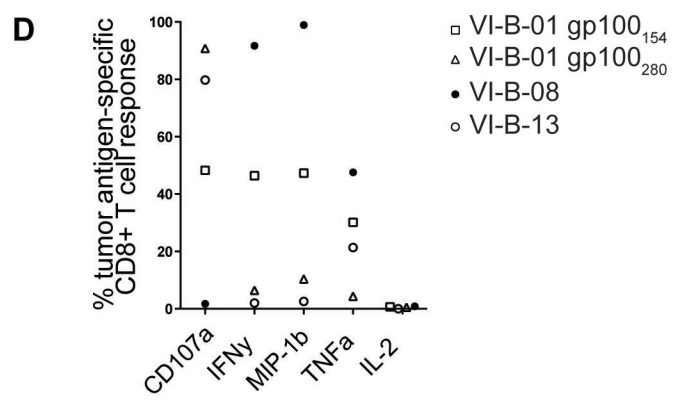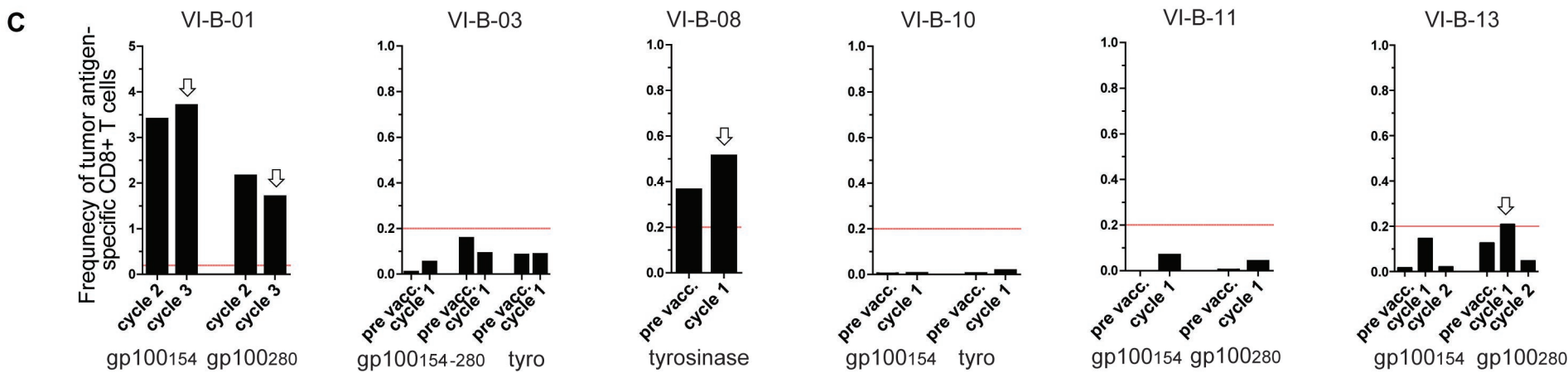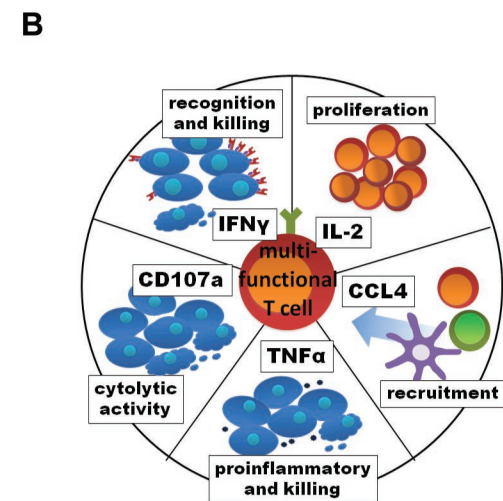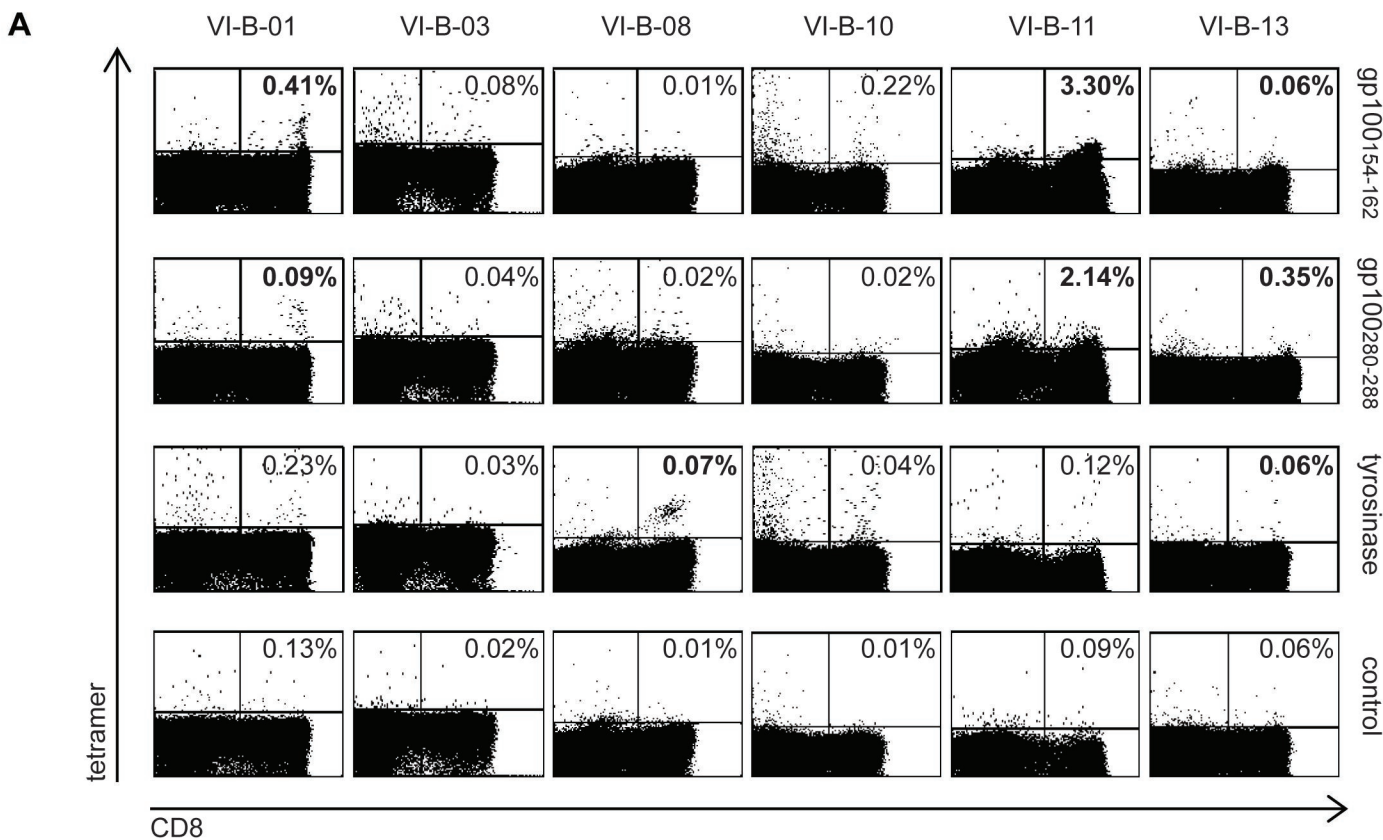
lymphocytes. Statistical significance between mDC-vaccinated patients with functional T cell responses compared to patients without functional T cell responses was determined by a log rank test.

Figure 1
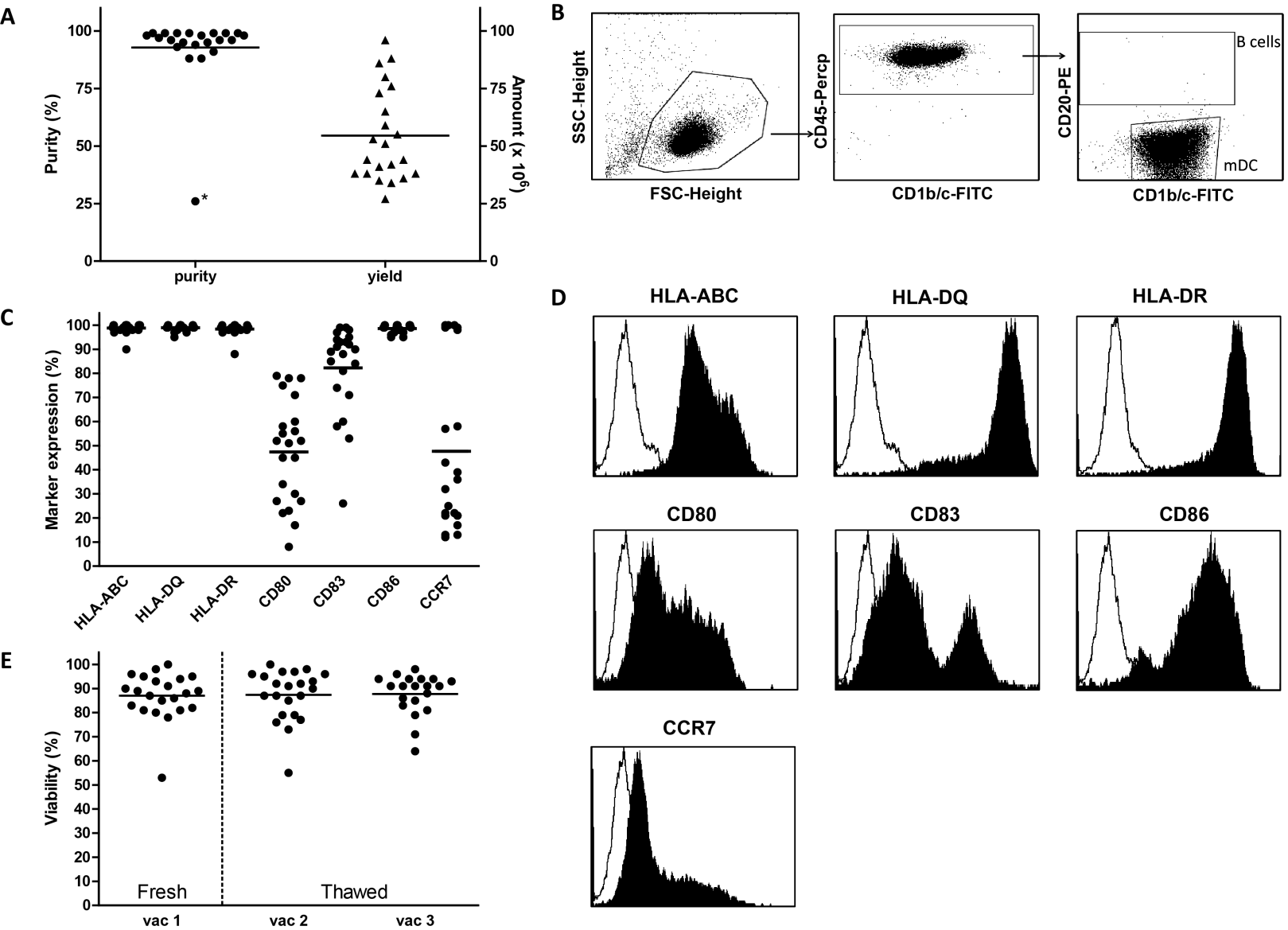
Figure 2

Figure 3

Figure 4

A

B