Vaccination with mRNA-electroporated dendritic cells induces robust tumor antigen-specific CD4+ and CD8+ T cells responses in stage III and IV melanoma patients

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Abstract

**Purpose** Electroporation of dendritic cells (DC) with mRNA encoding tumor associated antigens (TAA) has multiple advantages over peptide loading. We investigated the immunological and clinical responses to vaccination with mRNA-electroporated DC in stage III and IV melanoma patients.

**Experimental Design** Twenty-six stage III HLA*02:01 melanoma patients scheduled for radical lymph node dissection (stage III) and 19 melanoma patients with irresectable locoregional or distant metastatic disease (referred to as stage IV) were included. Monocyte-derived DC, electroporated with mRNA encoding gp100 and tyrosinase, were pulsed with keyhole limpet hemocyanin (KLH) and administered intranodally. TAA-specific T cell responses were monitored in blood and skin-test infiltrating lymphocyte (SKIL) cultures.

**Results** Comparable numbers of vaccine-induced CD8⁺ and/or CD4⁺ TAA-specific T cell responses were detected in SKIL cultures; 17/26 stage III patients and 11/19 stage IV patients. Strikingly, in this population, TAA-specific CD8⁺ T cells that recognize multiple epitopes and produce elevated levels of IFNγ upon antigenic challenge *in vitro*, were significantly more often observed in stage III patients; 15/17 versus 3/11 stage IV patients, p=0.0033. In stage IV patients, one mixed and one partial response were documented. The presence or absence of IFNγ-producing TAA-specific CD8⁺ T cells in stage IV patients was associated with marked difference in median OS of 24.1 months versus 11.0 months, respectively.

**Conclusion** Vaccination with mRNA-electroporated DC induces a broad repertoire of IFNγ producing TAA-specific CD8⁺ and CD4⁺ T cell responses, particularly in stage III melanoma patients.
**Translational relevance**

Electroporation of dendritic cells (DC) with mRNA encoding tumor associated antigens (TAA) has multiple advantages over the conventional peptide loading. The presentation of multiple naturally processed epitopes in both MHC Class I and II should broaden the repertoire of responding lymphocytes.

We studied in detail the immunological response to vaccination with mRNA-electroporated DC in 2 cohorts of melanoma patients: as palliative treatment for distant or irresectable locoregional metastatic disease and as adjuvant treatment following radical dissection of regional lymph nodes. A wide spectrum of tumor-specific IFNγ producing CD8⁺ T cells was detected, in particular in patients vaccinated in the adjuvant setting. Furthermore, vaccine-induced CD4⁺ T cells were shown to be FoxP3 negative.

In conclusion, vaccination with mRNA-electroporated DC successfully enhances anti-tumor cytotoxic T cell responses and appears to be a promising adjuvant treatment for stage III melanoma patients.
Introduction

Dendritic cells (DC) are the most effective antigen-presenting cells (APC) of the immune system, highly capable of stimulating naïve T cells. Immunotherapy with ex vivo-generated autologous DC pulsed with tumor peptides has provided proof of concept in clinical trials(1). We, and others, have demonstrated that tumor-specific immune responses can be induced in both stage III and IV melanoma patients(1-5). Since objective clinical responses are observed in a minority of patients, further optimization of DC based immunotherapy is warranted.

To date, the majority of clinical studies on DC based vaccinations have been performed with MHC class I restricted peptide-pulsed monocyte derived DC in patients with measurable distant metastatic disease. However, there are at least several theoretical disadvantages against these protocols, which might be improved to induce more effective and sustained immunological responses. First, the exploitation of MHC class I restricted peptide epitopes target CD8+ cytotoxic T cells (CTL) only, without involving CD4+ T helper cells to enhance and sustain anti-tumor CTL responses. Secondly, pulsing DC with peptide epitopes implicates the use of a given HLA type, with defined tumor associated antigens (TAA). Moreover, peptide-loaded DC expose the antigen only for a short period of time(6), since the peptides may readily dissociate from the MHC molecules(7). Importantly, peptide loading does not account for post-transcriptional modifications of peptide epitopes(8;9).

One strategy to circumvent most of these disadvantages of peptide pulsing is electroporation with synthetic mRNA encoding TAA, resulting in endogenous synthesis of the complete TAA. It has been shown previously that electroporation of DC with mRNA is effective and safe(7;10;11). DCs retain their phenotype and maturation potential upon electroporation, as well as their migratory capacities(10;12). Electroporated DC express TAA antigens, encoded by the electroporated mRNA and induce specific CD8+ T cell responses in melanoma


patients(10). Importantly, since mRNA lacks the potential to integrate into the host genome, it obviates safety concerns associated with gene therapy trials.

It is now widely recognized that high tumorload in end-stage cancer patients often induces local, or even systemic, immune suppression by the secretion of suppressive cytokines and attraction of regulatory T cells(13-15). This suppressive tumor microenvironment will hamper the effective anti-tumor responses. Melanoma patients with locoregional lymph node metastases are at high risk of relapse and currently no standard adjuvant treatment is available which results in overall survival benefit(16). Given the minimal burden of tumor; we hypothesized that vaccination of patients adjuvant to therapeutic radical lymph node dissection might enhance vaccine efficacy.

In this study we investigated in detail the immunological responses to intranodal vaccination with monocyte-derived DC electroporated with mRNA encoding gp100 and tyrosinase in 2 cohorts of melanoma patients; with distant metastatic or irresectable locoregional disease following radical regional lymph node dissection.
Patients and methods

Patient population

Melanoma patients with locoregional resectable disease (further referred to as stage III), before or within 2 months after radical dissection of regional lymph node metastases, and patients with irresectable locoregional or distant metastatic disease (further referred to as stage IV) were included. Additional inclusion criteria were HLA*02:01 phenotype, melanoma expressing the melanoma-associated antigens gp100 and tyrosinase, and WHO performance status 0 or 1. Patients with brain metastases, serious concomitant disease or a history of a second malignancy were excluded. The study was approved by our Institutional Review Board, and written informed consent was obtained from all patients. Clinical trial registration number is NCT00243529.

Study protocol

Patients received a DC vaccine intranodally, injected into a clinically tumor-free lymph node under ultrasound guidance. The DC vaccine consisted of autologous mature monocyte-derived DC electroporated with mRNA encoding for gp100 and tyrosinase protein, and pulsed with keyhole limped hemocyan (KLH) protein. Patients received three vaccinations with a biweekly interval. Ten patients received an extra vaccination 1 or 2 days before the radical lymph node dissection for additional imaging studies (manuscript in preparation). One to two weeks after the last vaccination a skin test was performed. In absence of disease progression or recurrence, patients received a maximum of two maintenance series at 6-month intervals, each consisting of three biweekly intranodal vaccinations (Supplementary figure 1). All vaccinations were administered between May 2006 and May 2010. Patients were considered evaluable when they had completed the first vaccination cycle.
specific immune response was the primary endpoint, clinical response was a secondary endpoint in stage IV patients. Progression-free and overall survival were calculated from the time from apheresis to recurrence (for stage III patients) or progression (for stage IV patients), or death.

**DC preparation and characterization**

DC were generated from peripheral blood mononuclear cells (PBMC) prepared from leukapheresis products as described previously(17). After leukapheresis, part of the PBMC was used for the generation of monocyte-conditioned medium (MCM)(18). Plastic-adherent monocytes or monocytes isolated by centrifugal elutriation were cultured for 5-7 days in X-VIVO 15™ medium (BioWhittaker, Walkersville, Maryland) supplemented with 2% pooled human serum (HS) (Bloodbank Rivierenland, Nijmegen, The Netherlands), IL-4 (500 U/ml) and GM-CSF (800 U/ml) (both from Cellgenix, Freiburg, Germany). Immature DC were pulsed at day 3 with Keyhole limpet hemocyanin (KLH, 10 μg/ml; Calbiochem, San Diego, CA). Two days prior the harvesting, cells were matured with autologous MCM, prostaglandin E2 (10 μg/ml; Pharmacia & Upjohn, Puurs, Belgium) and recombinant tumor necrosis factor alpha (10 ng/ml; provided by dr. G.Adolf, Bender Wien, Vienna, Austria)(19). This protocol gave rise to a mature phenotype meeting the release criteria described previously(20): low expression of CD14, high expression of MHC class I, MHC class II, CD83, CD80, CD86, and CCR7, and expression of gp100 and tyrosinase after electroporation with mRNA (Supplementary figure 2). Harvested DC were tested by FACS analysis as described below.

**Plasmids and in vitro mRNA transcription**

Plasmids have been sent to CureVac GmbH (Tübingen, Germany) for the production of documented GMP grade gp100 and tyrosinase RNA for *ex vivo* use in clinical DC
vaccination. The documented gp100 and tyrosinase mRNA was produced from the plasmids pGEM4Z/hgp100/A64 and pGEM4Z/tyrosinase/A64 (provided by Kris Thielemans, Free University Brussels, Belgium) according to GMP guidelines. CureVac mRNA contains a 5’ cap and 3’ poly A-tail that leads to high RNA stability and increased protein expression in transfected cells. The mRNA is purified by PUREmessenger™ technology. This chromatography method efficiently eradicates traces of DNA and proteins. The mRNA production process is performed in clean room facilities and is documented by in-process controls. RNA quality was verified by agarose gel electrophoresis, RNA concentration was measured spectrophotometrically, and RNA was stored at -80°C in small aliquots.

**Electroporation of DC**

Mature DC were electroporated as described previously(10). Briefly, DC were washed twice in PBS and once in OptiMEM without phenol red (Invitrogen, Breda, The Netherlands). Twenty micrograms of RNA encoding either gp100 or tyrosinase were transferred to a 4 mm cuvette (Bio-Rad, Hercules, CA) and 8×10⁶ cells were added in 200 µl OptiMEM and incubated for 3’ before being pulsed in a GenePulser Xcell (Bio-Rad) by an exponential decay pulse of 300 V, 150 µF, as described before (10). Immediately after electroporation, cells were washed and were transferred to warm (37°C) X-VIVO 15™ without phenol red (Cambrex Bio Science, Verviers, Belgium) supplemented with 5% HS and left for at least 2 hours at 37°C, before further manipulation. The first vaccination was given with fresh DC 4 hours after electroporation. DC for subsequent vaccinations were frozen 2 hours after electroporation, thawed at the day of vaccination and incubated for 2 more hours at 37°C before injection. Electroporation efficiency was analyzed by intracellular staining and flow cytometric analysis for each separate TAA, electroporated DC were mixed before vaccination.
Flow cytometric analysis

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 (BD Biosciences, Mountain View, CA), anti-CD14, anti-CD83 (both Beckman Coulter, Mijdrecht, The Netherlands), and anti-CD83 (BD Pharmingen, San Diego, CA). For intracellular staining of the TAA the following mAb were used: NKI/beteb (IgG2b) (purified antibody) against gp100, T311 (IgG2a) (Cell Marque Corp., Rocklin, CA) against tyrosinase. For intracellular staining cells were fixed for 4' on ice in 4% (w/v) paraformaldehyde (Merck, Darmstadt, Germany) in PBS, permeabilized in PBS/2%BSA/0.02% azide/0.5% saponin (Sigma-Aldrich) (PBA/saponin), and stained with mAb diluted in PBA/saponin/2%HS, followed by staining with allophycocyanin-labeled goat-anti-mouse (BD PharMingen). Flow cytometry was performed with FACSCalibur™ flow cytometer equipped with CellQuest software (BD Biosciences).

Flow cytometric analysis of T cells was performed using directly labelled mAbs against CD4, CD8, CD25, CD127, CTLA-4 (BD Pharmingen) and FoxP3 (eBioscience, San Diego, CA, USA), all according to the manufacturer's protocol. Tregs were defined as CD4⁺FoxP3⁺CD25⁺CD127low cells; percentage of Tregs was defined as the number of CD4⁺FoxP3⁺CD25⁺CD127low cells divided by the total number of CD4⁺ cells x100.

KLH-specific proliferation

KLH-specific cellular responses were measured before and after vaccination by proliferation assay. Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood by Ficoll-Paque density centrifugation. PBMC were stimulated with KLH (4 µg/2×10⁵ PBMC) in medium with 10% human AB serum. After 3 days, cells were pulsed with ³H-thymidine for 8 hours, and incorporation was measured with a betacounter. Experiments were performed in triplicate.
**KLH-specific antibody production**

KLH-specific antibodies were measured in the sera of patients before and after vaccination. Microtiter plates (96 wells) were coated overnight at 4°C with KLH (25 µg/ml in PBS). Different concentrations of patient serum (range 1:100 to 1:50,000) were added for 60’ at room temperature. After extensive washing, patient antibodies were detected with mouse anti-human IgG, IgA, or IgM antibodies labelled with horseradish peroxidase (Invitrogen, San Diego, CA). 3,3’ 5,5-tetramethyl-benzidine was used as a substrate and plates were measured with a microtiter plate reader at 450 nm. For quantification, an isotype-specific calibration curve for the KLH response was included in each microtiter plate.

**Skin-test infiltrating lymphocyte (SKIL) cultures**

0.2 - 1 x 10^6 DC pulsed with the gp100 and/or tyrosinase peptides and DC electroporated with gp100 and/or tyrosinase mRNA each were injected i.d. in the skin of the back of the patient at four or six different sites(21). The maximum diameter of induration was measured after 48 hours.1 From each site induration was measured and punch biopsies (6 mm) were obtained. Half of the biopsy was cryopreserved and the other part was manually cut and cultured in RPMI 1640 containing 7% HS and IL-2 (100 U/ml). Every 7 days, half of the medium was replaced by fresh medium containing HS and IL-2. After 2 to 4 weeks of culturing, SKILs were tested. In general, similar results were obtained per patient, regardless of the method of antigen-presentation (for example, Supplementary Figure 3).

**Tetramer staining**

SKILs and freshly isolated PBMC were stained with tetrameric-MHC complexes containing the HLA-A2-binding epitopes gp100:154-168, gp100:280-288 or tyrosinase:369-377 (Sanquin, Amsterdam, The Netherlands) or HLA-DR4-binding epitopes gp100:44-59 and tyrosinase:448-462 (provided by William Kwok, Benaroya Research Institute, Seattle, WA) as described previously(19). In addition, PBMCs of patients with tetramer positive CD4+ T cells were restimulated for 8 days with DR4-binding gp100 or tyrosinase peptides and stained with tetrameric-MHC complexes containing class II epitopes gp100:44-59 and tyrosinase:448-462. Tetrameric-MHC complexes recognizing HIV were used as controls; at least a two-fold increase of the double-positive population compared to control was regarded to be positive.

**Antigen and tumor recognition**

Antigen recognition was determined by the production of cytokines and cytotoxic activity of SKILs in response to T2 pulsed with the indicated peptides or BLM (a melanoma cell line expressing HLA^*02:01 but no endogenous expression of gp100 and tyrosinase), transfected with control antigen G250, with gp100 or tyrosinase, or an allogenic HLA^*02:01-positive, gp100-positive, and tyrosinase-positive tumor cell line (MEL624) were measured. Cytokine production was measured in supernatants after 16 hours of co-culture by a cytometric bead array (Th1/Th2 Cytokine CBA 1; BD PharMingen). Positive and specific cytokine production was defined as a two-fold increase compared to stimulation with the cell lines pulsed with an irrelevant peptide.

**Statistical analysis**

Differences between the groups were evaluated using the Fisher's exact test or one-way ANOVA. Statistical significance was defined as $p<0.05$. GraphPad Prism 5.0 was used for all analyses.
Results

Patient characteristics

A total of 48 HLA*02:01 positive melanoma patients were enrolled (supplementary figure 1), of which 3 patients (IV-D-13, IV-D-12, IV-D-07) were regarded as non-evaluable, since they did not complete the first cycle due to rapid progressive disease. Two stage IV patients were only evaluable for immunologic response; patient IV-C-05 had no measurable disease at baseline and patient IV-D-15 had proven brain metastasis after the second vaccination but completed the first cycle. Twenty-six stage III and 19 stage IV patients were included. Twenty-three stage III patients received 1 cycle of maintenance vaccinations and 20 patients completed the full three cycles. Three stage IV patients received 1 cycle of maintenance vaccinations; one patient completed the full three vaccination cycles. No unexpected or severe adverse events were observed. Patient characteristics are summarized in Table 1.

Vaccine characteristics

Phenotypic and functional release criteria were defined for DC vaccines to ensure minimal quality criteria and the usage of mature DC in clinical vaccination protocols(22). The phenotype of the ex vivo-generated DC was determined by flow cytometry and all produced vaccines met the standard release criteria, with respect to expression of MHC class I and II, and co-stimulatory molecules, CD83 and CCR7 (Supplementary figure 2a). Furthermore, we confirmed the intracellular expression of tumor associated antigens gp100 and tyrosinase after electroporation by flow cytometry (Supplementary figure 2b). Patients received on average 12×10^6 DC per vaccination with a maximum of 15×10^6 DC per vaccination.
**Immunological response to KLH**

For immunomonitoring purposes all DC have been loaded with the control antigen KLH. PBMC, isolated after each vaccination, showed increased proliferation upon stimulation with KLH after vaccination in almost all patients in the first cycle (Figure 1a). One patient first developed a proliferative response to KLH in the second cycle. Anti-KLH antibodies were detected in 9 out of 17 stage IV patients tested, and 15 out of 26 stage III patients tested (Figure 1b). These data demonstrate that the vaccine effectively induced *de novo* immune responses.

**Tumor-associated antigen specific responses in blood**

To investigate TAA-specific immune responses, PBMC were screened with tetrameric-MHC complexes before and after each cycle of 3 vaccinations at the timepoint of SKIL test. TAA-specific CD8\(^+\) T cells were only found in freshly isolated PBMC from 3 stage III and 3 stage IV patients after vaccination (Figure 2 and Table 2). Since it has been described that melanoma patients can already have a substantial number of TAA-specific T cells circulating in their blood, we analysed the presence of TAA-specific T cells in PBMC isolated prior to vaccination. Three out of these 6 patients had no detectable TAA-specific CD8\(^+\) T cells circulating before vaccination, suggesting that TAA-specific CD8\(^+\) T cells were newly induced, or at least enhanced, by the DC vaccinations in these patients, in concordance with previous reports(23).

Evidence is emerging that CD4\(^+\) T cells needs to be antigen-specific in order to potentiate the CD8\(^+\) immune response. Therefore we analysed the presence of TAA-specific CD4\(^+\) T cells in PBMCs of all 15 DR4\(^+\) patients. Four patients were positive after vaccination (patients IV-C-01, IV-C-02, IV-C-03, IV-C-08; Figure 3a and Table 2). Tetramer analysis of PBMCs restimulated *in vitro* with DR4-binding peptides confirmed the presence of TAA-specific CD4\(^+\) T cells (Figure 3B). TAA-specific CD4\(^+\) T cells were detectable before vaccination in only one
patient (IV-C-03), but only after in vitro restimulation of PBMCs with DR4-binding peptides, suggesting that tumor-specific CD4\(^+\) T cells were induced, or at least enhanced, by DC vaccinations in these patients. We identified concurrent TAA-specific CD8\(^+\) T cells in SKIL cultures in 3 of the 4 patients with tumor-specific CD4\(^+\) T cells in their blood (patients IV-C-01, IV-C-02, IV-C-03; Table 2). To exclude that the TAA-specific CD4\(^+\) T cells have a suppressor phenotype, we tested their FoxP3 expression, all were negative (Figure 3c).

**TAA-specific responses in skin-test infiltrating lymphocyte (SKIL) cultures**

Previously we showed that the presence of TAA-specific T cells in SKIL cultures positively correlates with clinical outcome in stage IV melanoma patients(22). Skin tests were performed after each cycle of vaccinations. Since performing skin tests and taking biopsies puts a great burden to the patient, and previously we observed in a series of patients who underwent pre-vaccination skin test analysis that none of the patients had detectable levels of TAA-specific T cells prior to vaccination, we choose not to perform pre-vaccination skin-tests, but rather perform in-depth analysis on the post-vaccination samples. Tetramer positive CD8\(^+\) SKILs were detected in 17 stage III patients and in 11 stage IV patients (p=0.7574 Fischer’s exact test). In 8 stage III patients and in 2 stage IV patients, CD8\(^+\) SKILs were specific for all 3 tested epitopes (Table 2, Figure 4a). Six stage III and 3 stage IV patients had TAA-specific CD8\(^+\) T cells against 2 of the 3 epitopes tested, while CD8\(^+\) T cells of the other patients recognized one epitope.

Merely the presence of TAA-specific CD8\(^+\) T cells is not necessary sufficient for effective anti-tumor responses. Therefore we tested whether the vaccine-induced TAA-specific CD8\(^+\) T cells were ‘functional’ in terms of selective IFN\(\gamma\) production upon coculture with peptide-, or protein-loaded target cells or tumor cells (Table 2, Figure 4b). Strikingly, although we detected tetramer-specific CD8\(^+\) T cells in both stage III and IV melanoma patients to similar extend, we found increased IFN\(\gamma\) production only in 3 out of 11 stage IV melanoma patients.
In contrast, in stage III patients with no measurable disease, we found IFNγ production in 15 out of 17 patients with tetramer-positive CD8+ SKIL cultures, (p=0.0033 Fisher’s exact test).

Interestingly, SKILs of three patients (IV-C-02, IV-C-10, IV-C-14) that did not produce cytokines upon co-culture with the HLA*02:01 binding peptides, produced IFNγ upon co-culture with the respective tumor protein, indicating that T cells recognized different epitopes. SKILs derived from three additional patients (II-E-04, II-E-05, IV-C-12) that produced IFNγ upon stimulation with only one of the HLA*02:01 binding peptides, produced IFNγ upon co-culture with gp100-expressing cell lines or tyrosinase-expressing cell lines (Table 2, Figure 4c), suggesting that TAA-specific T cells with another specificity than the epitopes used for peptide stimulation and tetramer staining were induced by the DC vaccine.

**Clinical outcome in stage III patients**

One patient had progressive disease within 4 months after start of vaccinations. As of June 2011, 15 out of 26 patients progressed at 3 to 37 months after the start of vaccination. Twelve of 26 patients are in ongoing remission for up to 45 months. The median progression free survival (PFS) is 34.3 months, and the median overall survival has not yet been reached. Extended follow-up is necessary to draw conclusions on the potency of vaccination with mRNA electroporated dendritic cells as an adjuvant therapy in melanoma patients.

**Clinical responses in stage IV patients**

All stage IV patients were evaluated for clinical response at 3-month intervals with CT scan. Five patients had stable disease up to 15 months and one patient (IV-C-02) showed a mixed response. One patient (IV-D-11) with irresectable primary melanoma of the nasal mucosa with bilateral lymph node metastases in the neck region and metastases in the maxillary sinus, showed a partial response of the primary tumor after three vaccinations, allowing
resection of the primary tumor. The lymph node metastases and sinus metastases completely regressed upon further vaccination and this patient is in ongoing complete remission for 52+ months (Figure 5). DR4 expression was not correlated with survival (data not shown) in the vaccinated patients.

We observed a trend towards improved PFS in patients with TAA-specific T cells in their blood or SKIL cultures compared to patients without TAA-specific responses, with 8.1 month versus 2.8 months, respectively (p= 0.062). Similarly, patients with TAA-specific T cells showed improved OS compared to patients without TAA-specific T cells, 24.1 months versus 11.0 respectively (p = 0.101).
Discussion

Early clinical trials have shown that vaccination with DC loaded with tumor peptides is feasible, safe and can induce tumor-specific immune responses in advanced cancer patients(1;5;24). Although these results are promising, further improvement is warranted before its use can be accepted in clinical practice. In the present study we show that DC presenting multiple naturally processed epitopes following mRNA electroporation, enhance tumor-specific CD8⁺ and CD4⁺ T cell responses in melanoma patients. Importantly, both the presence of TAA-specific CD8⁺ T cells and their capacity to produce IFNγ upon encounter of their cognate antigen was significantly increased in stage III patients treated in the adjuvant setting.

Long-lasting T cell receptor stimulation of several hours by fully matured DCs is necessary to activate naïve T cells to proliferate and differentiate into effector cells(25;26). The generated DCs highly and sustainably expressed gp100 and tyrosinase after electroporation with mRNA. In vitro, DC were able to activate gp100-specific CTL up to 48 hours after electroporation. Previously, we demonstrated that gp100 and tyrosinase protein can be detected inside lymph nodes up to 24 hours after intranodal injection of mRNA electroporated DC(10). In this study, TAA-specific T cells were induced in the majority of patients which clearly demonstrates that electroporated DC are indeed potent inducers of tumor-specific T cells.

We detected TAA-specific CD8⁺ T cells in peripheral blood of only 6 of the 45 patients. This is an underestimation, likely due to the low frequencies of these cells in the circulation and the observation that substantially more TAA-specific CD8⁺ T cells were detected in SKIL cultures. Still, the number of TAA-specific T cells measured in this study might be underestimated because we screened with HLA*02:01 binding tetramers only. Indeed, in six patients SKILs produced IFNγ upon co-culture with the protein gp100 and/or tyrosinase, while no IFNγ production was detected upon co-culture with the corresponding HLA*02-
binding peptide(s), supporting the notion that T cells with a broader specificity than the HLA*02:01 epitopes were induced. Recently, Bonehill et al. reported on the use of monocyte-derived DC electroporated with mRNA encoding multiple tumor antigens, CD40 ligand, active TLR4 and CD70 (TriMix-DC)(27). Although they monitored tumor-specific T cells by using autologous EBV-transformed B cells transfected with tumor antigens as target cells, comparable frequencies of gp100 and tyrosinase-specific CD8+ T cells were found.

In 4 out of 10 patients tested, mRNA-electroporated DC induced concomitant TAA-specific CD4+ T cells. The observation that these cells did not express FoxP3, suggest that these cells were T helper cells and not regulatory T cells. Initially, the main function of CD4+ T helper 1 cells was thought to be the production of cytokines providing growth and differentiation signals to precursor CTL to become effector CTL(28). However, CD4+ T cells have also been demonstrate to participate in the elimination of tumor and the maintenance of long-term protective immunity(29-31). In addition, activated T helper cells can stimulate precursor CTLs by reciprocal activation of APCs, for instance via CD40-CD40L interactions(32). Recently it was shown that CD4+ T cells enhance the recruitment of CD8+ T cells to the lymph nodes(33) and tumor(34-36). Moreover, a direct antitumor effect of T helper cells has been demonstrated(37-39). This may be of particular relevance for the antitumor response against melanoma since this tumor type frequently expresses class II molecules constitutively(40;41). Indeed, CD4+ T cell responses have been identified in peripheral blood from melanoma patients who remained disease-free after treatment for multiple relapses(39).

Our data suggests a trend towards improved overall survival, when compared to recently reported survival data in comparative arms from large randomized prospective studies on immunotherapy with anti-CTLA4 antibodies in irresectable metastatic melanoma patients(42;43). It is tempting to speculate that the observed clinical responses result from vaccine-induced immune responses. Indeed, stage IV melanoma patients with TAA-specific T cell responses showed increased clinical outcome after vaccination with mRNA-loaded DC
when compared to patients with no vaccine-enhanced TAA-specific T cell responses. These data confirm and extend our previous findings that the presence of tumor-specific T cells in SKIL cultures identifies a subgroup of patients that might benefit from immunotherapy(21). Moreover, these studies demonstrate that sustained disease control can be achieved in increasing numbers of patients, but objective anti-tumor responses might take several months to years to develop(44;45). The, in general, delayed response patterns in immunotherapy and the high response rates to novel targeted therapies in melanoma, obviously warrants future studies that combine both modalities to achieve durable tumor control. Such studies should implement SKIL culture analyses pre- and post-intervention in both active and comparative arms in order to elucidate the dynamics and nature of the induced immune responses.

The higher tumor burden in stage IV as compared to stage III melanoma patients may hamper the induction of effective immune responses but instead favour local immune suppression. The present study demonstrates that robust immunological responses are more frequently induced in patients with no evidence of disease compared to patients with macroscopic tumor burden. Based on the association of tumor-specific T cells and improved clinical outcome, this suggests that DC based vaccination is a promising adjuvant treatment for stage III melanoma patients. However, extended follow-up is warranted to draw conclusions on the clinical efficacy of DC based vaccination in this stage of disease.

In summary, the advantages of vaccination with DC electroporated with mRNA encoding TAA include lack of HLA-restriction, presentation of multiple TAA epitopes to both CD8⁺ and CD4⁺ T cells, and the subsequent induction of a large repertoire of TAA-specific T cells. We demonstrate that vaccination of melanoma patients with mRNA-electroporated DC induces robust tumor-specific CD4⁺ and CD8⁺ T cell responses, in particular in stage III melanoma patients treated adjuvant to radical lymph node dissection.
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References


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<th>Site of metastatic disease</th>
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IV-C-08  m 35  skin  N1a  LN  390  ++  ++  +  14.2  9  15  12  
IV-C-10  f  36  skin  N1a  LN  361  +++  ++  +  13.1  9  15  12  
IV-C-11  m  50  no primary  N1b  LN  379  +  +  +  14.0  11  15  12  
IV-C-12  f  38  no primary  N1b  LN  360  +++  +++  +  13.7  11  15  3  DTIC  
IV-C-14  f  54  skin  N1b  LN  458  +++  +  +  12.2  7  15  12  
IV-D-05  m  48  skin  N3  LN  388  ++  ++  -  7.0  8  8  
IV-D-08  f  54  skin  N2b  LN  416  +++  +  -  15.0  15  15  8  no  
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IV-D-14  m  63  skin  Nxb  LN  348  +++  +  -  13.3  10  15  12  
IV-D-16  m  31  skin  N2b  LN  517  +++  +++  -  12.1  8  15  12  
IV-D-19  m  62  skin  N1b  LN  280  +++  +  -  10.8  7  15  12  ITx  
IV-D-22  m  23  skin  N1b  LN  394  ++  -  -  13.3  12  15  3  DTIC, anti-CTLA4  
IV-D-24  m  65  skin  N2a  LN  350  ++  ++  -  14.2  12  15  12  no  

a LDH normalized after RLND  
b Patients stopped because of burden of skin test  
c gp100 and tyrosinase expression on the primary tumor was analyzed by immunohistochemistry. Intensity of positive cells were scored centrally and semi-quantitatively by a pathologist. Intensity was scored as low (+), intermediate (++), or high (+++).  
f=female, m=male, nr= number, LN=lymph node.
Table 2. Immunological and clinical responses

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<th>Clinical response</th>
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a Tetramer staining of freshly isolated PBMC or SKILs. - no recognition, + one epitope recognized, ++ two epitopes recognized, +++ three epitopes recognized.

b Antigen recognition of SKILs after stimulation with T2 cells loaded with HLA-A2.1-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) as analyzed by IFN-γ production. Responses were scored as the best immunological response after 1-3 cycles of DC vaccinations.

c Patient IV-C-14 had functional T cells without recognizing the tested epitopes (see Figure 4).
d not evaluable for clinical response because no target lesion at start of vaccination.
e not evaluable for clinical response because of symptomatic brain metastases during 1st cycle
f gp100-specific CD4+ T cells were found after in vitro restimulation with DR4-binding peptides
g SD = stable disease, PD= progressive disease, NED=no evidence of disease, PR= partial response, MR= mixed response

n.a. = not applicable; n.t. = not tested; SKIL= Skin-test Infiltrating Lymphocytes; PFS= Progression free survival; OS= Overall survival
Figure legends

Figure 1. KLH-specific immune responses before and after DC vaccination.

(A) KLH-specific T cell proliferation was analysed before the first vaccination and after each DC vaccination during the first vaccination cycle in PBMC of stage III (filled circles) and stage IV (open circles) melanoma patients. Per time point each dot represents one patient. Proliferative response to KLH is given as proliferation index (proliferation with KLH/proliferation w/o KLH). * p <0.05; NS, not significant. (B) KLH-specific IgG, IgA, and IgM antibodies were quantitatively measured after the first vaccination cycle in sera of vaccinated patients. Numbers indicate the number of patients without proliferative (A) or humoral (B) KLH-response.

Figure 2. Tumor antigen-specific CD8+ T cell responses in peripheral blood.

An example of tetramer analysis of PBMCs from patient II-E-08 is shown. Cells were stained with tetramers encompassing the HLA*02-specific gp100:154-168, gp100:280-288, tyrosinase:369-377 peptide or an irrelevant peptide and with anti-CD8 mAb. The irrelevant control peptide stained 0.01% of the PBMCs.

Figure 3. Tumor antigen-specific CD4+ T cell responses in peripheral blood.

An example of tetramer analysis of PBMCs of patient IV-C-02 after the first cycle of DC vaccination is shown. (A) Freshy isolated PBMCs were stained directly after isolation with tetramers encompassing the HLA-DR4-specific gp100:44-59 peptide, tyrosinase:448-462 peptide, or an irrelevant peptide and with anti-CD4 mAb. (B) Tetramer analysis of PBMC after 8 days of in vitro restimulation with DR4-binding gp100 or tyrosinase peptides. Note that
before restimulation (A), only gp100-specific CD4\(^+\) T cells are found, whereas after restimulation (B) both gp100- and tyrosinase-specific CD4\(^+\) T cells are detectable. (C) The in vitro stimulated population of gp100- or tyrosinase-specific CD4\(^+\) PBMC of patient IV-C-02 was further characterized for FoxP3 expression. TAA-specific CD4\(^+\) T cells did not express FoxP3.

**Figure 4. Tumor antigen-specific CD8\(^+\) T cell responses in post-treatment SKIL cultures.**

The presence and functionality of TAA-specific T cells were tested in lymphocytes cultured form skin-test biopsies (SKILs). (A) An example is shown of tetramer analysis of SKILs from patient II-E-07, cultured from a DTH reaction to DC pulsed with tumor peptides. Cells were stained with tetramers encompassing the gp100:154 peptide, gp100:280, tyrosinase or an irrelevant peptide (control) and with anti-CD8 mAb. The irrelevant control peptide stained 0.07% of the T cells. The biopsy contains gp100- and tyrosinase-specific CD8\(^+\) T cells. (B) IFN\(\gamma\) production by the same T cells after stimulation with T2 cells loaded with gp100:154-168 peptide and gp100:280-288 or tyrosinase:369-377 peptide (peptide stimulation), BLM cells expressing gp100- or tyrosinase protein (protein stimulation), or Mel624 cells expressing both gp100 and tyrosinase (tumor stimulation). (C) Example of functional responses of SKILs of patient IV-C-14, cultured from a biopsy of a DTH reaction to DC electroporated with tyrosinase mRNA, showing recognition of tyrosinase epitopes when presented by HLA-A2.1 positive tyrosinase-transfected BLM cells or endogenously tyrosinase expressing Mel624 cells, by the specific and elevated production of IFN\(\gamma\), although it does not recognize the specific epitopes used in previous vaccination studies. This indicates that a broad repertoire of TAA-specific T cells can be stimulated by vaccination with mRNA-transfected DC.
Figure 5. Partial response after three intranodal vaccinations with mRNA-transfected DC.

(A) Patient IV-D-11 presented with an unresectable primary melanoma from nasal mucosa with extension into the nasal septum, maxillary and ethmoid sinus, and bilateral lymphadenopathy of level 1, 2 and 5. (B) Three intranodal vaccinations with mRNA-transfected DC induce a partial response, allowing resection of the primary tumor. (C) Patient is in ongoing remission.
Figure 2

gp100:154-163

0.05%

gp100:280-288

1.17%
tyrosinase:369-377

0.00%

tetramer

CD8
Figure 3

A  
\[
\begin{array}{ccc}
\text{gp100:44-59} & \text{tyrosinase:448-462} & \text{control} \\
0.09\% & 0.03\% & 0.03\%
\end{array}
\]

B  
\[
\begin{array}{ccc}
\text{gp100:44-59} & \text{tyrosinase:448-462} & \text{control} \\
1.40\% & 0.44\% & 0.04\%
\end{array}
\]

C  
\[
\begin{array}{cc}
\text{gp100:44-59} & \text{tyrosinase:448-462} \\
0.05\% & 0.01\%
\end{array}
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Figure 4

A  
gp100:154-163  
2.62%  
gp100:280-288  
0.63%  
tyrosinase:369-377  
0.17%

B  
II-E-07

C  
IV-C-14
Vaccination with mRNA-electroporated dendritic cells induces robust tumor antigen-specific CD4+ and CD8+ T cell responses in stage III and IV melanoma patients


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