Immune response and long-term clinical outcome in advanced melanoma patients vaccinated with tumor-mRNA-transfected dendritic cells

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ABSTRACT
The most effective anticancer immune responses are probably directed against patient-specific neoantigens. We have developed a melanoma vaccine targeting this individual mutanome based on dendritic cells (DCs) loaded with autologous tumor-mRNA. Here, we report a phase I/II trial evaluating toxicity, immune response and clinical outcome in 31 metastatic melanoma patients. The first cohort \((n = 22)\) received the vaccine without any adjuvant; the next cohort \((n = 9)\) received adjuvant IL2. Each subject received four weekly intranodal or intradermal injections, followed by optional monthly vaccines. Immune response was evaluated by delayed-type hypersensitivity (DTH), T cell proliferation and cytokine assays. Data were collected for 10 y after inclusion of the last patient. No serious adverse events were detected. In the intention-to-treat-cohort, we demonstrated significantly superior survival compared to matched controls from a benchmark meta-analysis \((1 \text{ y survival } 43\% \text{ vs. } 24\%, 2 \text{ y } 23\% \text{ vs. } 6.6\%)\). A tumor-specific immune response was demonstrated in 16/31 patients. The response rate was higher after intradermal than intranodal vaccination \((80\% \text{ vs. } 38\%)\). Immune responders had improved survival compared to non-responders \((\text{median } 14 \text{ mo vs. } 6 \text{ mo; } p = 0.030)\), and all eight patients surviving \(>20\) mo were immune responders. In addition to the tumor-specific response, most patients developed a response against autologous DC antigens. The cytokine profile was polyfunctional and did not follow a Th1/Th2 dichotomy. We conclude that the favorable safety profile and evidence of a possible survival benefit warrant further studies of the RNA/DC vaccine. The vaccine appears insufficient as monotherapy, but there is a strong rationale for combination with checkpoint modulators.

Introduction
Malignant melanoma is among the leading causes of cancer-related death worldwide, and the incidence has increased considerably over the last decades. The vaccine trial reported herein was conducted in 2002–2006. At this time, the median survival for patients with visceral metastases was only 6–9 mo and the 5 y survival was about 5%.\textsuperscript{1,2} No chemotherapeutic drugs have shown a survival benefit for melanoma. In 2010, ipilimumab (anti-CTLA4) emerged as the first drug to improve survival, albeit to a moderate extent.\textsuperscript{3} More recently, the PD1 checkpoint inhibitors have produced remarkable clinical responses\textsuperscript{4-6} and highlighted the potential of immunotherapy.

We investigate melanoma vaccine therapy with dendritic cells (DCs) transfected with tumor-mRNA.\textsuperscript{7} The vaccine makes use of autologous tumor material as source of mRNA, thereby targeting the individual spectrum of tumor antigens in each patient, including the neoantigens. There is increasing evidence, suggesting that the most effective anticancer immune responses are directed against patient-specific neoantigens, often termed the mutanome.\textsuperscript{8-10} The breakthrough in melanoma therapy has arrived from checkpoint inhibitors and adoptive T-cell therapy with tumor-infiltrating lymphocytes.\textsuperscript{11,12} Both these approaches can initiate immune responses against a wide range of patient-specific antigens. Even with vaccines targeting defined antigens, there is evidence that clinical efficacy may depend on epitope spreading rather than a response against the vaccine antigen.\textsuperscript{13-15} In 2015, T-VEC became the first melanoma vaccine to be approved by the FDA and the EMA. Interestingly, even T-VEC is hypothesized to initiate a broad immune response against the individual mutanome.\textsuperscript{16}

The study reported herein was a first-in man trial in patients with advanced melanoma (NCT01278940), where the first cohort of 22 patients (DCM-1) received DC vaccine without any adjuvant. The protocol was amended to include a second cohort of nine patients (DCM-2) that received vaccination with adjuvant IL2. The standard immunomonitoring and short-term clinical outcome of the first cohort (DCM-1) has been reported previously.\textsuperscript{17} In this cohort, we observed no serious adverse effects and demonstrated a vaccine-specific immune response in 9/19 patients evaluated by T cell assays (T cell proliferation/IFN\textgamma/ELISPOT).\textsuperscript{17} Both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were recruited.\textsuperscript{18} Below, we report the results from the amendment cohort that
received adjuvant IL-2. Further, we have completed the data collection 10 y after the inclusion of the last patient and report long-term follow-up and survival data for both patient cohorts (DCM-1 and DCM-2). We also compare response rates after intradermal and intranodal vaccination and report studies of immunological reactivity that developed against DC antigens not included in the vaccine-mRNA.

Results

Patient characteristics

The first vaccine cohort DCM-1 included 22 patients (M01-M22; Table 1), and the subsequent DCM-2 cohort with adjuvant IL-2 included nine patients (M101-M109; Table 1). The majority had advanced stage IV disease at study entry with visceral involvement. Taking both cohorts together, 21/31 subjects belonged to metastasis category M1C (Table 1).

Vaccine production and feasibility

The good manufacturing practice (GMP) production of autologous tumor-mRNA and DCs was successful for all 34 patients that underwent leukapheresis. Three of these patients could not enter the vaccine study because of rapid disease progression. The DCM-1 production data has been reported previously.17 Vaccine production data for DCM-2 (n = 9) is given in Table 2. In DCM-2, the median yield of vaccine DCs was 2.6 \times 10^8 \text{ (range 7 \times 10^7–3.6 \times 10^8)}, and the median number of vaccine vials was 16 (range 7–18). The viability was >90% for all vaccine products (median 97%). The DC phenotype was analyzed before and after the 48 h maturation step, i.e., at day 5 (not shown) and at day 7 (Table 2). The results were consistent with DC maturation, including increased expression of CD83, CD208, CCR7, HLA-DR and CD86. There was no substantial difference in the phenotype or viability of mRNA-transfected DCs (tDCs) compared to non-transfected DCs (nDCs),

Table 1. Metastatic category, immune response and clinical outcome.

| Patient | Stage IV category | Injection site | tDC - nDC mm | tDC-specific | T-cell assay & DTH | Immune response | Tumor response | Survival months |
|---------|-------------------|---------------|--------------|-------------|------------------|----------------|---------------|----------------|
| DCM-1 (without IL-2) | | | | | | | | |
| M01 M1A | id | 50 | + | nd | + | NE (NED) | 85 | |
| M02 M1C | id | 8 | (-) | + | + | PD | 3 | |
| M03 M1C | id | 45 | + | + | + | SD (MR) | 23 | |
| M04 M1C | id | 55 | + | + | + | PD | 11 | |
| M05 Stage III | id | 35 | + | + | + | NE (NED) | 37 | |
| M06 M1C | in | nd | nd | - | - | PD | 8 | |
| M07 M1C | in | 5 | (+) | + | + | PD | 5 | |
| M08 M1B | in | <5 | - | nd | - | PD | 17 | |
| M09 M1C | in | <5 | - | + | + | PD | 15 | |
| M10 M1C | in | nd | nd | - | - | PD | 2 | |
| M11 M1C | in | nd | nd | - | + | PD | 3 | |
| M12 M1C | in | <5 | - | - | - | SD | 15 | |
| M13 M1C | in | <5 | - | - | - | PD | 6 | |
| M14 M1C | in | <5 | - | - | - | PD | 3 | |
| M15 M1C | in | 5 | (+) | nd | nc | PD | 6 | |
| M16 M1C | in | nd | nd | - | + | PD | 4 | |
| M17 M1C | in | <5 | - | - | - | PD | 10 | |
| M18 M1C | id | <5 | - | + | + | PD | 10 | |
| M19 M1B | id | 30 | + | + | + | PD | 30 | |
| M20 M1C | id | <5 | - | - | - | PD | 6 | |
| M21 M1A | id | <5 | - | - | - | PD | 16 | |
| M22 M1A | id | 5 | (+) | + | + | PD | 140 | |
| DCM-2 (with IL-2) | | | | | | | | |
| M101 M1C | in | <5 | - | - | - | PD | 13 | |
| M102 M1B | in | <5 | - | + | + | PD | 41 | |
| M103 M1B | in | <5 | - | + | + | PD | 30 | |
| M104 M1C | in | 5 | (+) | nc | PD | 5 | |
| M105 M1C | in | <5 | - | - | - | PD | 1.3 | |
| M106 M1B | in | <5 | - | - | - | SD | 18 | |
| M107 M1C | in | 5 | (+) | - | nc | PD | 3 | |
| M108 M1C | in | <5 | - | + | + | PD | 9 | |
| M109 M1B | in | 33 | + | + | + | PR | 121 | |

1Disease stage and metastasis category at study entry according to AJCC criteria. All patients except M05 were stage IV. M1A: Lymph node/subcutaneous metastasis. M1B: Lung metastasis. M1C: Visceral metastasis or elevated LDH.

2id = intradermal; in = intranodal; nd = not done.

3DTH: tDCs DTH to DCs after fourth vaccination, nDCs DTH to nDC controls after fourth vaccination.

4+: tDC-nDC = 10 mm; (+) tDC-nDC = 5–10 mm; − tDC-nDC = 5 mm.

5Moderate tDC-specific T-cell response in pre-vaccination samples, considerably increased in post-vaccination samples.

6tDC-specific response in pre-vaccination samples, unchanged in post-vaccination samples.

7tDC-specific response in follow-up samples (initial pre- and post-vaccination tests were negative).

8Combined immune response (T-cell assay & DTH). Patients M15, M104 and M107 were considered not conclusive (nc) because the DTH was weakly positive, and the T-cell assay negative or not done.

9Tumor response: PD, progressive disease; SD, stable disease; NE, not evaluable; NED, no evidence of disease.

10Survival: Months from first vaccination to Dec 2015 (+) to or death.
which were used as controls in the immunomonitoring (data not shown).

Safety

No treatment related grade III and IV toxicity was observed. This applied both to the DCM-1 cohort and to the DCM-2 cohort receiving IL-2 as local adjuvant. Some patients developed mild flu-like symptoms, pain in tumor or inflammatory reactions at the injection site (CTC grade I and II). One patient developed vitiligo (CTC grade I). Continued booster vaccination, as given to three subjects in DCM-2 (maximum 13 vaccines over 10 mo), did not produce increased toxicity. We have continued to monitor the long-term survivors, after end of study treatment. The follow-up has not revealed any long-term toxicity (maximum observation time 11.7 y).

Ultrasound monitoring of lymph nodes after intranodal vaccination

Twelve patients in cohort DCM-1 and nine patients in cohort DCM-2 received the vaccines by intranodal injection (Table 1). At each intranodal vaccination, the receiving lymph node was evaluated by ultrasound.\(^1\) We found that the lymph node macro-architecture remained well preserved in all patients through the vaccination period. The external capsule, the cortex/paracortex-region and the medulla appeared intact. Furthermore, we observed an increase in the width of the cortex/paracortex-region (i.e., the lymphocyte area) in most patients after vaccination. This applied both to initial the DCM-1 group\(^1\) and to the DCM-2 cohort receiving local IL-2 (data not shown).

Induction of vaccine-specific immune responses

T cells from pre- and post-vaccination samples were tested in proliferation assays for response to stimulation with tDCs or nDC controls. The proliferation assays were performed for all patients subject to availability of cells, i.e., 19/22 patients in DCM-1 and 9/9 subjects in DCM-2. To determine if a T-cell response was tDC-specific, we compared the T-cell proliferation counts elicited by tDCs, by nDCs and by T cells only. A response was considered tDC-specific only if the proliferation upon stimulation with tDCs was significantly higher (\(p < 0.05\)) than in the controls (ANOVA, Student Newman Keuls (SNK) test). The analysis demonstrated a tDC-specific T-cell proliferation response in 11/19 patients in cohort DCM-1, and in four out of nine subjects in cohort DCM-2 (Fig. 1; Table 1). In these 15 subjects, we conclude that a component of the responding T cells was specific for antigens encoded by the transfected tumor-mRNA.

The delayed-type hypersensitivity (DTH) results are shown in Table 1. In six patients, we detected a tDC-specific, vaccine-generated DTH reaction that was considered to be moderately/strongly positive, according to the criteria outlined below (Methods) and previously applied for cohort DCM-1.\(^1\) In six more patients, we measured vaccine-generated DTH reactions considered to be weakly positive. Twenty-four patients were evaluated both by DTH recordings and \textit{in vitro} T-cell assays (Table 1). Among these, all five subjects with a moderately/strongly positive DTH reaction also had a tDC-specific response in the T-cell assays, while three out of five with a weakly positive DTH-reaction tested positive \textit{in vitro}. 10 out of 14 DTH negative patients tested negative also \textit{in vitro}. In all, the results thus indicated a correlation between the DTH and \textit{in vitro} responses, which was most clear for subjects with a moderately/strongly positive DTH reaction. Based on both the \textit{in vitro} T-cell assays and the DTH-reactions, 16 out of 31 patients were considered as immune responders, 12 out of 31 as non-responders and 3 out of 31 as not conclusive (Table 1).

Two patients (M09 and M108) exhibited a substantial tDC-specific response both in pre-vaccination and post-vaccination samples, consistent with spontaneous reactivity against tumor antigens included in the vaccine. These patients were not classified as vaccine responders. However, their pre-vaccination responses indicate that the vaccine includes antigens that are naturally presented and relevant to the antitumor immune response.

Long-term development of immune responses

Clinical efficacy is likely to depend on durable immune responses. In cohort DCM-1, week-13 peripheral blood

Table 2. DCM-2 patients and vaccine characteristics.

| Age (years) | Sex | CD83\(^3\) | CD14 | CCR7 | HLA-DR | CD86 | DCs/vial\(^2\) | Viability\(^3\) | DC vials produced\(^4\) | # Vaccines\(^5\) |
|------------|-----|------------|------|------|--------|------|----------------|------------|------------------|----------------|
| M101       | 55  | Male       | 52%  | 0%   | 57%    | 58%  | nt            | 2.0        | 96               | 15             | 5               |
| M102       | 58  | Female     | 77%  | 0%   | 63%    | 77%  | nt            | 2.0        | 98               | 9              | 5               |
| M103       | 66  | Male       | 66%  | 0%   | 63%    | 78%  | nt            | 1.0        | 95               | 7              | 5 + 1 boost     |
| M104       | 35  | Female     | 66%  | 0%   | 53%    | 70%  | nt            | 69%        | 96               | 18             | 5               |
| M105       | 76  | Male       | 54%  | 0%   | 48%    | 62%  | nt            | 56%        | 90               | 18             | 5               |
| M106       | 55  | Male       | 87%  | 0%   | 48%    | 92%  | nt            | 88%        | 99               | 17             | 5 + 3 boosts    |
| M107       | 74  | Male       | 88%  | 0%   | 60%    | 90%  | 91%           | 2.0        | 96               | 18             | 5               |
| M108       | 55  | Female     | 84%  | 0%   | 55%    | 86%  | 85%           | 2.0        | 98               | 12             | 5               |
| M109       | 60  | Male       | 63%  | 4%   | 38%    | 71%  | 71%           | 2.0        | 97               | 13             | 5 + 8 boosts    |

\(^1\)DCs were analyzed by flow cytometry for expression of maturation markers. The data represent mRNA-transfected DCs.

\(^2\)Number of DCs per vial. One vial was used for each vaccine injection.

\(^3\)Viability in DC vaccine batches after freezing/thawing, assessed by Trypan blue staining.

\(^4\)Number of DC vaccine vials produced for each patient.

\(^5\)Number of vaccines administered per patient.
mononuclear cells (PBMCs) were available from six patients in which a tDC-specific T-cell response had been demonstrated. In four out of these six patients, a significant tDC-specific response was still detected at week 13.17 In cohort DCM-2, all four subjects with a response at week 6 exhibited retained T-cell response at the later time points measured (Fig. 1).

Patients M22 and M109 are alive to date, 11.7 and 10 y after start of vaccination. The first PBMC-test for patient M22 was negative, but a positive DTH reaction was observed. After 3 y, we obtained new PBMCs and demonstrated a tDC-specific T-cell response ($p < 0.05$; ANOVA/SNK-test). In patient M109, availability of tDCs/nDCs allowed for repeated testing of samples obtained at nine consecutive time points. A de novo tDC-specific response developed after vaccination. Long-term follow-up demonstrated a durable tDC-specific response, observed at all seven time points tested from week 10 onwards (Fig. 1).

**Vaccine response against antigens not encoded by the transfected mRNA**

The majority of patients developed T cell responses not only against tDCs, but also against nDCs after vaccination. Fig. 2 shows responses in freshly thawed T cells against nDCs for the patients in the DCM-2-cohort. As shown, all subjects
except M107 developed substantial and increased nDC-reactivity after vaccination. An increased nDC reactivity was also observed in the DCM-1 cohort (data not shown).

The nDC-responsive T-cell clones are activated in the same lymph nodes as the tDC-specific clones and are likely to modulate the vaccine response. We addressed this issue by characterizing the cytokine profile of 12 nDC-reactive T-cell clones from patient M03 (Table 3), established by limiting dilution. The results showed that all clones secreted multiple cytokines, consistent with polyfunctionality at the clonal level. Most clones secreted substantial amounts of key Th1 cytokines IFNγ and TNFα, with a high IFNγ/IL-4 ratio. However, the cytokine profiles did not follow a Th1/Th2-dichotomy, as high levels of the Th2-cytokines IL-5 and IL-13 were also detected. The levels of cytokine IL-10, associated with T regulatory cells, were highly variable between the T cell clones. The IFNγ/IL-10 ratios ranged from 0.1 to 270 and suggested that some nDC-responsive clones (# 48, 55, 67 and 101) had a regulatory T-cell cytokine pattern, while other clones exhibited an effector profile (# 8, 10, 12 and 125). Chemokine MIP-1b was secreted at high levels by all clones. Overall, the cytokine profiles resembled those previously observed for tDC-specific T-cell clones from the same patient.18

We further investigated whether the nDC-reactive clones recognized autologous antigens in the DCs or foreign antigens present during ex vivo culture. The nDC-reactive T cells were screened against the GMP DC culture medium and against the various cytokines supplemented during DC culture. No signs of reactivity were detected, though the T cells remained reactive against nDCs (data not shown). The results thus supported the hypothesis that the nDC-responses may represent an autologous mixed lymphocyte...
reaction (AMLR).\textsuperscript{19} We also performed a series of experiments showing that both the tDC-specific and the nDC-reactive T-cell responses could be blocked with antibodies against HLA (data not shown).

**Intranodal versus intradermal vaccination**

Previous studies have indicated that less than 5% of DCs migrate to regional lymph nodes after intradermal DC vaccination.\textsuperscript{20} Here, we compared intranodal and intradermal vaccine injection. The comparison was conducted in the melanoma DC-vaccine trial and a parallel trial in metastatic prostate cancer patients.\textsuperscript{21} Both trials evaluated vaccination with autologous DCs transfected with tumor-mRNA and applied the same vaccination schedule.

We compared the immune response rate after intradermal (i.d.) and intranodal (i.n.) vaccination, in terms of vaccine-generated T-cell-specific responses. Two melanoma subjects (M09 and M108) were excluded from the analysis because they exhibited post-vaccine T-cell responses that could not be classified as vaccine-generated, after comparison with pre-vaccine samples (Table 1). Surprisingly, the results demonstrated a higher response rate after i.d. vaccination (Fig. 3). In the melanoma trial, we observed immune responses in 80% of patients vaccinated i.d. compared to 38% after i.n. vaccination (Fig. 3A). The immune response rates for prostate cancer patients were 80% and 50% after i.d. and i.n. vaccination, respectively.\textsuperscript{21} The overall immune response rate in both trials taken together was 80% (16/20) after i.d. vaccination, compared to 42% (11/26 subjects) in the i.n. vaccine group (Fig. 3B). This difference was statistically significant (Fisher exact test, \(p = 0.016\)). We also performed a secondary statistical analysis including all subjects evaluable for post-vaccination responses, regardless of their pre-vaccine reactivity. The results showed that the i.d. response rate remained superior (80% vs. 46%; \(p = 0.035\), Fisher exact test).

To investigate whether the observed difference may be explained by other factors than the vaccination method, we performed logistic regression analyses. There was a considerable variation in the expression of DC-maturation marker CD83 between patients. However, the regression analyses indicated that this factor did not influence the immune response rate (hazard ratio (HR) 1.0). We further analyzed the effect of i.d. versus i.n. vaccination after adjusting for cancer form and disease dissemination, classified into three categories (melanoma: M1a, M1b, M1c; prostate cancer: PSA <20, 20–80, >80). The intradermal vaccination method remained superior with an adjusted HR of 5.0 (\(p = 0.041\)) for melanoma and prostate cancer taken together. The adjusted HR was 11.7 (\(p = 0.053\)) and 3.2 (\(p = 0.29\)) for melanoma and prostate cancer, respectively.

**Clinical follow-up of long-term survivors**

Two patients are alive to date, patient M22 and M109. Patient M22 received six vaccines over a period of 5 mo. She developed a tDC-specific immune response (Table 1). The patient had stage IV M1a disease at study entry, with multiple subcutaneous metastases located at the back, thorax and the left leg. She developed new subcutaneous lesions after start of vaccination, i.e., progressive disease (PD) according to response evaluation criteria in solid tumors (RECIST). However, only small subcutaneous lesions appeared, and other lesions disappeared. CT scans revealed no involvement of lymph nodes or visceral organs, until a 1 cm suprarenal lesion appeared after 2.5 y. She remained in a good general condition. Three years after start of DC vaccination, patient M22 started therapy with ipilimumab. The patient achieved complete clinical response that was confirmed by a negative PET-CT scan. She continued ipilimumab for 4 y. The patient has since been observed without treatment and has maintained a complete response, 11.7 y after start of DC vaccination and 8.7 y after start of ipilimumab.

Patient M109 received a total of 14 vaccine injections over 10 mo. The vaccination induced a durable tDC-specific response, detectable in all T-cell assays from week 10 onwards (Fig. 1). DTH recordings also demonstrated a tDC-specific response, with an erythema diameter of 40 mm for tDCs, compared to 7 mm for nDC controls. At study entry, M109 had multiple metastases involving lymph nodes and both lungs (Fig. 4). Three out of six lesions were >10 mm and thus defined as target lesions. After vaccination, he developed complete regression of all six pre-study lesions (Fig. 4B). Interestingly, the response developed gradually over a long-time frame with lymph node lesions only.

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**Figure 3.** Intranodal versus intradermal vaccination. The patients in the melanoma and prostate cancer DC-vaccination trials were allocated to intradermal (i.d.) or intranodal (i.n.) vaccine injection. The figure shows the percentage of immune responders and non-responders in the i.d. and i.n. vaccination groups, for the melanoma patients (A) and for the melanoma and prostate cancer trials taken together (B). The response rates after i.d. and i.n. vaccination were compared by use of Fishers exact test, two-sided \(p\)-values are displayed.
starting to regress substantially after month 6 (Fig. 4B). At month 9, the patient developed a new pararenal lesion, defining PD according to RECIST. However, he maintained the clinical response in other regions. According to immune-related response criteria (irRC), he had a partial response for 4.5 y. Thereafter, a gradual growth of the pararenal lesion and abdominal lymph nodes developed, and the patient then (2010) received four injections of ipilimumab (anti-CTLA4).

In 2012, the pararenal lesion and abdominal lymph nodes were removed by surgery. The patient has later developed limited disease in the thorax. He started treatment with pembrolizumab (anti-PD1) in 2015 and remains in good general health, 10 y after start of DC vaccination.

**Survival of study subjects compared to matched controls from meta-analysis**

Table 1 lists overall survival (OS) from start of vaccination. Taking both cohorts together (n = 31), median OS was 10 mo by intention-to-treat analysis (range: 1–140+ mo). The median OS was 10 mo in the first cohort, and 13 mo in the IL-2 cohort. Only two out of the 31 study subjects, M22 and M109, received treatment with agents known to improve survival in metastatic melanoma patients. As described above, these two patients started treatment with ipilimumab 3 and 5 y after vaccination, respectively.

Patients included in clinical trials may differ from other patients due to selection processes. Korn et al. have conducted a meta-analysis of 2100 trial patients and suggested that survival in phase I/II melanoma studies is compared with their data set. They also outlined a method for calculating predicted survival. We applied this method on our cohort, as intention-to-treat analysis. Briefly, prognostic factors (sex, ECOG status, metastatic category) from our patients were inserted into a formula generating predicted survival. The calculation was based on trials where brain metastases were not excluded, because we did not perform screening for brain involvement. A 95% confidence interval was calculated for the predicted survival. The analysis suggested a difference in favor of the RNA/DC-vaccine treatment (p = 0.036).

**Figure 4.** Tumor response in patient M109. (A) Total diameter of all measurable lesions (corresponding to RECIST) at time points for CT evaluation. Three lesions were >10 mm at study entry and thus defined as target lesions. (B) Response of individual lesions at different time points (mo = months). The six measurable pre-study lesions all completely regressed. The three target lesions (>10 mm at study entry) are shown in the top half of the diagram. A new pararenal lesion (NL) appeared at month 9. (C) CT scans before and after vaccination, showing complete regression of the three target lesions defined at study entry. The displayed post-vaccination scans are from month 3 (subpleural lesion) or month 12 (lymph node I and II).

**Figure 5.** (A) Survival in study patients compared to benchmark meta-analysis. Blue line: Overall survival for all enrolled study patients calculated from study entry. Dashed lines represent the 90% confidence interval (CI). Green line: Predicted survival for our patients (fixed estimate), calculated from Korn’s meta-analysis. The calculation corrects for prognostic factors (performance status, site of metastases etc.) and was performed as recommended by Korn et al. The analysis suggested a difference in favor of the RNA/DC-vaccine treatment (p = 0.036). (B) Survival versus immune response. Kaplan–Meier analysis comparing overall survival between patients with/without a vaccine-specific T-cell response. The analysis suggested a difference in favor of the immune responders (log rank test; p = 0.030).
confidence interval was generated for survival of the study group, while the predicted survival curve was regarded as a fixed estimate (Fig. 5A).

The analysis demonstrated a survival advantage for our DC-vaccine cohort with the following estimates: 1 y survival 43% versus 24%, 2 y 23% versus 6.6%, 3 y 13% versus 3.5%. Korn et al. suggested declaring a treatment worthy of further study if the 1-y survival was better than predicted, with a p value less than 0.10. This criterion was met (p = 0.036).

**Immune response was associated with prolonged survival**

We compared survival (OS) in immune-responders and non-responders. The analysis demonstrated that OS was significantly increased in immune responders compared to non-responders (p = 0.030; log-rank test; Fig. 5B), with a median OS of 14 mo versus 6 mo. To investigate whether the immune response represented an independent prognostic factor, we performed Cox-regression analyses. When not adjusting for other factors, Cox-regression indicated an HR of 2.8 for non-responders compared to immune responders. The metastatic category (M1a/M1b/M1c) and ECOG performance status represented the most recognized prognostic factors. After adjusting for these factors, the immune response retained an HR of 2.7.

The survival difference between immunological vaccine responders and non-responders was particularly evident within the tail of the survival curve, i.e., the subpopulation with substantially extended survival. Interestingly, the eight patients with survival >20 mo all belonged to the immune responders (Fig. 5B and Table 1).

**Discussion**

The described RNA/DC vaccine was designed to target a wide spectrum of antigens in each individual patient’s tumor, including the neoantigens. We found that about 50% of patients developed a T-cell response specific for the vaccine antigens and that this immune response was associated with improved survival. The long-term follow-up demonstrated durable immune responses and showed that the vaccine is safe and well tolerated. The benign safety profile is of particular importance for this vaccine concept, as a wide range of unknown antigens are included in the vaccine.

The reported group of patients survived longer than expected, compared to predicted survival from Korn’s benchmark meta-analysis. This finding should be interpreted with utmost caution, but is of interest, in particular as the “controls” had been selected for clinical trials and displayed matched performance status and disease characteristics. Further, the controls had received potentially active treatment. We calculated survival from study entry, while Korn’s meta-analysis gives survival from a possibly earlier point (time of registration). The survival advantage may thus be larger than estimated. The emergence of checkpoint inhibitor therapy means that the data from Korn’s meta-analysis are not representative for currently treated patients. Our DC-vaccine trial, however, was conducted before checkpoint inhibitors were available. The subsequent administration of ipilimumab to patient M22 and M109 may have prolonged the survival of these individuals. However, as only two patients received checkpoint inhibitors, and none of them received it until several years after vaccination, this therapy cannot substantially have influenced the survival outcome for the study group. We thus consider that the survival data in the report from Korn et al. is relevant to our study cohort. The interpretation of our findings is still complex. The apparent survival benefit from DC vaccination needs to be evaluated and reproduced with a randomized control group. It has been hypothesized that cancer vaccines may be more efficient in patients with less tumor burden. Interestingly, Bol et al. have reported favorable survival in stage III melanoma patients receiving adjuvant DC vaccination after radical lymph node dissection, compared to matched controls. This finding also needs to be validated in a randomized trial.

There is no established standard assay for readout of immune responses in cancer vaccine trials. In our protocol, we applied both in vitro T-cell assays and DTH recordings. The results indicated a correlation between the DTH recordings and the T-cell assays, though not a perfect concordance. DTH represents an in vivo parameter, but is difficult to measure accurately. There is a need not only to standardize the methods for measuring vaccine responses, but also to discriminate between effective and pointless immune activation. This aim is difficult to achieve until cancer vaccines with documented clinical activity have been developed. The long-term survivors in the present protocol had durable T-cell memory responses. We have also observed memory responses among survivors in other vaccine studies, including a melanoma trial were all 10 patients with a durable immune response survived longer than those rapidly losing their immune response. Others have reported from DC melanoma vaccination, the generation of memory CD8+ T cells lasting up to 10 y and with a polyfunctional cytokine profile associated with long-term survival.

The vaccine induced a considerable nDC reactivity that tended to obscure the tDC-specific response. This two-component response reflected the activation of two distinct sets of T-cell clones. As previously reported, some clones responded exclusively to stimulation by tDCs, i.e., to antigens encoded by the transfected tumor-mRNA. The nature of the antigens recognized by tDC-specific T cells is not known. The transfected mRNA may encode both antigens from tumor cells and antigens expressed by stromal, endothelial or infiltrating cells. Some stromal antigens may represent appropriate vaccine targets. Importantly, the antigens recognized were expressed in the tumor of each individual patient, at least at the mRNA level.

The reactivity against nDCs may represent an AMLR, known to be readily elicited by mature DCs. Considering the evident nDC-reactivity, it is worth noting that the long-term follow-up only uncovered autoimmune side effects in one patient (vitiligo). We found that the nDC-reactive T-cell clones produced a wide range of cytokines. This observation suggests that the nDC-response may exert an important regulatory influence on the tDC-specific response, in particular as the both sets of T-cell clones will be activated in the same lymph nodes. The cytokine profile did not follow a Th1/Th2-diochotomy. This is in line with our previous observation of mixed Th1/Th2 patterns for vaccine-specific responses in several trials, across different cancer forms and vaccine strategies.
The Th1/Th2 paradigm was established based on elegant studies in mouse models.\textsuperscript{28} Many vaccine trials employ INF\textgamma/IL4 assays to determine a Th1 or Th2 profile. Our data suggest that this practice may be misleading, as an INF\textgamma/IL-14 response may include high levels of other Th2-cytokines (IL-5 and IL-13) as well as IL-10. We have observed this both in T-cell bulk cultures and in T-cell clones. These observations suggest that it may be better to avoid Th1/Th2-lables, and rather focus on polyfunctionality or the ratio between key cytokines, like INF\textgamma/IL-10.

Most reports from previous DC-vaccine trials have not described any nDC reactivity, while a few studies have uncovered considerable nDC-responses.\textsuperscript{29} This apparent contradiction may be due to the chosen experimental settings. Several important studies have not included nDC controls,\textsuperscript{30-33} while other reports focus on readout assays without DC controls, though nDC-reactivity has been observed in DTH-reactions.\textsuperscript{34} Furthermore, some studies only include cytotoxicity assays that may not reveal an AMLR.\textsuperscript{35} Finally, several early trials made use of immature DCs,\textsuperscript{36-38} while DC maturation is considered to be important for the initiation of AMLR.

We compared i.d. and i.n. DC vaccine administration. Only a limited number of studies have compared T-cell responses after different routes of DC vaccination, and the overall interpretation is not clear.\textsuperscript{14,20,39-43} We found that the immune response rate was higher in the i.d. group, in both the melanoma and prostate cancer trials. It is conceivable that confounding factors have affected the immune response rates. Several factors may, however, favor i.d. vaccination. First, the lymph node micro-architecture might be disrupted by i.n. injections, though the ultrasound monitoring indicated retained macro-architecture. Second, a high number of DCs were used per batch for injection, so that even a small fraction of successfully migrating DCs may be sufficient. Third, the in vitro DC maturation may not result in functionally optimal cells, and the DCs injected i.d. may receive further maturation signals during migration. Finally, the successfully migrating DCs after i.d. vaccination will be a selected DC population, whereas the functionally immature DCs reaching the lymph node after i.n. injection may not optimally stimulate a vaccine response. Our finding is not necessarily relevant for other DC vaccination protocols. Probably, the injection method should be optimized for each protocol, as DC migration and functionality will be influenced by the in vitro milieu and the expression of chemokine receptors on the DCs. The tumor-mRNA was obtained from one metastatic lesion in each patient. This may be a concern with regard to tumor heterogeneity. One may though note that recent data from MacGranahan et al. suggest that the most efficient anticancer responses are directed against clonal neoantigens present in all lesions.\textsuperscript{10} The mRNA amount from each patient varied depending on the tumor size. We used all tumor-mRNA available from each patient for vaccine production, provided that the mRNA passed the quality control. Furthermore, in patients were high numbers of DCs were generated, we made additional vaccine batches to allow for booster vaccination. This meant that the DC and mRNA concentration at electroporation varied considerably between the patients, depending on the mRNA and DC yield. Patient M08 had the lowest mRNA concentration, 8 µg/mL, among those developing a tDC-specific immune response. Based on this observation, we have set an mRNA concentration of 10 µg/mL as minimum requirement in subsequent trials. Furthermore, we have in later quality control studies observed that high DC-concentrations may impair the transfection efficiency and decided on a maximum DC concentration at electroporation. In the patient cohorts reported above, these limits had not been established. It is hence possible that some of the subjects received less efficiently transfected DCs.

The RNA/DC-vaccine platform described herein is in principle applicable to any cancer form. At our institution, we have used the platform for developing vaccines against glioblastoma,\textsuperscript{44} prostate cancer (NCT01197625) and acute myeloid leukemia (NCT02405338). Based on the observations reported above, we have chosen i.d. vaccine administration in these trials. The mRNA strategy allows recruiting both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, as observed in our studies and reported by others.\textsuperscript{18,45} It may be beneficial to increase the tumor-mRNA concentration at electroporation, though the translation machinery may at some point be overloaded. In the glioblastoma studies, we use amplified tumor mRNA from stem-cell-like neurospheres.\textsuperscript{44} In the prostate cancer trial NCT01197625, we combine tumor-mRNA with synthetic mRNA encoding selected tumor antigens. Our in-house mRNA expression vector includes certain features enhancing mRNA expression and stability.\textsuperscript{46} Others have explored different strategies for improving the mRNA-construct and the antigen presentation on HLA class I and II.\textsuperscript{47-49} The vaccine concept may be further developed by novel strategies for enhancing DC-migration and immune stimulation.\textsuperscript{50-54} We currently test the clinical use of DCs generated with a 3D protocol, based on a collaboration with Prof. Schendel and colleagues.\textsuperscript{55} This protocol includes a TLR seven out of eight agonist that enhance the IL-12 secretion of the DCs and their ability to generate Th1-like responses in vitro and in mouse models.\textsuperscript{56} Of note, it remains to be seen if this or other modifications yield the desired effect in cancer patients.

The success of checkpoint inhibitors suggests that it may be wise to let the immune system choose its targets, rather than to target defined antigens.\textsuperscript{8,10} The mRNA/DC approach applied in our study represents a feasible strategy for inducing broad T-cell responses against neoantigens. We have not identified the nature of the antigens recognized. Interestingly, Carrero and colleagues have elegantly demonstrated a diverse neoantigen-specific response after DC vaccination.\textsuperscript{57} The clinical efficacy of most vaccines is though limited. In the study reported herein, the majority of patients rapidly developed PD. Still, a few patients obtained prolonged tumor control and two are still alive. It is important to identify biomarkers for clinical responders. In a recent study, Figdor and colleagues found that T-cell infiltration into primary melanoma predicted prolonged survival after DC vaccination.\textsuperscript{58} This observation is in line with studies suggesting that checkpoint inhibitors and immunogenic chemotherapy are most effective in tumors with evidence of spontaneous immunological activity.\textsuperscript{59-61} Several investigators, including our group, have reported that Treg and MDSC levels may be of predictive value for cancer vaccination.\textsuperscript{6,26-65} In the present tumor-based mRNA approach, it would also be
interesting to investigate if the neoantigen burden can be used for selecting patients, as appears to be the case for checkpoint inhibitors.8-10,57

To improve clinical efficacy, it is probably necessary to combine vaccination with checkpoint modulators or other strategies countering immunological tolerance.6,25,52,66-71 As a combination strategy will increase the risk of side effects, it is encouraging that our vaccine study indicated low toxicity, even after long-term follow-up. One may further note that the long-term survivors (M22 and M109) responded well to treatment with ipilimumab. Other groups have also reported favorable responses in patients receiving other therapies after vaccination.72 These observations strengthen the hypothesis that these therapies may synergize. The checkpoint inhibitor therapy was started 3 and 5 y after vaccination, respectively, in patient M22 and M109. Their long-term survival thus cannot be explained only by checkpoint inhibitor therapy. The present study design though does not allow for any conclusions on a causal relationship between the vaccine and clinical outcome.

We conclude that the mRNA/DC vaccine was safe, feasible and induced detectable immune responses in about 50% of the patients. The immune response was associated with prolonged survival. We also demonstrated improved survival compared to a benchmark meta-analysis of patients from other trials. Based on these findings, we consider that further studies are warranted. The vaccine appears insufficient as monotherapy, but there is a strong rationale for investigating combination strategies with immunological checkpoint modulators.

Materials and methods

Patients

The patients were enrolled between 2002 and 2005 into trial NCT01278940. The study included an original cohort (DCM-1; n = 22) and an amendment cohort (DCM-2; n = 9) that received adjuvant IL-2. There was no pause between enrollments into the two cohorts. Apart from the administration of adjuvant IL-2, the protocol remained unchanged throughout both cohorts. Key inclusion criteria: Historically confirmed metastatic melanoma, evidence of disease progression, age >18 y, ECOG performance status 0 or 1, adequate hematologic, renal and hepatic function. Any prior radiotherapy or chemotherapy had to be completed a minimum of 4 weeks prior to study entry.

The original study protocol (2001) and the amendment (2004) were approved by the Norwegian Medicines Agency, the Regional Committee for Medical Research Ethics and the Hospital Internal Review Board. The trial was performed in compliance with the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all patients.

Tumor material and RNA extraction/evaluation

Tumor biopsies were stored on “RNA Later” solution (Sigma-Aldrich, St. Louis, MO, USA). Tumor-RNA extraction was performed as previously described.7 The RNA quality was evaluated by electrophoresis on agarose gels and on Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA).7

DC generation, transfection and phenotyping

DC generation was performed as previously described.7 Briefly, PBMCs were harvested by leukapheresis. Monocytes were enriched and cultured for 5 d in CellGro DC medium (CellGenix, Freiburg, Germany) with GM-CSF (2,500 U/mL; Schering-Plough, Kenilworth, NJ) and IL-4 (1,000 U/mL; CellGenix). The immature DCs were transfected with RNA and then cultured for two more days with cytokines facilitating maturation (IL-1β 10 ng/mL, IL-6 1,000 U/mL, TNFα 10 ng/mL; CellGenix) and prostaglandin E2 (1 µg/mL) (Sigma-Aldrich). Finally, matured DCs were frozen to −80°C and transferred to liquid nitrogen. The RNA-transfection was performed by electroporation, as described earlier.7,46 Through DC generation, we evaluated the cell quality, phenotype and yield by flow cytometry, automated counting, sterility and viability testing, as previously described.7,17

Vaccine administration

Each patient received four weekly i.n. or i.d. injections, followed by an i.d. injection at week 6 used for DTH recording. Additional booster vaccines were provided monthly thereafter, depending on the availability of vaccine DCs and the condition of the patient. Each vaccine injection generally contained 20 × 10⁶ DCs. If required, because of lower DC yield, the number of DCs per injection was adjusted (Table 2).

In cohort DCM-1, 12 patients had the DCs injected by ultrasound-guidance into an inguinal lymph node. The other 10 patients were vaccinated by intradermal injection 10 cm below the inguinal ligament, i.e., in a region where the lymphatic vessels drain to inguinal lymph nodes. The DCM-1 patients were allocated to the intranodal and intradermal vaccine group according to the order of inclusion; the first five and last five patients included were vaccinated i.d.

In cohort DCM-2, all nine patients received the vaccines by intranodal injection in the groin, followed by IL-2 injected into the same lymph node for 5 d, starting 1–3 d after the last vaccine. The first five patients (M101-M105) received 5 million units IL-2 (1 mill units per day). The next four patients (M106-M109) received 10 million units (2 mill units per day).

T-cell cultures and assays

PBMCs were obtained prior to vaccination, after five weeks (i.e., Two weeks after last vaccine) and after 13 weeks. Some patients received monthly booster vaccinations thereafter. In these subjects, PBMCs were also obtained on the day of these vaccinations. PBMCs were routinely obtained from blood samples drawn before the vaccine injection. The PBMCs were isolated, frozen and cultured as previously described.7,17 Thawed PBMCs were stimulated twice with TDCs. For each patient, the T cells from pre- and post-vaccination samples were cultured under identical conditions.

The T-cell proliferation assays were performed as described earlier,7,17 on freshly thawed PBMCs, and after one and two in
vitro stimulations. The assays were performed in triplicates. Irradiated tDCs and nDC controls (electroporated without mRNA) were used as antigen presenting cells (APCs). Negative controls with T cells only and positive controls with T cells + nDCs + Staphylococcus enterotoxin C3 (SEC3; Toxin Technologies, USA) were included.

Interferon-γ (IFNγ) ELISPOT assays were performed on selected patients, subject to availability of cells, as described previously.2,17 Responder T cells were seeded as duplicates at three different concentrations and stimulated with tDCs or nDCs. Negative controls with T cells only and positive controls with T cells + nDCs + SEC3 were included.

Bioplex cytokine assay: Supernatants were harvested from triplicate T-cell cultures at day 3 and analyzed by Bioplex assays (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturers protocol. We analyzed the supernatants for IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFNγ, TNFα, GM-CSF, MIP-1α and MCP-1.

Delayed-type hypersensitivity (DTH) reaction

The DTH reactions were recorded for tDCs and nDC controls injected at separate sites after completion of the vaccination schedule. For i.d. vaccinations, the DTH-reaction to each vaccine was registered. The DTH was measured as diameter of the erythema 48 h after injection. The DTH was scored as moderately/strongly positive if the erythema diameter for tDCs was ≥10 mm larger than for the nDCs. Erythema differences of 5–10 mm were considered weakly positive.

Clinical monitoring

Adverse events were graded according to the National Cancer Institute common toxicity criteria (NCI-CTC) version 2 and considered as being related to treatment if the relationship was reported as probable or suspected. Objective tumor response was assessed by clinical examination and CT scans at study entry and after 3 mo. The tumor response was classified according to the RECIST version 1.0. The patients were followed at the study hospital and data collected for 10 y after inclusion of the last subject. Beyond month 3, radiological scans and laboratory tests were performed as clinically indicated.

Statistics

Statistical analysis of T-cell proliferation assays and Bioplex assays was performed with one-way ANOVA, followed by SNK test. In the ELISPOT assays, the different T-cell concentrations were analyzed together by two-way ANOVA, followed by SNK test.

OS was estimated by the Kaplan–Meier method and compared with predicted survival from a large meta-analysis which was regarded as fixed. In addition, OS was compared between immune responders and non-responders using log-rank test. The comparison was conducted as a landmark analysis, calculating survival from week 6. Patients with a pre-existing immune response that did not substantially increase after vaccination (M09, M108) were excluded from the analysis. Patient M022 was also excluded, as she was weakly positive in DTH tests, negative in week 5 PBMCs and positive in later PBMC tests.

The statistical analyses were performed using SPSS 21 for Windows (IBM, USA) and Stata 13 (Metrika Consulting, Sweden). All tests were two-sided, with a 5% significance level.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

1. Korn EL, Liu PY, Lee SJ, Chapman JA, Niedzwiecki D, Suman VJ, Moon J, Sondak VK, Atkins MB, Eisenhauer EA et al. Meta-analysis of phase II cooperative group trials in metastatic stage IV melanoma to determine progression-free and overall survival benchmarks for future phase II trials. J Clin Oncol 2008; 26(4):527–34; PMID:18253113; http://dx.doi.org/10.1200/JCO.2007.12.7837
2. Eigentler TK, Caroli UM, Radny P, Garbe C. Palliative therapy of disseminated malignant melanoma: a systematic review of 41 randomised clinical trials. Lancet Oncol 2003; 4(12):748–59; PMID:14662431; http://dx.doi.org/10.1016/S1470-2045(03)01280-4
3. Hodi FS, O’Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010; 363(8):711–23; PMID:20525992; http://dx.doi.org/10.1056/NEJMoa1003466
4. Robert C, Ribas A, Wolchok JD, Hodi FS, Hamid O, Kefford R, Weber JS, Joshua AM, Hwu WJ, Gangadhar TC et al. Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. Lancet 2014; 384(9948):1109–17; PMID:25034862; http://dx.doi.org/10.1016/S0140-6736(14)60958-2
5. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, Schadendorf D, Dummer R, Smylie M, Rutkowski P et al. Combined nivolumab and ipilimumab or monoclonal antibodies in untreated melanoma. N Engl J Med 2015; 373(1):23–34; PMID:26027431; http://dx.doi.org/10.1056/NEJMoa1504030
6. Robert C, Ribas A, Wolchok JD, Hodi FS, Hamid O, Kefford R, Weber JS, Joshua AM, Hwu WJ, Gangadhar TC et al. Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. Lancet 2014; 384(9948):1109–17; PMID:25034862; http://dx.doi.org/10.1016/S0140-6736(14)60958-2
7. Kyte JA, Kvalheim G, Aamdal S, Saebø-Larssen S, Gaudernack G. Preclinical full-scale evaluation of dendritic cells transfected with...
autologous tumor-mRNA for melanoma vaccination. Cancer Gene Ther 2005; 12(6):579-91; PMID:15818380; http://dx.doi.org/10.1038/sj.cgt.7700837.

8. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel J, Lee W, Yuan J, Wong P, Ho TS et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 2015; 348(6230):124-8; PMID:25765070; http://dx.doi.org/10.1126/science.aaa1348.

9. Schumacher TN, Schreiber RD. Neoadtigens in cancer immunother-apy. Science 2015; 348(6230):69-74; PMID:25833875; http://dx.doi.org/10.1126/science.aaa4971.

10. McGranahan N, Furness AJ, Rosenthal R, Ramskov S, Lyngaa R, Morse MA, Coleman RE, Akabani G, Niehaus N, Coleman D, Wang HJ, Elashoff RM, McBride WH, Mukherji B et al. Determi-nation with tumor-mRNA transfected dendritic cells in androgen-resistant prostate cancer patients. Br J Cancer 2005; 93(7):749-56; PMID:16136047; http://dx.doi.org/10.1038/sj.bjc.6602761.

11. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res 2011; 17(13):4550-7; PMID:21498395; http://dx.doi.org/10.1158/1078-0432.CCR-11-0116.

12. Andersen R, Donia M, Ellebaek E, Borch TH, Kongsted P, Iversen TZ, Holmich LR, Hendel HW, Met O, Andersen MH et al. Long-lasting complete responses in patients with metastatic melanoma after adoptive cell therapy with tumor-infiltrating lymphocytes and an attenuated IL2 regimen. Clin Cancer Res 2016; 22(15):3734-45; PMID:27006492; http://dx.doi.org/10.1158/1078-0432.CCR-15-1879.

13. Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, Coulie PG, Boon T. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med 2005; 201(2):249-57; PMID:15960315.

14. Butterfield LH, Ribas A, Dissette VB, Amarnani SN, Yu HT, Osguerra D, Wang HJ, Elashoff RM, McBride WH, Mukherji B et al. Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. Clin Cancer Res 2003; 9(3):998-1008; PMID:12631598.

15. Inderberg-Suso EM, Trachsel S, Lislerud K, Rasmussen AM, Gaudernack G. Widespread CD4+ T cell reactivity to novel hTERT epitopes following vaccination of cancer patients with a single hTERT peptide GV1001. Oncoimmunology 2012; 1(5):670-8; PMID:22934259; http://dx.doi.org/10.4161/onci.20426.

16. Andibacka RH, Kaufman HL, Collichio F, Amatruda T, Senzer N, Jacobs JF, Tel J, de Vries II. Long-lasting multifunctional CD8+ T cell responses in end-stage melanoma patients can be induced by dendritic cell vaccination. Oncoimmunology 2016; 5(1): e1067745; PMID:26942087; http://dx.doi.org/10.1080/2162402X.2015.1067745.

17. Kyte JA, Trachsel S, Risberg B, thor Straten P, Lislerud K, Gaudernack G. Unconventional cytokine profiles and development of T cell mem-ory in long-term survivors after cancer vaccination. Cancer Immunol Immunother 2009; 58(10):1609-26; PMID:19221745; http://dx.doi.org/10.1007/s00262-009-0670-2.

18. Kyte JA, Gaudernack G, Dueland S, Trachsel S, Julsrud L, Aamdal S. Telomerase peptide vaccination combined with temozolomide: a clinical trial in stage IV melanoma patients. Clin Cancer Res 2011; 17(13):4568-80; PMID:21586625; http://dx.doi.org/10.1158/1078-0432.CCR-11-0184.

19. Wimmers F, Aarnethen EH, Duiveman-deBoer T, Figdor CG, Jacobs JF, Tel J, de Vries II. Long-lasting multifunctional CD8+ T cell responses in oral cavity squamous cell carcinoma following vaccination of cancer patients with a single hTERT peptide GV1001. Oncoimmunology 2012; 1(5):670-8; PMID:22934259; http://dx.doi.org/10.4161/onci.20426.

20. Fassnacht M, Lee J, Milazzo C, Boczkowski D, Su Z, Nair S, Gilboa E. Induction of CD4(+) and CD8(+) T-cell responses to the human stromal antigen, fibroblast activation protein: implication for cancer immunotherapy. Clin Cancer Res 2005; 11(15):5566-71; PMID:16061874; http://dx.doi.org/10.1158/1078-0432.CCR-05-0699.

21. Mosmann TR, Chernwinski H, Bond MW, Giedlman MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986; 136(7):2348-57; PMID:22934259; http://dx.doi.org/10.1080/2162402X.2015.1067745.

22. Hirschowitz EA, Foody T, Krysico R, Dickson L, Sturgill J, Yanneli J. Autologous dendritic cell vaccines for non-small-cell lung cancer. J Clin Oncol 2004; 22(14):2808-15; PMID:15254048; http://dx.doi.org/10.1200/JCO.2004.01.074.

23. Brossart P, Wirthes S, Stuhler G, Reichardt VL, Kanz L, Brugger W. Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. Blood 2000; 96(9):3102-8; PMID:10856173.

24. Di Nicola M, Carlo-Stella C, Mortarini R, Baldassari P, Guidetti A, Gallino GF, Del Vecchio M, Ravagnani F, Magni M, Chaplin P et al. Boosting T cell-mediated immunity to tyrosinase by vaccinia virus-transduced, CD8(+)-derived dendritic cell vaccination: a phase I trial in metastatic melanoma. Clin Cancer Res 2004; 10(16):5381-90; PMID:15328176; http://dx.doi.org/10.1158/1078-0432.CCR-04-0602.

25. Villena R, Benitez D, Mila J, Lozano M, Vilana R, Pomes J, Tomas X, Costa J, Vilalta A, Malvehy J et al. Pilot study of treatment of bioche-motherapy-refractory stage IV melanoma patients with autologous dendritic cells pulsed with a heterologous melanoma cell line lysate. Cancer Immunol Immunother 2004; 53(7):651-8; PMID:14999431; http://dx.doi.org/10.1007/s00262-003-0495-3.

26. Boczkowski D, Nair SK, Nam JH, Kyte JA, Gaudernack G. Induction of tumor immunity and cytotoxic T lymphocyte responses using
36. Caruso DA, Orme LM, Amor GM, Neale AM, Radcliff FJ, Downie P, Tang ML, Ashley DM. Results of a Phase I study utilizing monoclonally derived dendritic cells pulsed with tumor RNA in children with Stage 4 neuroblastoma. Cancer 2005; 103(6):1280-91; PMID:15693021; http://dx.doi.org/10.1001/jp.2005.1002/cncr.2091.

37. Nestle FO, Aljagic S, Gillette M, Sun Y, Grabbe S, Dummer R, Burg G, Schadendorf D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998; 4(3):328-32; PMID:9506067; http://dx.doi.org/10.1038/30938-328.

38. Su Z, Dannull J, Heiser A, Yancey D, Pruitt S, Madden J, Coleman D, Niedzwiecki D, Gilboa E, Viecek J. Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. Cancer Res 2003; 63(9):2127-33; PMID:12727829.

39. Lambert LA, Gibson GR, Maloney M, Durell B, Noelle RJ, Barth RJ, Jr. Intratumoral immunization with tumor lysate-pulsed dendritic cells enhances protective antitumor immunity. Cancer Res 2001; 61(2):641-6; PMID:11212262.

40. Brown GA, Alper S, Trichel A, Murphy-Corb M, Watkins SC, Gambotto A, Barratt-Boyes SM. Adenovirus-transduced dendritic cells injected into skin or lymph node prime potent simian immunodeficiency virus-specific T cell immunity in monkeys. J Immunol 2003; 171(12):6875-82; PMID:14662894; http://dx.doi.org/10.4049/jimmunol.171.12.6875.

41. Bedrosian I, Mick R, Xu S, Nisenbaum H, Faries M, Zhang P, Cohen PA, Koski G, Czerniecki BJ. Intratumoral administration of peptide-pulsed mature dendritic cell vaccine results in superior CD8+ T-Cell function in melanoma patients. J Clin Oncol 2003; 21(20):3826-35; PMID:14551301; http://dx.doi.org/10.1200/JCO.2003.04.042.

42. Lesterhuis WJ, de Vries JJ, Schreibelt G, Lambbe AJ, Aarnntzen EH, Jacobs JF, Scharenberg NM, van de Rakt MW, de Boer AJ, Croockewit PM, et al. Route of administration modulates the induction of dendritic cell vaccine-induced antigen-specific T cells in advanced melanoma patients. Clin Cancer Res 2011; 17(17):5725-35; PMID:21771874; http://dx.doi.org/10.1158/1078-0432.CCR-11-1261.

43. Bol KP, Figdor CG, Aarnntzen EH, Welzen ME, van Rossum MM, Blokk WA, van de Rakt MW, Scharenberg NM, de Boer AJ, Pots JM et al. Intratumoral vaccination with mRNA-optimized dendritic cells in metastatic melanoma patients. Oncoimmunology 2015; 4(8): e1019197; PMID:26405571; http://dx.doi.org/10.1080/2162402X.2015.1019197.

44. Vik-Mo EO, Nyakas M, Mikkelsen BV, Moe MC, Due-Tonnesen T et al. Route of administration modulates the induction of dendritic cell vaccine-induced antigen-specific T cells in metastatic melanoma patients. J Immunol 2011; 185(1):738-47; PMID:20511554; http://dx.doi.org/10.4049/jimmunol.1000600.

45. Spranger S, Frankenberger B, Schendel DJ, NOD/scid IL-2(-)/2g(null) mice: a preclinical model system to evaluate human dendritic cell-based vaccine strategies in vivo. J Transl Med 2010; 12:80; PMID:20236422; http://dx.doi.org/10.1186/1479-5876-10-30.

46. Carreno BM, Magrini V, Becker-Hapak M, Kaibinejadian S, Kundal J, Petti AA, Ly A, Lie WR, Hildebrand WH, Mardis ER et al. Cancer immunotherapy. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. Science 2015; 348(6236):803-8; PMID:25837513; http://dx.doi.org/10.1126/science.aaa3828.

47. Carreno BM, Magrini V, Becker-Hapak M, Kaibinejadian S, Kundal J, Petti AA, Ly A, Lie WR, Hildebrand WH, Mardis ER et al. Cancer immunotherapy. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. Science 2015; 348(6236):803-8; PMID:25837513; http://dx.doi.org/10.1126/science.aaa3828.

48. Vasaturo A, Hailovic A, Bol KP, Verweij DJ, Blokk WA, Punt CJ, Groenen PJF, van Krieken JH, Tejedor J, de Vries JJ et al. T-cell landscape in a primary melanoma predicts the survival of patients with metastatic disease after their treatment with dendritic cell vaccines. Cancer Res 2016; 76(12):3496-506; PMID:27197179; http://dx.doi.org/10.1158/0008-5472.CAN-15-3211.

49. Kroemer G, Senovilla L, Galluzzi L, Andre F, Zitvogel L. Natural and therapy-induced immunosurveillance in breast cancer. Nat Med 2015; 21(10):1128-38; PMID:26444637; http://dx.doi.org/10.1038/nm.3944.

50. Sistigu A, Yamazaki T, Vacchelli E, Chaba K, Enot DP, Adam J, Vitale Petti A, Ly A, Lie WR, Hildebrand WH, Mardis ER et al. Cancer immunotherapy. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. Science 2015; 348(6236):803-8; PMID:25837513; http://dx.doi.org/10.1126/science.aaa3828.

51. Tophalin SL, Taube JM, Anders RA, Pardoll DM. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. Nat Rev Cancer 2016; 16(5):275-87; PMID:27098902; http://dx.doi.org/10.1038/nrc.2016.36.

52. Hansen GL, Gaudernack G, Brunsvig PF, Cvanacarova M, Kye JA. Immunological factors influencing clinical outcome in lung cancer patients after telomerase peptide vaccination. Cancer Immunol.
63. Iclozan C, Antonia S, Chiappori A, Chen DT, Gabrilovich D. Therapeutic regulation of myeloid-derived suppressor cells and immune response to cancer vaccine in patients with extensive stage small cell lung cancer. Cancer Immunol Immunother 2013; 62(5):909-18; PMID:23589106; http://dx.doi.org/10.1007/s00262-013-1396-8

64. Pohla H, Buchner A, Stadlbauer B, Frankenberger B, Stevanovic S, Walter S, Frank R, Schwacha T, Olek S, Kopp J et al. High immune response rates and decreased frequencies of regulatory T cells in metastatic renal cell carcinoma patients after tumor cell vaccination. Mol Med 2012; 18:1499-508; PMID:23269976; http://dx.doi.org/10.2119/molmed.2012.00221

65. Dang Y, Wagner WM, Gad E, Rastetter L, Berger CM, Holt GE, Disis ML. Dendritic cell-activating vaccine adjuvants differ in the ability to elicit antitumor immunity due to an adjuvant-specific induction of immunosuppressive cells. Clin Cancer Res 2012; 18(11):3122-31; PMID:22510348; http://dx.doi.org/10.1158/1078-0432.CCR-12-0113

66. Sanchez-Paulote AR, Cueto FJ, Martinez-Lopez M, Labiano S, Morales-Kastresana A, Rodriguez-Ruiz ME, Jure-Kunkel M, Azpiqueta A, Aznar MA, Quetglas JJ et al. Cancer immunotherapy with immunomodulatory anti-CD137 and anti-PD-1 monoclonal antibodies requires BATF3-dependent dendritic cells. Cancer Discov 2016; 6(1):71-9; PMID:26493961; http://dx.doi.org/10.1158/2159-8290.CD-15-0510

67. Rech AJ, Mick R, Martin S, Recio A, Aqui NA, Powell DJ, Jr, Collignon TA, Trosko JA, Leinbach LI, Fletcher CH et al. CD25 blockade depletes and selectively reprograms regulatory T cells in concert with immuno-therapy in cancer patients. Sci Transl Med 2012; 4(134):134ra162; PMID:22593175; http://dx.doi.org/10.1126/scitranslmed.3003330

68. Peng W, Chen JQ, Liu C, Malu S, Creasy C, Tetzlaff MT, Xu C, McKenzie JA, Zhang C, Liang X et al. Loss of PTEN promotes resistance to T cell-mediated immunotherapy. Cancer Discov 2016; 6(2):202-16; PMID:26645196; http://dx.doi.org/10.1158/2159-8290.CD-15-0283

69. Ellebaek E, Engell-Noerregaard L, Iversen TZ, Froesig TM, Munir S, Hadrup SR, Andersen MH, Svane IM. Metastatic melanoma patients treated with dendritic cell vaccination, Interleukin-2 and metronomic cyclophosphamide: results from a phase II trial. Cancer Immunol Immunother 2012; 61(10):1791-804; PMID:22426890; http://dx.doi.org/10.1007/s00262-012-1242-4

70. Ridolfi L, Petrini M, Granato AM, Gentilcore G, Simeone E, Ascieri PA, Pancisi E, Ancarani V, Fiammenghi L, Guidoboni M et al. Low-dose temozolomide before dendritic-cell vaccination reduces (specifi- cally) CD4+CD25+++Foxp3+ regulatory T-cells in advanced melano- noma patients. J Transl Med 2013; 11:135; PMID:23725550; http://dx. doi.org/10.1186/1479-5876-11-135

71. Poschke I, Lovgren T, Adamson L, Nystrom M, Andersson E, Hans- son J, Tell R, Masucci GV, Kiessling R. A phase I clinical trial combin- ing dendritic cell vaccination with adoptive T cell transfer in patients with stage IV melanoma. Cancer Immunol Immunother 2014; 63 (10):1061-71; PMID:24993563; http://dx.doi.org/10.1007/s00262-014-1575-2

72. Ridolfi L, Petrini M, Fiammenghi L, Granato AM, Ancarani V, Pancisi E, Scarpi E, Guidoboni M, Migliori G, Sanna S et al. Unexpected high response rate to traditional therapy after dendritic cell-based vaccine in advanced melanoma: update of clinical outcome and subgroup analysis. Clin Dev Immunol 2010; 2010:504979; PMID:20936106; http://dx.doi.org/10.1155/2010/504979