

# *In situ* Expression of Tumor Antigens by Messenger RNA–Electroporated Dendritic Cells in Lymph Nodes of Melanoma Patients

Danita H. Schuurhuis,<sup>1</sup> Pauline Verdijk,<sup>1</sup> Gerty Schreibelt,<sup>1</sup> Erik H.J.G. Aarntzen,<sup>1,2</sup> Nicole Scharenborg,<sup>1</sup> Annemiek de Boer,<sup>1</sup> Mandy W.M.M. van de Rakt,<sup>1</sup> Marieke Kerkhoff,<sup>1</sup> Marie-Jeanne P. Gerritsen,<sup>3</sup> Femke Eijckeler,<sup>4</sup> Johannes J. Bonenkamp,<sup>5</sup> Willeke Blokkx,<sup>4</sup> J. Han van Krieken,<sup>4</sup> Otto C. Boerman,<sup>6</sup> Wim J.G. Oyen,<sup>6</sup> Cornelis J.A. Punt,<sup>2</sup> Carl G. Figdor,<sup>1</sup> Gosse J. Adema,<sup>1</sup> and I. Jolanda M. de Vries<sup>1,7</sup>

Departments of <sup>1</sup>Tumor Immunology, <sup>2</sup>Medical Oncology, <sup>3</sup>Dermatology, <sup>4</sup>Pathology, <sup>5</sup>Surgery, <sup>6</sup>Nuclear Medicine, and <sup>7</sup>Pediatric Hemato-Oncology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands

## Abstract

**Electroporation of dendritic cells (DC) with mRNA encoding tumor-associated antigens (TAA) for cancer immunotherapy has been proved efficient and clinically safe. It obviates prior knowledge of CTL and Th epitopes in the antigen and leads to the presentation of multiple epitopes for several HLA alleles. Here we studied the migration capacity and the antigen expression of mRNA-electroporated DC (mRNA-DC) in lymph nodes after vaccination in melanoma patients. DC were electroporated with mRNA encoding gp100 or tyrosinase, labeled with indium-111 and superparamagnetic iron oxide particles, and injected intranodally in melanoma patients 24 to 48 hours before scheduled dissection of regional lymph nodes. Immunohistochemical analysis of the lymph nodes after surgery revealed that mRNA-DC migrated from the injection site into the T-cell areas of the same and subsequent lymph nodes, where they expressed the antigen encoded by the electroporated mRNA. Furthermore, vaccine-related CD8<sup>+</sup> T-cell responses could be detected in 7 of 11 patients vaccinated with mRNA-DC. Together these data show that mature DC electroporated with mRNA encoding TAA migrate and express antigens in the lymph nodes and induce specific immune responses. [Cancer Res 2009;69(7):2927–34]**

## Introduction

Immunotherapy targeted against cancer aims at activating the immune system to destroy tumor cells. Many tumors express tumor-associated antigens (TAA), which can be exploited as targets for immunotherapy. Because antigen presentation by tumor cells themselves is insufficient to induce an adequate immune response, more potent antigen-presenting cells (APC) are required. Dendritic cells (DC) are professional APC, with the ability to initiate and maintain immune responses (1). Early clinical studies reported the feasibility and safety of DC vaccinations (2). Whereas immunologic responses have been reported frequently, the number of clinical responses is still low. One crucial aspect of DC-based vaccines concerns the efficacy of

antigen loading of DC. Thus far, mostly DC loaded with defined tumor peptides have been used for the induction of antitumor immunity (3–6). These DC are exogenously loaded by incubation with peptides binding directly to MHC class I and/or MHC class II molecules on the cell surface. Because peptide-loaded DC expose antigen only for a short period of time (7), various other methods have been explored to introduce whole TAA into DC. These include viral vectors (8), RNA encoding tumor antigen (9–14), whole tumor cell contents (apoptotic tumor cells or tumor cell lysate; refs. 15–17), and fusion of tumor cells with DC (18, 19). The use of DNA or RNA encoding tumor antigens, whole tumor RNA, or tumor lysates to load DC is attractive because no knowledge of nominal epitopes in the tumor antigen is required, nor is there a restriction for the HLA type of the patient. Proteolytic processing of the (endogenously produced) antigen within the DC will result in loading of suitable peptides onto MHC molecules expressed by the DC. Furthermore, the use of whole antigens instead of peptides has the advantage that multiple epitopes for both CTL and Th cells can be presented by the DC. DC transfected with mRNA encoding TAA or with whole tumor mRNA have been shown to induce potent antigen- and tumor-specific T-cell responses (reviewed in refs. 20, 21). The advantage of the use of synthetic mRNA is that transfection with RNA is not considered gene therapy and does not induce the risk of adverse events due to integration in the host genome. Furthermore, autologous tumor is often not available and the use of whole tumor lysates or RNA may increase the risk of induction of autoimmunity.

Antigen-loading by mRNA electroporation of monocyte-derived DC was shown to be very efficient (22, 23). We previously observed that electroporation efficiency was highest when using mature DC and that electroporated DC show the same immunostimulatory capacities as untreated cells *in vitro* (24). To induce an adequate immune response, it is important that DC migrate to draining lymph nodes and into the T-cell area (25). We therefore investigated the *in vivo* migratory behavior of mRNA-electroporated DC (mRNA-DC) in a phase I/II clinical trial with melanoma patients. We included patients with melanoma that were scheduled for lymph node dissection, which provided a unique opportunity to study not only *in vivo* migration (by scintigraphy) but also the localization and *in situ* antigen expression of the mRNA-DC within the lymph node by immunohistochemistry of isolated nodes. We here show that DC electroporated with mRNA encoding TAA actively migrate into T-cell areas after intranodal injection and express the encoded antigens

**Note:** D.H. Schuurhuis and P. Verdijk contributed equally to this work.

**Requests for reprints:** I. Jolanda M. de Vries, Department of Tumor Immunology, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands. Phone: 31-24-3617600; Fax: 31-24-3540339; E-mail: j.devries@ncmls.ru.nl  
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*in situ* for at least 24 hours. Furthermore, DC vaccine-related CD8<sup>+</sup> T-cell responses could be detected in melanoma patients vaccinated with mRNA-DC.

## Materials and Methods

**Peptides.** The following HLA-A2.1-restricted peptides were used: gp100-derived peptides gp100<sub>154</sub> (amino acids 154–162, KTWGQYWQV) and gp100<sub>280</sub> (amino acids 280–288, YLEPGPVTA) and tyrosinase-derived peptide tyrosinase<sub>369</sub> (amino acids 369–377, YMNGTMSQV).

**Plasmids and *in vitro* mRNA transcription.** Plasmid pGEM4Z-5'UT-hgp100-3'UT-A64 was provided by Kris Thielemans (Free University of Brussels, Belgium). pGEM4Z-5'UT-tyrosinase-3'UT-A64 was constructed by digestion of pGEM4Z-5'UT-tNGFR-3'UT-A64 with *Bgl*III and *Xba*I and digestion of pcDNA1.amp/tyrosinase with *Bam*HI and *Xba*I and insertion of the tyrosinase into the cut pGEM4Z. Plasmids were linearized with *Spe*I (pGEM4Z-5'UT-CEA-3'UT-A64) or *Not*I enzyme (pGEM4Z-5'UT-tNGFR-3'UT-A64, pGEM4Z-5'UT-hgp100-3'UT-A64, and pGEM4Z-5'UT-tyrosinase-3'UT-A64), purified with phenol/chloroform extraction and ethanol precipitation, and used as DNA templates. mRNA was obtained from CureVac GmbH. RNA quality was verified by agarose gel electrophoresis; RNA concentration was measured spectrophotometrically; and RNA was stored at -40°C in small aliquots.

**Patients.** Eligibility criteria included stage III melanoma according to the 2001 American Joint Committee on Cancer Staging criteria (26), planned regional lymph node dissection for lymph node metastases, HLA-A2.1 phenotype, melanoma expressing the melanoma-associated antigens gp100 and tyrosinase, and WHO performance status of 0 or 1. Additional eligibility criteria are described previously (3). The study was approved by our Institutional Review Board, and written informed consent was obtained from all patients. Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria. In total, 11 stage III melanoma patients were included. Toxicity was similar to previous DC vaccination studies (27) and consisted of low-grade fever, mild flu-like symptoms, and irritation at the site of injection after the vaccination in some patients.

**Treatment schedule.** At day -8, peripheral blood mononuclear cells were obtained by leukapheresis for DC culturing. At day 0, patients received an intranodal injection of DC, labeled with both <sup>111</sup>In and SPIO and electroporated with either gp100- or tyrosinase-encoding mRNA (as shown in Table 1) directly into a lymph node of the region that was to be resected using a 21-gauge sterile needle (0.8 × 50 mm; Microlance, Becton Dickinson). Intranodal injections were done under ultrasound guidance 1 or 2 d before the scheduled radical lymph node dissection. Scintigraphic images of the injection depot and corresponding lymph node basin were made directly after injection and before surgery. For one patient, surgery was postponed for >2 wk and was therefore not evaluable. One patient did not have superficial lymph nodes in the designated lymph node area, and one patient received DC labeled with SPIO only. All patients received three subsequent intranodal vaccinations with mRNA-DC at days 14, 28, and 42.

**DC culture and labeling.** Monocytes were enriched from leukapheresis products by counterflow elutriation using Elutra-cell separator (Gambro BCT, Inc.) and single-use, functionally sealed disposable Elutra sets, as described before (28) and according to the manufacturer's instructions. Monocytes were cultured in the presence of interleukin (IL)-4 (500 units/mL), granulocyte macrophage colony-stimulating factor (800 units/mL; both from Cellgenix), and keyhole limpet hemocyanin (KLH; 10 µg/mL; Calbiochem) as described (27). Immature DC were labeled with SPIO 1 d before maturation induction by adding 200 µg/mL ferumoxide (Endorem, Laboratoire Guerbet; ref. 29). DC were matured with autologous monocyte-conditioned medium (30%, v/v) supplemented with prostaglandin E<sub>2</sub> (10 µg/mL, Pharmacia & Upjohn) and 10 ng/mL tumor necrosis factor α (Cellgenix) for 48 h (30, 31). DC met the release criteria (2): more than 80% of the DC expressed high levels of CD80 and CD86. Mature DC were electroporated with mRNA encoding gp100 or tyrosinase as described below. For the first vaccination, mature, SPIO-labeled mRNA-DC were labeled 2 h after electroporation with <sup>111</sup>In-oxine (Covidien) as described previously (30, 32).

**Electroporation of DC.** Mature DC were washed twice in PBS and once in OptiMEM without phenol red (Invitrogen). Twenty micrograms of RNA were transferred to a 4-mm cuvette (Bio-Rad) and 12 × 10<sup>6</sup> cells were added in 200 µL OptiMEM and incubated for 3 min before being pulsed in a GenePulser Xcell (Bio-Rad) by an exponential decay pulse of 300 V, 150 µF, as described before (24). For vaccination purposes, electroporation was done under good manufacturing practice conditions. For the first vaccination, 2 to 3 aliquots of 12 × 10<sup>6</sup> SPIO-labeled DC were electroporated with either gp100- or tyrosinase-encoding mRNA. Electroporation efficiency was analyzed by intracellular staining and fluorescence-activated cell sorting (FACS) analysis and by immunohistochemistry for each separate TAA. DC for the first vaccination were labeled with <sup>111</sup>In as described above and injected 4 h after electroporation. DC for subsequent vaccinations were frozen 2 h after electroporation, thawed at the day of vaccination, and incubated for an additional 2 h at 37°C before injection.

**Flow cytometric analysis (FACS analysis).** Flow cytometry was done using the following monoclonal antibodies (mAb) or appropriate isotype controls: anti-HLA class I (W6/32), anti-HLA DR/DP (Q5/13), anti-HLA DR, anti-CD80 (all BD Biosciences), anti-CD14, anti-CD83 (both Beckman Coulter), anti-CD86 (BD Pharmingen), and anti-CCR7 (kind gift of Martin Lipp, Max Planck Institute, Berlin, Germany). For intracellular staining, NK1/beteb (IgG2b; purified antibody) against gp100 and T311 (IgG2a; Cell Marque Corp.) against tyrosinase were used. Flow cytometry was done with FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences).

**Delayed type hypersensitivity test.** Two weeks after four DC vaccinations, a delayed-type hypersensitivity (DTH) skin test was done (3). Briefly, DC pulsed with both gp100<sub>154</sub> and gp100<sub>280</sub>, tyrosinase<sub>369</sub>, or all three peptides or DC electroporated with gp100 mRNA, tyrosinase mRNA, or both were injected intradermally. From these DTH sites, punch biopsies (6 or 3 mm) were obtained and cut in half: one part was cryopreserved and the other part was cultured for 2 to 4 wk in interleukin 2 (IL)-2 (3). T cells were tested for binding allophycocyanin-labeled tetrameric MHC complexes (Sanquin).

**Scintigraphic imaging.** *In vivo* and *ex vivo* planar scintigraphic images were acquired with a gamma camera (Siemens ECAM) equipped with medium energy collimators, at day 0 and 18 h later (30, 32). Migration was quantified by region-of-interest analysis of the individual nodes visualized on the images and expressed as the relative fraction of <sup>111</sup>In-labeled DC that had migrated from the injection depot.

**Iron staining and immunohistochemistry of cytopins and histopathologic sections.** Radioactive lymph nodes were dissected from the surgical specimen under guidance of a gamma probe (Europrobe, Eurorad) and then fixed in Unifix (Klinipath). Sections (5 µm) of the resected radiolabeled lymph nodes were stained with Prussian blue to detect SPIO-labeled cells as described previously (29). Immunohistochemical staining was done on paraffin-embedded tissue sections using mAbs against CD83 (Novocastra), HMB-45 against gp100 (DAKO), and T311 against tyrosinase (Cell Marque Corp.). 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 h. The avidin-biotin complex (Vector) method was used for visualization with 3-amino-9-ethyl-carbazole or 3,3'-diaminobenzidine hydrochloride solution. Slides were counterstained with hematoxylin solution or nuclear fast red, dehydrated, and mounted in Permount (Fisher Chemicals).

**Migration assays.** For random migration on fibronectin, we used our previously established migration assay to study migration of DC (33). For CCR7-mediated migration, a standard *in vitro* transwell migration assay was used (34, 35).

## Results

**Labeling of mRNA-DC for cell tracking *in vivo*.** Tracking of DC *in vivo* requires cell labeling. Therefore, we first tested whether

**Table 1.** Overview of patients

| Patient             | mRNA first vaccination | Time after injection (h) | %Redistribution | TAA protein expression | T-cell specificity DTH: mRNA-DC*  | T-cell specificity DTH: peptide-loaded DC*          |
|---------------------|------------------------|--------------------------|-----------------|------------------------|---|---|
| 2E-01               | Gp100                  | 24                       | 63              | +                      | None  | None  |
| 2E-02               | Gp100                  | 24                       | 10              | +                      | None  | None  |
| 2E-03               | Gp100                  | 24                       | 5.3             | +                      | Control: 0.09%<br>Gp100: 0.45%<br>Tyrosinase: 0.56%                         | Control: 0.12%<br>Gp100: 0.71%<br>Tyrosinase: 1.13% |
| 2E-04 <sup>†</sup>  | Gp100                  | nd                       | nd              | nd                     | None  | None  |
| 2E-05 <sup>‡</sup>  | Gp100                  | 48                       | nd              | —                      | None  | None  |
| 2E-06               | Gp100                  | 24                       | 38              | +                      | Control: 0.06%<br>Tyrosinase: 0.45%   | Control: 0.07%<br>Tyrosinase: 1.01%                 |
| 2E-07 <sup>§</sup>  | Gp100                  | 48                       | 7.6             | nd                     | Control: 0 pg/mL<br>Tyrosinase: 90 pg/mL                                    | Control: 0 pg/mL<br>Tyrosinase: 21 pg/mL            |
| 2E-08 <sup>  </sup> | Gp100                  | nd                       | nd              | nd                     | Control: 0.05%<br>Gp100: 0.28%  | Control: 0.03%<br>Gp100: 0.39%                      |
| 2E-09               | Tyrosinase             | 24                       | 0               | —                      | Control: 0.03%<br>Gp100: 0.28%<br>Control: 0 pg/mL<br>Tyrosinase: 492 pg/mL | Control: 0.05%<br>Gp100: 0.33%                      |
| 2E-10 <sup>¶</sup>  | Tyrosinase             | 24                       | 0               | nd                     | Control: 0.08%<br>Tyrosinase: 0.23%   | Control: 0.03%<br>Gp100: 0.06%<br>Tyrosinase: 0.46% |
| 2E-11               | Tyrosinase             | 24                       | 65              | +                      | Control: 0.10%<br>Gp100: 0.71%  | Control: 15 pg/mL<br>Tyrosinase: 924 pg/mL          |

Abbreviation: nd, not done.

\*Percentage of tetramer-positive cells in DIL cultures from biopsies of DTH sites induced by either mRNA-DC or peptide-loaded DC. Due to technical problems with tetramers in some cases, IFN $\gamma$  production after stimulation with irrelevant peptide (control) or relevant peptide is depicted.

<sup>†</sup> No superficial lymph nodes due to previous sentinel lymph node removal; no accessible lymph nodes present in axillary lymph node region.

<sup>‡</sup> No <sup>111</sup>In radiolabeling.

<sup>§</sup> No lymph nodes isolated.

<sup>||</sup> Surgery was cancelled due to high blood pressure; no scintigraphy.

<sup>¶</sup> Cells were injected in perinodal fat; lymph node was missed.

mRNA electroporation of mature DC was compatible with both SPIO and <sup>111</sup>In labeling according to our current protocols (30, 32, 35). In previous studies, mature DC were labeled with <sup>111</sup>In directly before vaccination. Because electroporation allows the mRNA to enter the cell by temporarily increasing the cell membrane permeability, <sup>111</sup>In may not be sufficiently retained within the electroporated cell and may leak out. We observed that in DC labeled with <sup>111</sup>In 1 hour after electroporation, <sup>111</sup>In retention during the first 24 hours was comparable to that of untreated cells (Fig. 1A). Similarly, we checked if SPIO taken up by the DC interfered with mRNA electroporation efficiency and subsequent protein expression. For this, expression of TAA after electroporation was analyzed in SPIO-labeled and unlabeled cells by flow cytometry and immunohistochemistry (Fig. 1B and C). Labeling of DC with SPIO did not affect the efficacy of mRNA electroporation or the viability and phenotype of the DC (35). Expression of gp100 measured 4 hours after electroporation was comparable in SPIO-labeled and unlabeled cells (Fig. 1B). Thus, SPIO-labeled DC can be efficiently electroporated with TAA-encoding mRNA and these cells can be stably labeled with <sup>111</sup>In for tracking *in vivo*.

**Efficient migratory capacity of mRNA-DC.** Stimulation of T cells not only requires expression of the antigen of interest and of costimulatory molecules by the mRNA-DC, but it is also essential that DC migrate from the injection site into the T-cell areas of the

draining lymph nodes. Therefore, we first studied *in vitro* both the random and the directional migration of labeled and unlabeled DC 4 hours after electroporation. Random migration on the extracellular matrix protein fibronectin (33) was determined by analyzing the tracks of single cells measured by time lapse microscopy (Fig. 2A). No significant differences were detected in both the percentage of migrating cells (Fig. 2B) and the velocity of movement (Fig. 2C) for mRNA-DC and control DC. SPIO labeling resulted in an ~15% lower velocity of the SPIO-DC, as was observed previously (35), but did not impede migration (Fig. 2B and C). Directional migration toward chemokine CCL21 was slightly diminished when DC were either loaded with SPIO or electroporated with mRNA as compared with controls (Fig. 2D). These results indicate that after labeling with SPIO, mRNA electroporation, and labeling with <sup>111</sup>In, DC still had sufficient migratory capacities and can be used for studying migration *in vivo* and detection *in situ*.

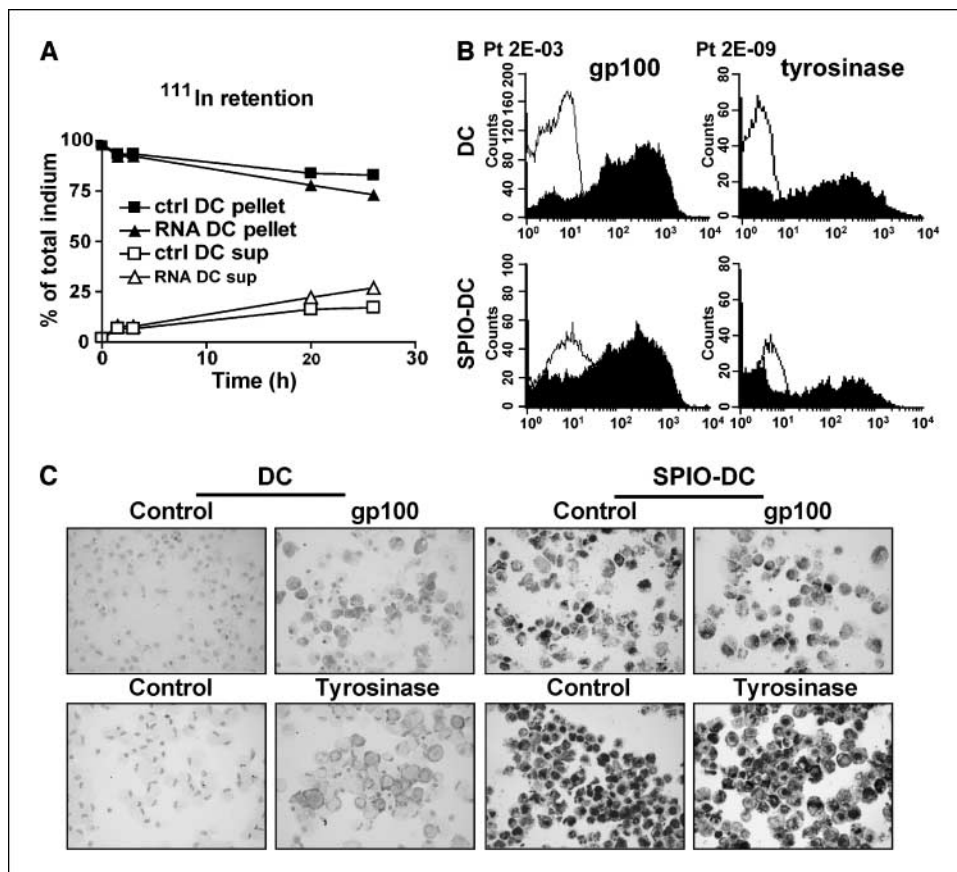
**Biodistribution of mRNA-DC after intranodal injection in melanoma patients.** For the first intranodal vaccination, DC were electroporated with either gp100 or tyrosinase mRNA and labeled with both SPIO and <sup>111</sup>In. The biodistribution of the DC was imaged after vaccination and before surgery (8 of 11 patients; Table 1). Analysis of the scintigraphs revealed major differences in the biodistribution between the various patients. Redistribution of

DC over regional lymph nodes other than the injected node varied between none (0%, 2 of 8 patients), intermediate (>0-10%, 2 of 8 patients), and high ( $\geq 10\%$ , 4 of 8 patients; Table 1). Representative images are shown in Fig. 3. Thus, mRNA-DC were detected in multiple lymph nodes after intranodal injection, maximizing the possibility for DC to enter the T-cell areas and to activate specific T cells.

**mRNA-DC express TAA in the T-cell area.** We next evaluated the migratory capacity and antigen expression of mRNA-DC within the lymph node. For this, individual lymph nodes that contained radiolabeled cells were isolated from the dissected lymph node basin from seven of eight evaluable patients. Although many DC remained at the injection site and lost the expression of CD83 (Fig. 4A), single SPIO<sup>+</sup> cells expressing CD83 had migrated into the T-cell areas of lymph nodes from all seven patients (Fig. 4C). More important, SPIO<sup>+</sup> DC present in the T-cell area strongly expressed the TAA protein encoded by the mRNA (Fig. 4B and D: gp100, tyrosinase). Gp100 expression by SPIO-labeled DC was clearly detectable in the lymph nodes 24 hours after intranodal injection with gp100 mRNA-DC (four of four patients; Table 1). We could not detect gp100 expression when lymph nodes were removed after 48 hours, but thus far, only one patient was tested. Additionally, tyrosinase expression by mRNA-DC was observed in the T-cell areas of lymph nodes from one of two patients (Table 1) after 24 hours, although *in vitro* expression of tyrosinase mRNA reached its maximum after 2 to 4 hours and was almost undetectable 24 hours after electroporation (24). These results show that mRNA-DC not only are capable of migrating from the injection depot into the T-cell areas of injected lymph nodes but also expressed the TAA protein encoded by the mRNA.

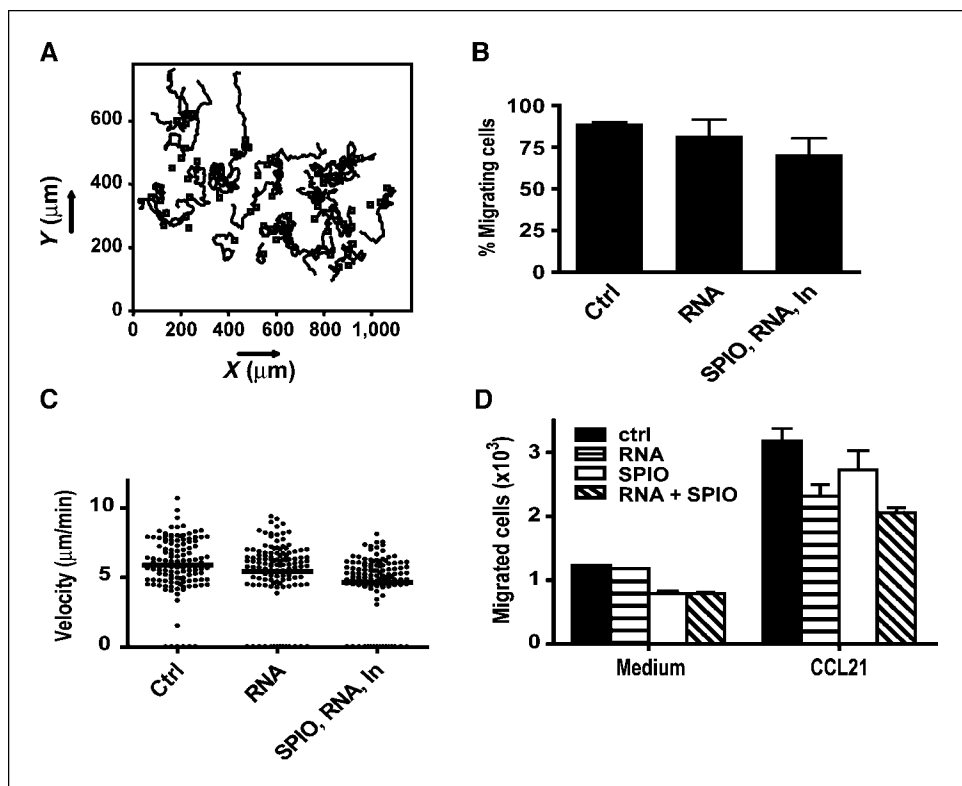
**Activation of CD8<sup>+</sup> T cells after vaccination with mRNA-DC.** To investigate the immune-stimulatory capacities of mRNA-DC, all patients received three additional vaccinations. Measuring antibody responses against KLH, with which all DC had been loaded for immunomonitoring purposes, we detected anti-KLH antibodies (total IgG) in the serum of 6 of 10 patients after vaccination (data not shown). In one patient, antibodies reacting with KLH were already detected before vaccination and were not further analyzed. Furthermore, in all 11 patients KLH-specific T-cell proliferation was detected in the peripheral blood (data not shown).

Next, we studied TAA-specific immune responses by staining of T cells with HLA-A2.1 tetramers with known TAA epitopes (gp100<sub>154</sub>, gp100<sub>280</sub>, and tyrosinase<sub>369</sub>; ref. 3). Tetramer-binding T cells were found in freshly isolated peripheral blood in one patient after four vaccinations (patient 2E-08: 1.2% gp100<sub>280</sub>). Because the frequency of peptide-specific T cells in the blood is usually very low and often undetectable, we further analyzed the presence of TAA-specific T cells by inducing DTH responses with intradermally injected mRNA-DC or peptide-loaded DC. We have shown previously that sampling of DTH sites is an effective approach to detect vaccine-related CD8<sup>+</sup> T cells and that the specificity of the DTH-infiltrated T cells (DIL) corresponds with the antigen loaded on the injected DC (3). Indeed, TAA-specific CD8<sup>+</sup> DIL were found in biopsies of DTH reactions from 7 of 11 patients (Table 1). Both gp100- and tyrosinase-specific CD8<sup>+</sup> DIL could be detected by tetramer analysis (Fig. 5). In four patients, CD8<sup>+</sup> DIL were specific for both tyrosinase and gp100. In three patients, either gp100-specific CD8<sup>+</sup> DIL (one patient) or tyrosinase-specific CD8<sup>+</sup> DIL (two patients) were detected. When the specific T cells were cocultured with gp100- or tyrosinase peptide-loaded target



**Figure 1.** RNA electroporation of DC is compatible with both SPIO and <sup>111</sup>In labeling. **A**, <sup>111</sup>In retention of electroporated and control DC. DC were electroporated (RNA DC) or not (ctrl DC) and, 1 h later, labeled with <sup>111</sup>In. At different time points after labeling, cells were spun down and the amounts of <sup>111</sup>In in the cell pellet and the supernatant (sup) were measured. Data shown are the percentages of <sup>111</sup>In in the pellet and the supernatant. **B** and **C**, electroporation of (SPIO-labeled) DC with mRNA encoding TAA results in expression of the respective proteins. SPIO-labeled (described in Materials and Methods) or unlabeled DC were electroporated with mRNA encoding gp100 or tyrosinase. **B**, intracellular FACS analysis was done 4 h after electroporation. Histograms are shown for isotype control antibody (open curves) and specific antibody for gp100 and tyrosinase (filled curves). **C**, cytopins of unlabeled and SPIO-labeled mRNA-DC were prepared 4 h after electroporation and stained with isotype control antibody or specific antibody for gp100 or tyrosinase and with Prussian blue to detect SPIO-labeled cells (all  $\times 200$ ).

**Figure 2.** Electroporated cells maintain their migratory capacity *in vitro* and can be labeled with <sup>111</sup>In. DC were left untreated (*Ctrl*); electroporated (*RNA*); labeled with SPIO (*SPIO*); SPIO labeled and electroporated (*RNA + SPIO*); or SPIO labeled, electroporated, and labeled with <sup>111</sup>In (*SPIO, RNA, In*). *A* to *C*, random migration on fibronectin was measured as described in Materials and Methods. *A*, example of the traversed path in 1 h of mRNA-DC migrating on fibronectin. The x and y axes represent the coordinates of the imaged field. *B* and *C*, percentage of migrating DC (*B*) and velocity of migration (*C*). *B*, columns, mean percentages; bars, SE. *C*, points, individual cells; horizontal bars, mean. Data shown are pooled data from DC preparations from two independent patients. *D*, chemotaxis toward 10 ng/mL CCL21 was measured in a standard transwell migration assay. Representative data of three experiments.



cells, they produced significant amounts of IFN $\gamma$  (data not shown; Table 1, patients 2E-07 and 2E-09). We did not observe differences in the outcome of DIL screening whether the DTH was induced with peptide-loaded DC or mRNA-DC (Table 1). These data show that mRNA-DC can induce a *de novo* immune response (as shown by the KLH responses) and are capable of inducing or expanding functional TAA-specific CD8<sup>+</sup> T-cell responses in melanoma patients.

**Discussion**

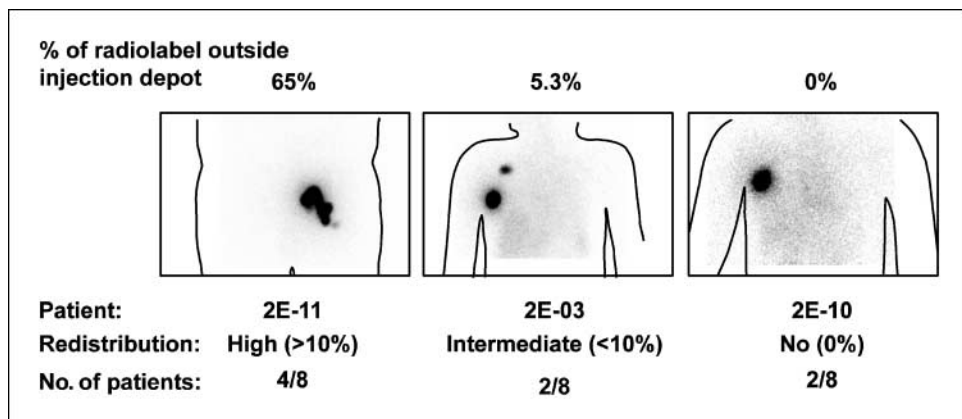
Although early clinical trials clearly show the potency of DC therapy, it is clear that the therapy needs further optimization before it can be introduced as a standard cancer treatment (2). Here we studied the effect of electroporation on the migratory and immune-stimulatory capacities of the mRNA-DC in melanoma patients. We found that electroporation had no effect on the

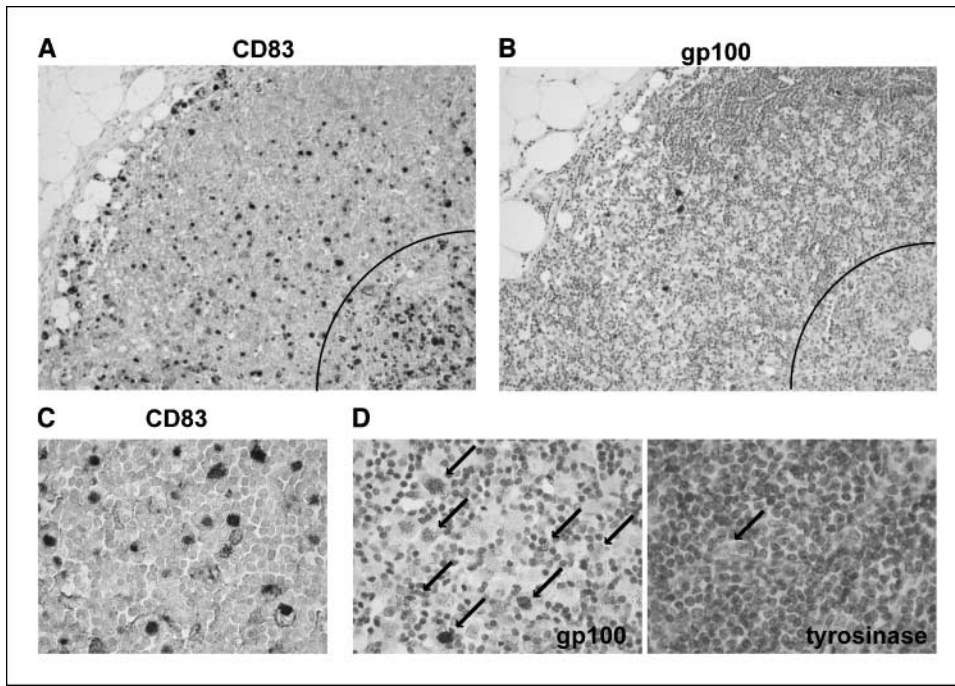
migratory behavior of mRNA-DC not only *in vitro* but also *in vivo*, as shown by the effective migration of mRNA-DC into the T-cell areas of lymph nodes from melanoma patients. Importantly, these mRNA-DC still expressed the tumor antigen encoded by the electroporated mRNA and were capable of inducing TAA-specific CD8<sup>+</sup> T cells.

Our data confirm and extend previous studies showing that electroporation of mature DC with mRNA is an effective method to introduce expression of whole TAA (13, 24, 36). Furthermore, we show that it does not affect the random and directional migratory capacity of DC *in vitro*. To the best of our knowledge, this is the first time that *in situ* expression of TAA and *in vivo* migration of mRNA-DC have been shown after intranodal injection in patients.

A major advantage of injecting DC directly into the lymph node is that all cells are immediately inside the target organ, minimizing the requirements for migration. Scintigraphic imaging revealed that in six of eight patients, DC spread to more than one lymph

**Figure 3.** Biodistribution of mRNA-DC after intranodal injection. Patients with metastatic melanoma were injected intranodally with SPIO- and <sup>111</sup>In-labeled DC electroporated with gp100- or tyrosinase-encoding mRNA. Scintigraphic images showing the distribution of <sup>111</sup>In-labeled cells 18 h after injection are depicted for a patient with high migration ( $\geq 10\%$  of injected DC left injection depot, observed in 4 of 8 patients), a patient with intermediate migration (<10% of injected DC left injection depot, observed in 2 of 8 patients), and a patient with no migration (100% of injected DC remained in the injection depot, observed in 2 of 8 patients).

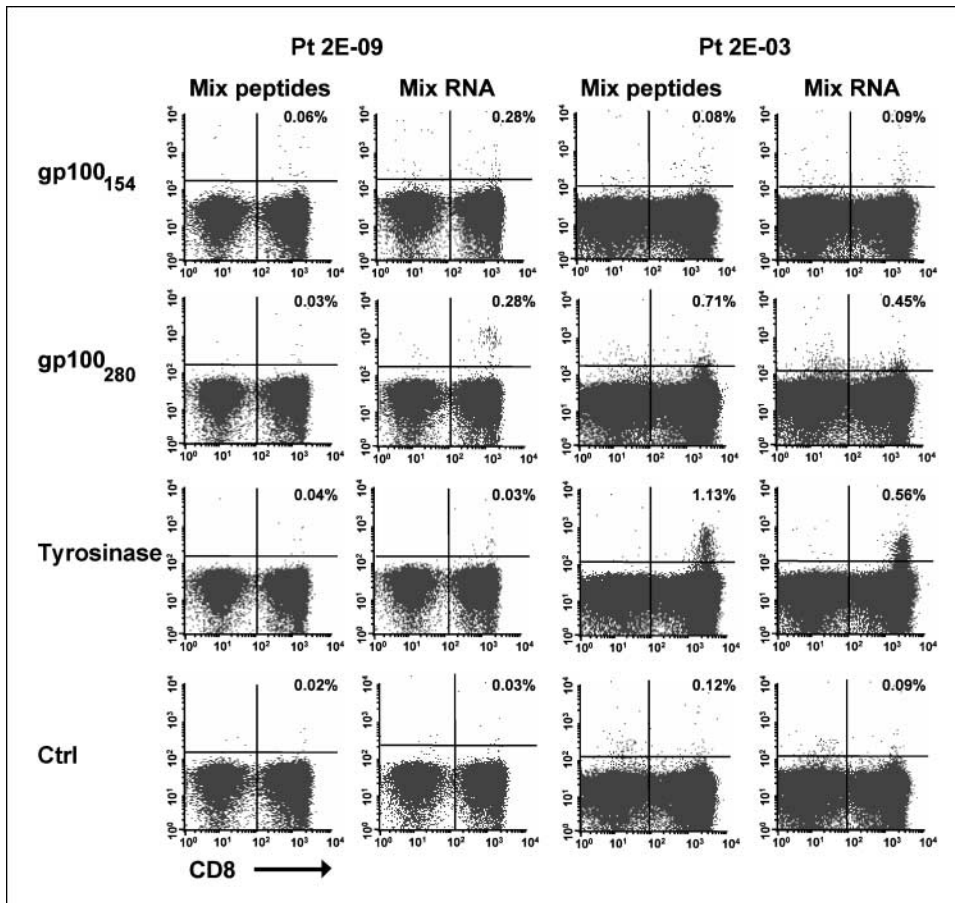




**Figure 4.** mRNA-DC migrate into the T-cell areas of the lymph node and express TAA *in situ*. Eighteen hours after intranodal injection of mRNA-DC, the lymph node basin was resected. Radioactive lymph nodes were isolated with a probe and processed for immunohistochemistry. A to D, paraffin-embedded sections of these lymph nodes were stained for CD83 (brown; A and C), gp100 protein (red; B and D), or tyrosinase protein (red; D). The images show part of the injection site (A and B, circular line) and the paracortex (magnified in C and D) of a lymph node containing SPIO- and <sup>111</sup>In-labeled mRNA-DC. SPIO was stained with Prussian blue (blue; A and C) or left untreated (light brown; B and D). Nuclei were counterstained with nuclear fast red (A and C) or hematoxylin (B and D). Original magnifications, ×100 (A and B) and ×400 (C and D). Arrows, SPIO-labeled cells expressing the TAA.

node after injection. In two patients, the vaccine remained localized in one spot. Scanning the resected lymph node basin for the presence of radiolabeled lymph nodes showed that in only one of these patients were DC not correctly injected into a lymph

node, whereas in the other patient DC were inside a lymph node but had not migrated to subsequent nodes. This is an improvement in comparison with our previous study wherein we showed that ~ 50% of intranodal DC vaccinations were not correctly injected



**Figure 5.** TAA-specific CD8<sup>+</sup> T-cell responses are detected in DTH biopsies. Two weeks after the fourth vaccination, a DTH test was done to monitor specific CD8<sup>+</sup> T-cell responses. DC loaded with gp100- or tyrosinase-derived peptides or electroporated with gp100- or tyrosinase-encoding mRNA were injected intradermally. After 2 d, biopsies were taken, which were cultured for 3 to 4 wk in low-dose IL-2. Specificities of the T cells derived from these biopsies were analyzed by tetramer binding. FACS plots are depicted for 2 patients showing CD8<sup>+</sup> DIL binding gp100<sub>154</sub>, gp100<sub>280</sub>, and tyrosinase<sub>369</sub> tetramers. Tetramer-binding T cells were detected in 8 of 11 patients.

into the lymph node (29) despite ultrasound guidance of the injection needle by a highly experienced radiologist. In this study, the success rate for the first injection was >87%. Because subsequent vaccinations are injected in another, often easier accessible and nonpathologic lymph node region, we hypothesize that success rates for subsequent vaccination are higher.

Not all injected DC migrated into the T-cell area. Many DC remained at the site of injection and expression of CD83 was lost. Further analysis revealed that DC had lost viability and were phagocytosed by infiltrating macrophages (37). However, viable DC had migrated into the T-cell areas and expressed the TAA protein encoded by the mRNA inside the lymph nodes of vaccinated patients. We previously showed that of the two TAA mRNA tested, gp100 mRNA resulted in the most stable protein expression (24). Gp100 protein could readily be detected inside the lymph nodes 24 hours after intranodal injection in all four patients studied. No gp100 expression was found in the lymph nodes 48 hours after injection, but thus far, we were able to test merely one patient. *In vitro* tyrosinase protein expression peaked 4 hours after mRNA electroporation (24). Nevertheless, expression of tyrosinase protein by mRNA-DC was found *in situ* in lymph nodes from one of two patients 24 hours after administration. High steady-state levels of a protein are neither a prerequisite nor a guarantee for recognition by CTL, but the rate of protein processing determines recognition (38). Fast degradation or processing of protein might lead to efficient presentation of peptides derived from this protein. Indeed, despite low expression levels, specific T cells against the tyrosinase<sub>369</sub> peptide were detected in biopsies from six of the seven patients with TAA-specific T cells. T cells specific for one or both gp100 epitopes were detected in biopsies of five of seven patients. Altogether, T cells specific for one of the HLA-A2.1 binding peptides were present in DTH biopsies taken after four vaccinations in 7 of 11 patients. However, it is likely that T cells of more specificities are induced, both for class I and class II molecules of different HLA alleles. Because the DC are loaded with mRNA encoding the whole TAA, immune responses will not be restricted to HLA-2.1, but the endogenous processing of the TAA and antigen presentation of multiple peptides in both MHC class I and MHC class II molecules will lead to the induction of a considerably larger

repertoire of TAA-specific T cells. This could be tested by specific IFN $\gamma$  production by DIL stimulated with long overlapping peptides of gp100 or tyrosinase presented by autologous APC or by electroporating these cells with the mRNA encoding these proteins. The broad specificity of T cells will greatly enhance the potency of DC vaccination and may improve the clinical efficacy of the DC immunotherapy. To date, 9 of 11 stage III patients treated are still disease-free, 1 had stable disease but progressed 8.5 months after inclusion in the study, and 1 had progressive disease. However, in patients with stage III melanoma, a follow-up time of more than 7 years is required before any conclusions about clinical responses can be drawn.

Taken together, after intranodal injection in melanoma patients, mRNA-DC are capable of migrating from the injection depot into the T-cell areas of both the injected and subsequent lymph nodes, while expressing the TAA protein encoded by the mRNA-DC. Moreover, mRNA-DC are capable of activating vaccine-related CD8<sup>+</sup> T-cell responses in melanoma patients after vaccination. Thus, electroporation of mRNA encoding TAA is an effective way of loading DC with antigen for vaccination purposes. Clinical results from the ongoing trials in melanoma patients are awaited to address the question of clinical benefit.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## References

- Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;392:245-52.
- Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. *Nat Med* 2004;10:475-80.
- de Vries IJ, Bernsen MR, Lesterhuis WJ, et al. Immunomonitoring tumor-specific T cells in delayed-type hypersensitivity skin biopsies after dendritic cell vaccination correlates with clinical outcome. *J Clin Oncol* 2005;23:5779-87.
- Fay JW, Palucka AK, Pacesny S, et al. Long-term outcomes in patients with metastatic melanoma vaccinated with melanoma peptide-pulsed CD34(+) progenitor-derived dendritic cells. *Cancer Immunol Immunother* 2006;55:1209-18.
- Nestle FO, Aljagid S, Gilliet M, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998;4:328-32.
- Schuler-Thurner B, Schultz ES, Berger TG, et al. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J Exp Med* 2002;195:1279-88.
- Laverman P, de Vries IJ, Scharenborg NM, et al. Development of <sup>111</sup>In-labeled tumor-associated antigen peptides for monitoring dendritic-cell-based vaccination. *Nucl Med Biol* 2006;33:453-8.
- Marshall JL, Hawkins MJ, Tsang KY, et al. Phase I study in cancer patients of a replication-defective avipox recombinant vaccine that expresses human carcinoembryonic antigen. *J Clin Oncol* 1999;17:332-7.
- Pecher G, Haring A, Kaiser L, Thiel E. Mucin gene (MUC1) transfected dendritic cells as vaccine: results of a phase I/II clinical trial. *Cancer Immunol Immunother* 2002;51:669-73.
- Bonehill A, Heirman C, Tuytaerts S, et al. Messenger RNA-electroporated dendritic cells presenting MAGE-A3 simultaneously in HLA class I, class II, molecules. *J Immunol* 2004;172:6649-57.
- Nair SK, Morse M, Boczkowski D, et al. Induction of tumor-specific cytotoxic T lymphocytes in cancer patients by autologous tumor RNA-transfected dendritic cells. *Ann Surg* 2002;235:540-9.
- Saebøe-Larsen S, Fossberg E, Gaudernack G. mRNA-based electrotransfection of human dendritic cells and induction of cytotoxic T lymphocyte responses against the telomerase catalytic subunit (hTERT). *J Immunol Methods* 2002;259:191-203.
- Schaft N, Dorrie J, Thumann P, et al. Generation of an optimized polyvalent monocyte-derived dendritic cell vaccine by transfecting defined RNAs rather than before maturation. *J Immunol* 2005;174:3087-97.
- Su Z, Dannull J, Yang BK, et al. Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses in patients with metastatic prostate cancer. *J Immunol* 2005;174:3798-807.
- Salcedo M, Bercovici N, Taylor R, et al. Vaccination of melanoma patients using dendritic cells loaded with an allogeneic tumor cell lysate. *Cancer Immunol Immunother* 2006;55:819-29.
- Hirschowitz EA, Foody T, Kryscio R, Dickson L, Sturgill J, Yannelli J. Autologous dendritic cell vaccines for non-small-cell lung cancer. *J Clin Oncol* 2004;22:2808-15.
- Lee WC, Wang HC, Hung CF, Huang PF, Lia CR, Chen MF. Vaccination of advanced hepatocellular carcinoma patients with tumor lysate-pulsed dendritic cells: a clinical trial. *J Immunother* 2005;28:496-504.
- Avigan D, Vasir B, Gong J, et al. Fusion cell vaccination of patients with metastatic breast and renal cancer induces immunological and clinical responses. *Clin Cancer Res* 2004;10:4699-708.

19. Wei Y, Sticca RP, Holmes LM, et al. Dendritoma vaccination combined with low dose interleukin-2 in metastatic melanoma patients induced immunological and clinical responses. *Int J Oncol* 2006;28:585-93.
20. Grunebach F, Muller MR, Brossart P. New developments in dendritic cell-based vaccinations: RNA translated into clinics. *Cancer Immunol Immunother* 2005;54:517-25.
21. Ponsaerts P, Van Tendeloo VF, Berneman ZN. Cancer immunotherapy using RNA-loaded dendritic cells. *Clin Exp Immunol* 2003;134:378-84.
22. Grunebach F, Muller MR, Nencioni A, Brossart P. Delivery of tumor-derived RNA for the induction of cytotoxic T-lymphocytes. *Gene Ther* 2003;10:367-74.
23. Tuyaerts S, Michiels A, Corthals J, et al. Induction of Influenza Matrix Protein 1 and MelanA-specific T lymphocytes *in vitro* using mRNA-electroporated dendritic cells. *Cancer Gene Ther* 2003;10:696-706.
24. Schuurhuis DH, Lesterhuis WJ, Kramer M, et al. Polyinosinic polycytidylic acid prevents efficient antigen expression after mRNA electroporation of clinical grade dendritic cells. *Cancer Immunol Immunother*. Epub 2008 Nov 19.
25. Adema GJ, de Vries IJ, Punt CJ, Figdor CG. Migration of dendritic cell based cancer vaccines: *in vivo* veritas? *Curr Opin Immunol* 2005;17:170-4.
26. Balch CM, Buzaid AC, Soong SJ, et al. Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol* 2001;19:3635-48.
27. de Vries IJ, Lesterhuis WJ, Scharenborg NM, et al. Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. *Clin Cancer Res* 2003;9:5091-100.
28. Berger TG, Strasser E, Smith R, et al. Efficient elutriation of monocytes within a closed system (Elutra) for clinical-scale generation of dendritic cells. *J Immunol Methods* 2005;298:61-72.
29. de Vries IJ, Lesterhuis WJ, Barentsz JO, et al. Magnetic resonance tracking of dendritic cells in melanoma patients for monitoring of cellular therapy. *Nat Biotechnol* 2005;23:1407-13.
30. de Vries IJ, Eggert AA, Scharenborg NM, et al. Phenotypical and functional characterization of clinical grade dendritic cells. *J Immunother* 2002;25:429-38.
31. Thurner B, Haendle I, Roder C, et al. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999;190:1669-78.
32. de Vries IJ, Kroochoop DJ, Scharenborg NM, et al. Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. *Cancer Res* 2003;63:12-7.
33. Kroochoop DJ, Torensma R, van den Bosch GJ, et al. An automated multi well cell track system to study leukocyte migration. *J Immunol Methods* 2003;280:89-102.
34. Boullart AC, Aarntzen EH, Verdijk P, et al. Maturation of monocyte-derived dendritic cells with Toll-like receptor 3 and 7/8 ligands combined with prostaglandin E2 results in high interleukin-12 production and cell migration. *Cancer Immunol Immunother* 2008;57:1589-97.
35. Verdijk P, Scheenen TW, Lesterhuis WJ, et al. Sensitivity of magnetic resonance imaging of dendritic cells for *in vivo* tracking of cellular cancer vaccines. *Int J Cancer* 2007;120:978-84.
36. Michiels A, Tuyaerts S, Bonehill A, et al. Electroporation of immature and mature dendritic cells: implications for dendritic cell-based vaccines. *Gene Ther* 2005;12:772-82.
37. Verdijk P, Aarntzen EMJG, Lesterhuis WJ, et al. Limited amounts of dendritic cells migrate into the T cell area of lymph nodes but have high immune activating potential in melanoma patients. *Clin Cancer Res*. In press 2009.
38. Vierboom MP, Zwaveling S, Bos GMJ, et al. High steady-state levels of p53 are not a prerequisite for tumor eradication by wild-type p53-specific cytotoxic T lymphocytes. *Cancer Res* 2000;60:5508-13.