Cancer Therapy: Clinical

Clinical Cancer Research

Route of Administration Modulates the Induction of Dendritic Cell Vaccine–Induced Antigen-Specific T Cells in Advanced Melanoma Patients

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Abstract

Purpose: It is unknown whether the route of administration influences dendritic cell (DC)-based immunotherapy. We compared the effect of intradermal versus intranodal administration of a DC vaccine on induction of immunologic responses in melanoma patients and examined whether concomitant administration of interleukin (IL)-2 increases the efficacy of the DC vaccine.

Experimental Design: HLA-A2.1$^+$ melanoma patients scheduled for regional lymph node dissection were vaccinated four times biweekly via intradermal or intranodal injection with 12×10^6 to 17×10^6 mature DCs loaded with tyrosinase and gp100 peptides together with keyhole limpet hemocyanin (KLH). Half of the patients also received low-dose IL-2 (9 MIU daily for 7 days starting 3 days after each vaccination). KLH-specific B- and T-cell responses were monitored in blood. gp100- and tyrosinase-specific T-cell responses were monitored in blood by tetramer analysis and in biopsies from delayed-type hypersensitivity (DTH) skin tests by tetramer and functional analyses with ^{51}Cr release assays or IFNγ release, following coculture with peptide-pulsed T2 cells or gp100- or tyrosinase-expressing tumor cells.

Results: In 19 of 43 vaccinated patients, functional tumor antigen–specific T cells could be detected. Although significantly more DCs migrated to adjacent lymph nodes upon intranodal vaccination, this was also highly variable with a complete absence of migration in 7 of 24 intranodally vaccinated patients. Intradermal vaccinations proved superior in inducing functional tumor antigen–specific T cells. Coadministration of IL-2 did not further augment the antigen-specific T-cell response but did result in higher regulatory T-cell frequencies.

Conclusion: Intradermal vaccination resulted in superior antitumor T-cell induction when compared with intranodal vaccination. No advantage of additional IL-2 treatment could be shown. *Clin Cancer Res; 17(17); 5725–35. ©2011 AACR.*

Introduction

Over the past decade, vaccines consisting of autologous dendritic cells (DC) loaded with tumor antigens have proven to be safe and capable of inducing tumor antigen–specific immune responses in a substantial part of the vaccinated patients. However, clinical efficacy is still limited, underlining the necessity to further optimize different parameters such as DC subtype, DC maturation and activation status, and the route, dose, and frequency of administration (1, 2).

Because DCs are the main antigen-presenting cells of the immune system (3), various DC vaccines have been evaluated for the induction of antitumor immune responses *in vivo* (4–12). Their unique ability to take up antigens, migrate to the lymph nodes, and (cross)present the antigens in context

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-11-1261

Translational Relevance

Dendritic cell (DC) vaccination constitutes a promising novel immunotherapy in cancer. For optimal T-cell induction, it is crucial for the DCs to interact with T cells in the lymph nodes. Although previous studies have shown that after intranodal administration more DCs spread throughout the lymphatic system as compared with intradermal administration, robust clinical trials comparing the different routes of administration are lacking. Here, we directly compared the 2 routes of administration in 43 melanoma patients with locoregional lymph node metastases. We found that upon intradermal vaccination, the induced T cells were more often able to recognize endogenously processed tumor antigens as compared with intranodal vaccination. Thus, the more laborious and variable intranodal route of administration does not offer an advantage over intradermal vaccination.

of the appropriate costimulatory molecules to T cells and B cells stimulates the induction of potent tumor-specific T cells. Most DC vaccines to date have been derived from patient-derived monocytes cultured in the presence of interleukin (IL) 4 and granulocyte macrophage colony-stimulating factor (GM-CSF) and subsequently matured and loaded with peptides derived from tumor antigens (4–12).

We and others previously showed that maturation of the DC is essential to develop their migratory capacity and their capacity to induce antigen-specific T cells (13–15). In previous studies, we showed that although the majority of DCs remain localized at the injection site and are phagocytosed by macrophages, a small number migrates to the T-cell areas within the lymph nodes. Within the lymph node, these DCs associate with T cells and are capable of inducing antigen-specific T-cell responses *in vivo* (16, 17). The existence of circulating antigen-specific T cells after DC vaccination could be shown in skin biopsies taken from delayed-type hypersensitivity (DTH) reactions, indicating that vaccine-induced T cells are indeed capable of homing to sites where antigen is exposed by DC (5). Furthermore, the presence of such antigen-specific T cells at DTH sites clearly correlated with improved survival (5), showing that skin biopsies taken from DTH sites are a representative compartment for immunomonitoring.

The route of administration clearly directs the distribution of a DC vaccine upon injection and consequently may lead to differences in immunologic responses (8,18–22). Although we have shown that intranodal (IN) administration results in a much higher migration of injected DCs to the draining lymph nodes compared with intradermal (ID) vaccination (14; 17), it is not clear whether intranodal administration also results in a superior immunologic and clinical response. Therefore, the aim of this clinical study was to compare intranodal and intradermal DC vaccine administration to determine the effect of route of administration on the induction of immunologic responses in melanoma patients with locoregional lymph node metastases.

Although true clinical benefit of low-dose IL-2 has not been unequivocally proven in melanoma (23–26), it has been suggested that low-dose IL-2 may enhance proliferation of antigen-specific T cells after DC vaccination (27, 28). Therefore, we also examined whether concomitant treatment with low-dose IL-2 increases the efficacy of the DC vaccine.

Materials and Methods

Study protocol and patient population

In this study, stage III and IV melanoma patients (according to American Joint Committee on Cancer criteria) who were scheduled for regional lymph node dissection with either curative or palliative intention were included. Additional inclusion criteria included HLA-A2.1 phenotype, melanoma expressing the melanoma-associated antigens gp100 and tyrosinase, and WHO performance status 0 or 1. Patients with brain metastases, serious concomitant disease, or a history of a second malignancy were excluded. The study was approved by the Regional Review Board, and written informed consent was obtained from all patients. Clinical trial registration number is NCT00243594.

Patients received a DC vaccine via intradermal or intranodal injection, either with or without systemically administered IL-2. The first 20 patients were assigned to either the IN + IL-2 or ID + IL-2 arm in an alternating manner, the next cohort of patients was assigned to either the intranodal without IL-2 or the intradermal without IL-2 arm in an alternating manner. Assignment was conducted by I.J.M. de Vries who had no knowledge about the clinical characteristics of the patients. Intranodal vaccination was conducted in a clinically tumor-free lymph node under ultrasound guidance. Intradermal vaccination was conducted at 5 to 10 cm distal from a (preferably inguinal) clinically tumor-free lymph node, by clinicians with extensive experience with the procedure (W.J. Lesterhuis, E.H.J. G. Aarntzen, C.J.A. Punt). Because the first vaccination was administered 1 day before regional lymph node dissection, presumably a significant benefit to the patient could not be expected. For this reason, the first vaccination always consisted of an injection of radionuclide-labeled, but not peptide-pulsed and not keyhole limpet hemocyanin (KLH)-loaded DCs on the side of the lymph node dissection, and an injection of peptide-pulsed DCs on the contralateral side. The latter vaccine could be radionuclide labeled or not. The DC vaccine consisted of autologous mature DCs pulsed with gp100 and tyrosinase peptides and KLH. Patients received 1 cycle consisting of 4 DC vaccinations administered at a biweekly interval. IL-2 was administered by subcutaneous injections (at 9 MIU) once daily for 1 week starting 3 days after each DC vaccination. Twenty-four to 48 hours after the first vaccination, a radical lymph node dissection was conducted. One to 2 weeks after the fourth vaccination, a DTH test was conducted. All patients who remained free of disease progression after

the first vaccination cycle were eligible for 2 maintenance cycles, each at 6-month intervals and each consisting of 3 biweekly intranodal vaccinations without IL-2 (Supplementary Fig. S1). Patients were considered evaluable when they had completed the first vaccination cycle. Vaccine-specific immune response was the primary endpoint. Clinical response was a secondary endpoint. Progression-free survival was defined as the time from apheresis to recurrence (for stage III patients) or progression (for stage IV patients).

DC preparation and characterization

KLH-loaded DCs were generated from peripheral blood mononuclear cells (PBMC) and matured with autologous monocyte-conditioned medium containing prostaglandin E_2 (10 μg/mL; Pharmacia & Upjohn) and recombinant TNF-α (10 ng/mL; provided by Dr. G. Adolf, Bender Wien GmbH), as described (29, 30). This procedure gave rise to mature DCs meeting the release criteria (29).

Peptide pulsing

DCs were pulsed with the HLA class I gp100-derived peptides $gp100_{154–167}$ and $gp100_{280–288}$ and the tyrosinase-derived peptide $tyrosinase_{369–376}$ (31–33). Peptide pulsing was conducted as described (13), and cells were resuspended in 0.1 mL for injection.

DC migration

DC migration was measured after the first vaccination by scintigraphic imaging as described (16). During the first vaccination, patients received DC labeled with ^{111}In (16). One hour after injection, the first scintigraphic image was acquired. At day 2, a second scintigraphic image was acquired followed by lymph node dissection. Migration was quantified by region-of-interest analysis of the individual nodes visualized on the images and expressed as the fraction of ^{111}In-labeled DCs that had migrated from the injection depot to following lymph nodes after 2 days (16). Patients simultaneously received peptide-loaded DCs in a contralateral clinically tumor-free lymph node, which was not to be resected.

KLH-specific proliferation

KLH-specific cellular responses were measured by proliferation assay. PBMCs were isolated from heparinized blood by Ficoll-Paque density centrifugation. PBMCs were stimulated with KLH (4 μg/2×10^5 PBMCs) in medium with 10% human AB serum (Sanquin blood bank). After 3 days, cells were pulsed with ^{3}H-thymidine for 8 hours and incorporation was measured with a β-counter. Experiments were carried out in triplicate.

KLH-specific antibody production

Antibodies against KLH were measured in the serum of vaccinated patients by ELISA (34). Microtiter plates (96 wells) were coated overnight at 4°C with KLH (25 μg/mL in PBS per well). After washing the plates, different concentrations of patient serum were allowed to bind at room temperature for 1 hour. After extensive washing, patient antibodies were detected with mouse antihuman IgG, IgA, or IgM antibodies labeled with horseradish peroxidase (Invitrogen). 3,3′,5,5-Tetramethyl-benzidine was used as a substrate, and plates were measured in a microtiter plate reader at 450 nm. For quantification, an isotype-specific calibration curve for the KLH response was included in each microtiter plate (Jacobs and colleagues, manuscript in preparation).

DTH test

Previously, we have developed a monitoring tool to assess T-cell responses following vaccination, using DTH-infiltrated T cells (13). We have shown that the presence of DTH-infiltrated, vaccine-specific T cells correlates with clinical outcome (5). In this study, DTH skin tests were conducted approximately 2 weeks after the fourth vaccination, as described previously (5). DCs pulsed with gp100 and tyrosinase peptides (2×10^6–10×10^6 DC each) were injected intradermally in the skin of the back of the patient at 4 different sites. The maximum diameter of induration was measured after 48 hours. From positive DTH sites (>2 mm), punch biopsies (6 mm) were obtained. Half of the biopsy was cryopreserved, and the other part was manually cut and cultured in RPMI 1640, containing 7% human serum and IL-2 (100 U/mL). Every 7 days, half of the medium was replaced by fresh medium containing human serum and IL-2. After 2 to 4 weeks of culturing, T cells were tested.

Outgrowth of DTH-infiltrating lymphocytes was defined as at least 1×10^5 cells per biopsy. Although insufficient numbers of T cells could be derived from the DTH biopsies of patients II-A-02, II-A-03, II-A-04, II-A-06, II-A-09, and II-A-10 after the first vaccination cycle, we obtained sufficient numbers of T cells after the second and/or third vaccination cycle. Therefore, for all patients, the presence of tumor antigen–specific T cells was analyzed over all received cycles of DC vaccinations. From patients II-A-01, II-B-04, and II-B-05, who only received 1 cycle of vaccinations, insufficient numbers of T cells could be derived from their DTH biopsies.

Tetramer staining

PBMCs and DTH-derived cells were stained with tetrameric MHCs containing $gp100_{154–167}$, $gp100_{280–288}$, or $tyrosinase_{369–376}$ peptide (Sanquin), as described (5). Patients were scored as having tetramer-positive cells when the percentage of tetramer-positive $CD8^+$ T cells was at least twice the background staining.

Antigen and tumor recognition

Antigen recognition was determined by the production of cytokines or cytotoxic activity of DTH-derived cells in response to T2 cells pulsed with the indicated peptides or the melanoma cell line BLM (HLA-A2.1–positive melanoma cell line without endogenous expression of gp100 or tyrosinase), transfected with control antigen G250, gp100, or tyrosinase or an allogeneic HLA-A2.1–positive,

gp100-, and tyrosinase-positive tumor cell line (Mel624) were measured. Cytotoxic activity was measured using a chromium release assay (35). Cytokine production was measured in supernatants after 16 hours of coculture by cytometric bead array (Th1/Th2 cytokine CBA1; BD Pharmingen). The reason we used 3 different types of target cells is because it has been shown previously that peptide-induced T cells not necessarily also recognize the corresponding endogenously processed antigen (36).

FOXP3 staining

Cells were stained with anti-CD3, anti-CD4, and anti-CD25 (BD Biosciences), fixed and permeabilized and stained with anti-FOXP3 (eBiosciences). Flow cytometric analysis was conducted with a FACSCalibur (Becton Dickinson).

Statistical analysis

Differences between groups were evaluated using ANOVA (KLH-specific proliferation and antibodies, percentage tetramer$^+$ cells), unpaired Student's t test (CCR7 expression, percentage migration), or the Mann–Whitney U test (number of targeted lymph nodes). Frequency distributions were analyzed by Fisher's exact test. All statistical tests were 2 sided and significance was defined as $P < 0.05$.

Results

Patient characteristics and treatment

Of a total of 47 patients enrolled in this study, 4 patients were excluded from the analysis, 2 patients due to rapid disease progression before completing the first vaccination cycle, 1 patient was HLA-A2.1 negative, and 1 patient developed brain metastases prior to initiation of treatment. The patients were assigned to 4 different groups (Supplementary Figs. S1 and 2). Thirteen patients received only 1 cycle consisting of 4 vaccinations, 7 patients received 1 additional maintenance cycle, consisting of 3 intranodal vaccinations, and 22 patients completed the full treatment schedule of 2 additional maintenance cycles of 3 intranodal vaccinations each. Patient characteristics are summarized in Table 1 and were comparable between the groups.

Treatment outcome

The 3 patients with distant metastasis at inclusion had progression-free intervals of 3, 4, and 7 months, respectively. The median time to recurrence for stage III patients was 32 months (range: 2–61 months) for intradermally vaccinated patients not treated with IL-2, 27 months (range: 6–115 months) for intradermally vaccinated patients treated with IL-2, 42 months (range: 7–74 months) for intranodally vaccinated patients not treated with IL-2, and 14 months (range: 4–83 months) for the intranodal group treated with IL-2. At a median follow up of 61 months (range: 20–115 months), 15 of the 40 patients with stage III at inclusion have no evidence of disease (Table 1). Clinical data are summarized in Table 1.

Characteristics and distribution of injected DCs

Patients in the different groups received on average 12×10^6 to 17×10^6 DCs per vaccination during the first cycle (Table 2). After maturation, DCs of all patients showed a mature phenotype exemplified by high HLA, CD80, CD83, and CD86 expression (Table 2). There was no difference in the amount or maturation status of injected DCs between the different groups. Furthermore, there were no significant differences in CCR7 expression between intranodally and intradermally injected DCs (Fig. 1A). The distribution of ^{111}In-labeled DCs was determined 24 to 48 hours after the first intradermal or intranodal vaccination by scintigraphic imaging (Fig. 1). The mean overall redistribution of injected cells from the intradermal injection depot to draining lymph nodes was relatively constant, with a median migration of 1.0% (range: 0.2%–4.0%; Fig. 1B). Although the percentage of migrating cells from the injection depot was significantly higher when the cells were injected intranodally, this was also much more variable (median migration: 3.2%, range: 0%–84%): in all intradermally vaccinated patients, at least a small fraction of the injected DCs actively migrated to regional lymph nodes, whereas in 7 of 24 intranodally vaccinated patients, no migration was observed at all. This may be caused by incorrect injection of intranodal vaccines during the first of 4 injections, as we have shown previously (16).

It is possible that upon intranodal injection, part of the redistribution may have taken place as the result of passive lymphatic flow rather than active migration if the DCs were injected in an efferent lymphatic vessel. When only the percentage of migrating cells is taken into account, this might therefore not be a true representation of the amount of viable, migrating DCs. For this reason, we also analyzed the number of lymph nodes that were targeted upon injection and found no difference between intranodal (median: 1, range: 0–6) and intradermal vaccination (median: 1, range: 1–5).

KLH-specific immune responses

To investigate whether the differences in distribution after intranodal and intradermal vaccination resulted in differences in immunologic responses, humoral and cellular responses against the control antigen KLH were measured in peripheral blood of the patients after the first cycle of vaccinations. In all groups, levels of KLH-specific antibodies increased to a similar extent upon vaccination (Fig. 2A–D). KLH-specific T-cell proliferation was measured in PBMCs of patients after the first cycle of vaccinations by proliferation assay. KLH-specific proliferation was higher after vaccination than before vaccination in all treatment groups and was induced in all vaccinated patients including patients that did not show DC migration after the first intranodal DC vaccination (Fig. 3A–D). The magnitude of KLH-specific T-cell proliferation was similar in all treatment groups. In summary, comparable KLH-specific immune responses are induced irrespective of the route of DC administration or concomitant treatment with IL-2.

Table 1. Patient characteristics

Patient	Age, y/ Sex	AJCC stage at inclusion	N status	M status	gp100[a]		Tyrosinase[a]		Cycles[b], *N*	Relapse-free (stage III) or progression-free (stage IV) interval, mo	Overall survival, mo
					Intensity	%	Intensity	%			
Intradermal −IL-2											
II-D-01	51/M	IIIA	N1a	M0	+++	80	++	50	3	61+	61+
II-D-02	57/F	IIIA	N1a	M0	+++	10	++	10	3	59+	59+
II-D-03	65/M	IIIB	N1a	M0	+++	90	+++	40	3	21	60+
II-D-04	53/F	IIIA	N1a	M0	++	80	++	80	3	56+	56+
II-D-05	58/M	IIIA	N1b	M0	+++	75	+	5	3	43	47
II-D-06	57/M	IIIC	N3	M0	++	90	++	25	3	14	56+
II-D-07	73/M	IV	N3	M1c	+++	60	+++	60	1	3	4
II-D-08	50/F	IIIC	N3	M0	+	n.d	n.d.	n.d.	1	3	7
II-D-09	39/F	IIIA	N2a	M0	+++	50	+++	50	2	50	50+
II-D-10	51/M	IIIC	N3	M0	+++	20	++	90	1	2	4
II-D-12	48/M	IIIC	N3	M0	+++	33	++	75	3	11	49+
Intradermal +IL-2											
II-A-01	55/M	IIIC	N1b	M0	+	10	++	40	1	10	118+
II-A-02	55/M	IIIA	N1a	M0	++	90	+++	90	3	44	48
II-A-03	44/F	IIIA	N1a	M0	+++	80	+++	90	3	115+	115+
II-A-04	59/F	IIIC	N2b	M0	++	40	+	30	3	22	50
II-A-05	59/M	IIIC	N3	M0	++	na	+	na	3	16	21
II-A-06	68/M	IIIC	N3	M0	++	80	+	20	3	23	29
II-A-08	38/F	IIIC	N1b	M0	++	5	++	70	3	44	59
II-A-09	51/F	IIIA	N1a	M0	++	30	++	30	3	31+	31+
II-A-10	40/F	IIIC	N1b	M0	++	10	++	90	3	6	25
II-A-11	64/M	IIIA	N1a	M0	+++	80	+++	80	3	33+	33+
Intranodal −IL-2											
II-C-01	71/M	IIIC	N3	M0	+++	15	++	50	2	9	12
II-C-02	54/M	IIIC	N3	M0	++	10	+	20	2	17	21
II-C-03	59/M	IIIB	N1b	M0	+++	100	+++	80	3	66+	66+
II-C-04	35/M	IIIA	N1a	M0	+++	30	+++	80	3	74+	74+
II-C-06	58/M	IIIB	N1a	M0	++	90	−	100	3	49	73+
II-C-07	56/M	IIIA	N2b	M0	+++	60	+++	90	3	74+[c]	74+
II-C-08	34/F	IIIB	N1b	M0	+	na	+	na	2	72+	72+
II-C-09	60/F	IV	N3	M1b	+++	25	+++	25	1	4	6
II-C-10	65/F	IIIB	N1a	M0	++	90	++	90	2	7	31
II-C-12	43/M	IIIB	N1b	M0	+++	80	++	80	3	35+	35+
II-C-13	19/F	IV	N3	M1a	+++	60	+++	90	1	7	14
II-C-14	64/F	IIIB	N1b	M0	+	na	+	na	3	34+	34+
Intranodal +IL-2											
II-B-01	55/F	IIIC	N3	M0	+++	na	+++	10	1	4	14
II-B-02	64/M	IIIA	N2b	M0	+	na	+	na	1	19	21
II-B-03	54/M	IIIC	N3	M0	+	70	+	5	3	46	47
II-B-04	78/F	IIIC	N3	M0	+	na	+	na	1	6	10
II-B-05	67/M	IIIC	N2b	M0	+++	30	++	10	1	9	12
II-B-06	41/F	IIIC	N3	M0	+	na	+	30	1	4	6
II-B-07	39/M	IIIB	N1b	M0	++	80	+++	70	3	83+	83+
II-B-08	72/F	IIIB	N1b	M0	++	70	+	50	1	8	11
II-B-09	36/M	IIIB	N1b	M0	+++	40	+++	90	3	78+	78+
II-B-10	56/F	IIIB	N2b	M0	+++	na	+++	na	3	20+	20+

Abbreviations: n.d., not determined; na, not applicable; AJCC, American Joint Committee on Cancer.

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

[b]Number of received vaccination cycles [1 cycle consists of 4 (first cycle) or 3 (second and third cycle) vaccinations and a DTH test].

[c]Ongoing progression-free and overall survival after surgery of local metastasis.

Table 2. Characteristics of injected DCs

Group	Injected DC[a] ($\times 10^6$)	CD80, %	CD83, %	CD86, %	HLA-ABC, %	HLA-DR/DP, %	HLA-DQ, %
IN + IL-2	15 (±5)	83 (±14)	82 (±14)	98 (±3)	96 (±7)	98 (±3)	91 (±11)
ID + IL-2	17 (±12)	82 (±14)	81 (±15)	97 (±2)	89 (±8)	94 (±6)	73 (±22)
IN − IL-2	12 (±3)	92 (±5)	81 (±14)	96 (±6)	96 (±7)	99 (±1)	79 (±14)
ID − IL-2	13 (±3)	87 (±7)	78 (±12)	94 (±8)	84 (±15)	96 (±4)	68 (±24)

Abbreviations: IN, intranodal; ID, intradermal.
[a]Average number of DCs injected per vaccination (mean ± SD) during the first cycle. Patients received a total of 4 vaccinations.

Effect of route of administration on tumor antigen–specific responses in DTH-derived T cells

The frequencies of tumor antigen-specific T cells in blood are generally very low. In our previous studies, we were often unable to detect tumor antigen–specific T cells in peripheral blood by tetramer staining, whereas functional tumor antigen–specific T cells were present in biopsies taken from DTH challenges (5, 37). Because the presence of antigen-specific T cells in DTH sites correlates with clinical outcome (5), we monitored tumor antigen–specific responses in lymphocyte cultures from DTH sites. In 32 of 40 tested patients, we were able to retrieve gp100- or tyrosinase-specific T cells from their DTH biopsies, as measured by tetramer staining (Supplementary Table S1 and Fig. 4). In intradermally vaccinated patients, tetramer-positive $CD8^+$ T cells were detected in the DTH of 90% of the patients, compared with 70% in the intranodally vaccinated group ($P = 0.2$). Although there were also no significant differences in tumor peptide recognition (90% intradermal vs. 70% intranodal, $P = 0.2$) or protein recognition (60% intradermal vs. 37% intranodal, $P = 0.2$), DTH-derived $CD8^+$ T cells from intradermally vaccinated patients more frequently recognized tumor cells expressing gp100 and tyrosinase (53% intradermal vs. 16% intranodal, $P < 0.05$), as indicated by IFNγ production or cytolytic activity. Specific production of IL-2 and IL-5 in DTH-derived T-cell cultures of intradermally and

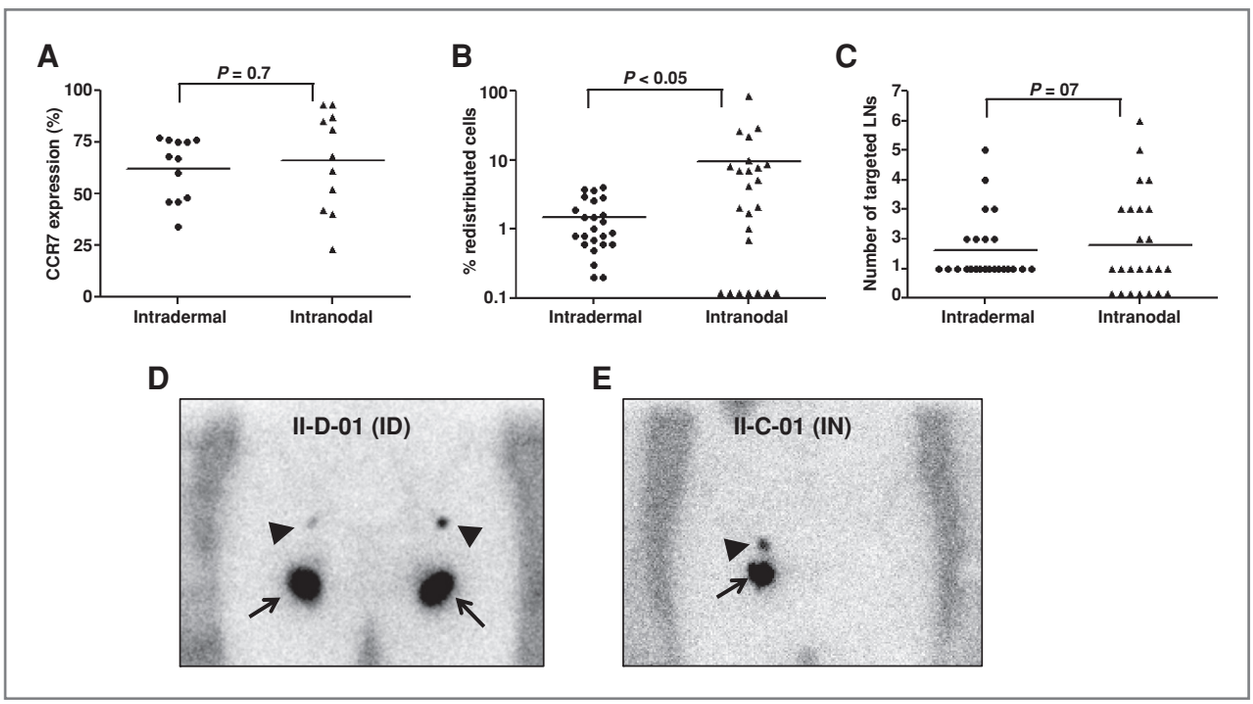

Figure 1. DC migration after intranodal or intradermal injection. A, expression of CCR7 measured by flow cytometry on mature DCs for the first vaccination. Data are shown as percentage of cells expressing CCR7. One symbol represents 1 patient; horizontal line represents mean percentage. B and C, percentage of cells redistributed to nearby lymph nodes (B) and number of targeted lymph nodes (C) 24 to 48 h after intradermal and intranodal injection of ^{111}In-labeled DCs imaged by scintigraphy of the lymph node region. One symbol represents 1 injection of 15×10^6 cells; horizontal lines represent mean redistribution. D and E, scintigraphic images showing the redistribution of ^{111}In-labeled cells from the injection depot (arrows) to nearby lymph nodes (arrow heads) 48 hours after injection are depicted from a patient after intradermal (ID; D; patient II-D-01) and intranodal (IN; E; patient II-C-01) vaccination. LN, lymph node.

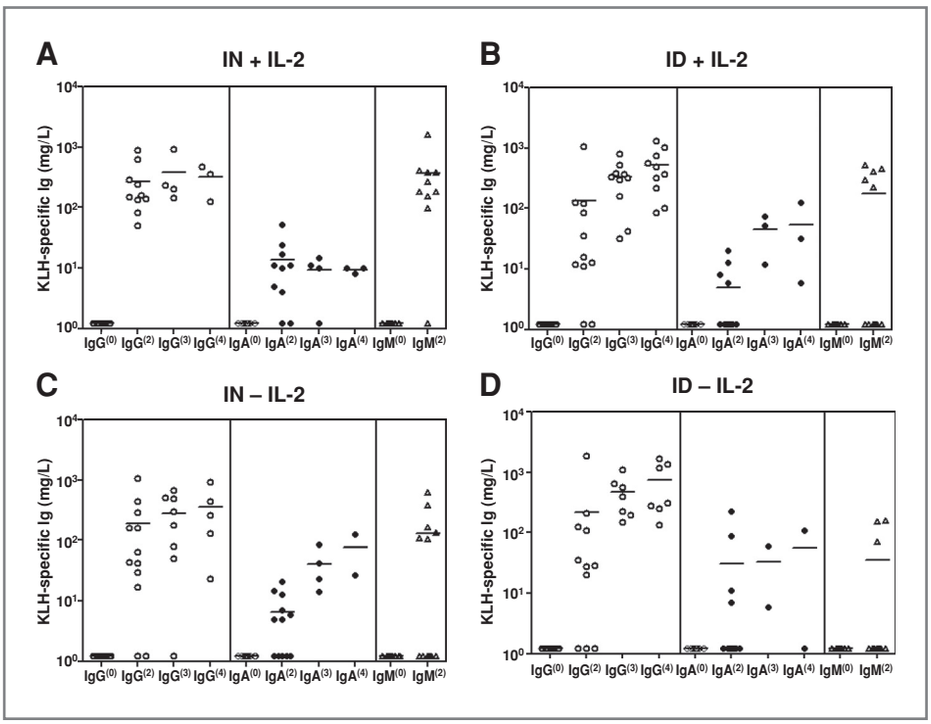

Figure 2. Induction of humoral KLH-specific responses by DC vaccination. KLH-specific IgG and IgA antibodies were quantitatively measured after each DC vaccination during the first vaccination cycle in sera of patients vaccinated intranodal (A; IN) or intradermal (B; ID) with concomitant IL-2 treatment and patients vaccinated intranodal (C) or intradermal (D) without IL-2 treatment. IgM antibodies were analyzed after the first vaccination only. Per time point each dot represents 1 patient. Horizontal lines represent group averages per time point. Numbers in parentheses indicate after which vaccination serum antibodies were analyzed.

intranodally vaccinated patients was comparable (data not shown). IL-4 or IL-10 production was low upon stimulation of DTH-derived T cells in all groups (data not shown). Together, these data suggest that intradermally injected DCs are more potent in inducing functional antigen-specific $CD8^+$ T cells as compared with intranodally injected DC.

Effect of IL-2 administration on tumor antigen–specific responses in DTH-derived T cells

In intranodally vaccinated patients, treatment with IL-2 resulted in tetramer-positive (58% −IL-2 vs. 88% +IL-2) and peptide-specific (50% −IL-2 vs. 86% +IL-2) DTH-derived T cells in a higher percentage of patients. In addition, IL-2 treatment significantly increased the percentage

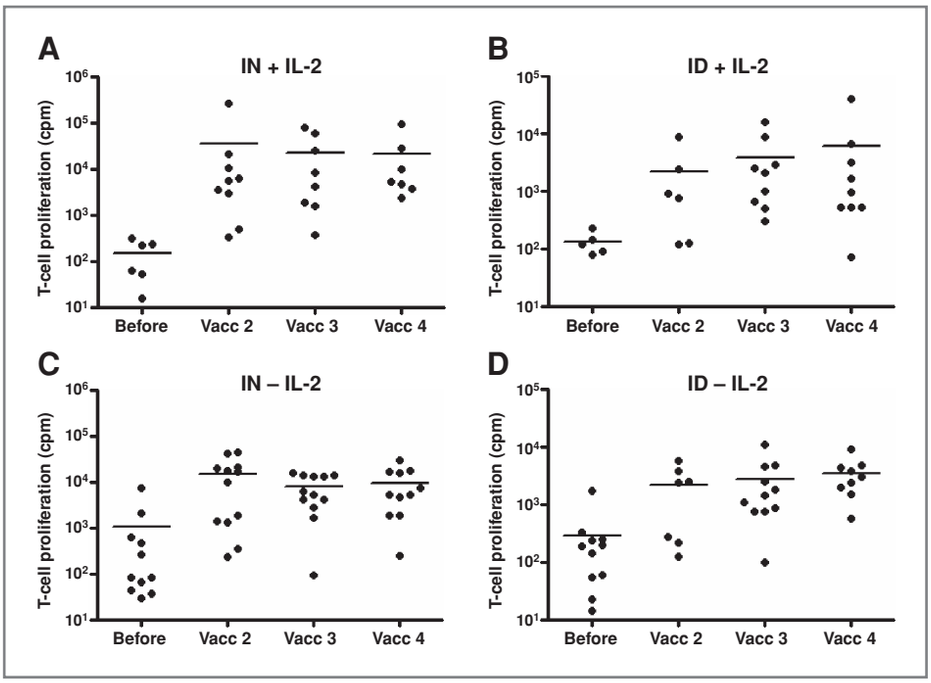

Figure 3. Induction of KLH-specific T-cell responses by DC vaccination. KLH-specific T-cell proliferation was analyzed before vaccination and after each DC vaccination (vacc) during the first vaccination cycle in PBMCs of patients vaccinated intranodally (A; IN) or intradermally (B; IN) with concomitant IL-2 treatment and patients vaccinated intranodally (C) or intradermally (D) without IL-2 treatment. Per time point each dot represents 1 patient. Horizontal lines represent group averages per time point.

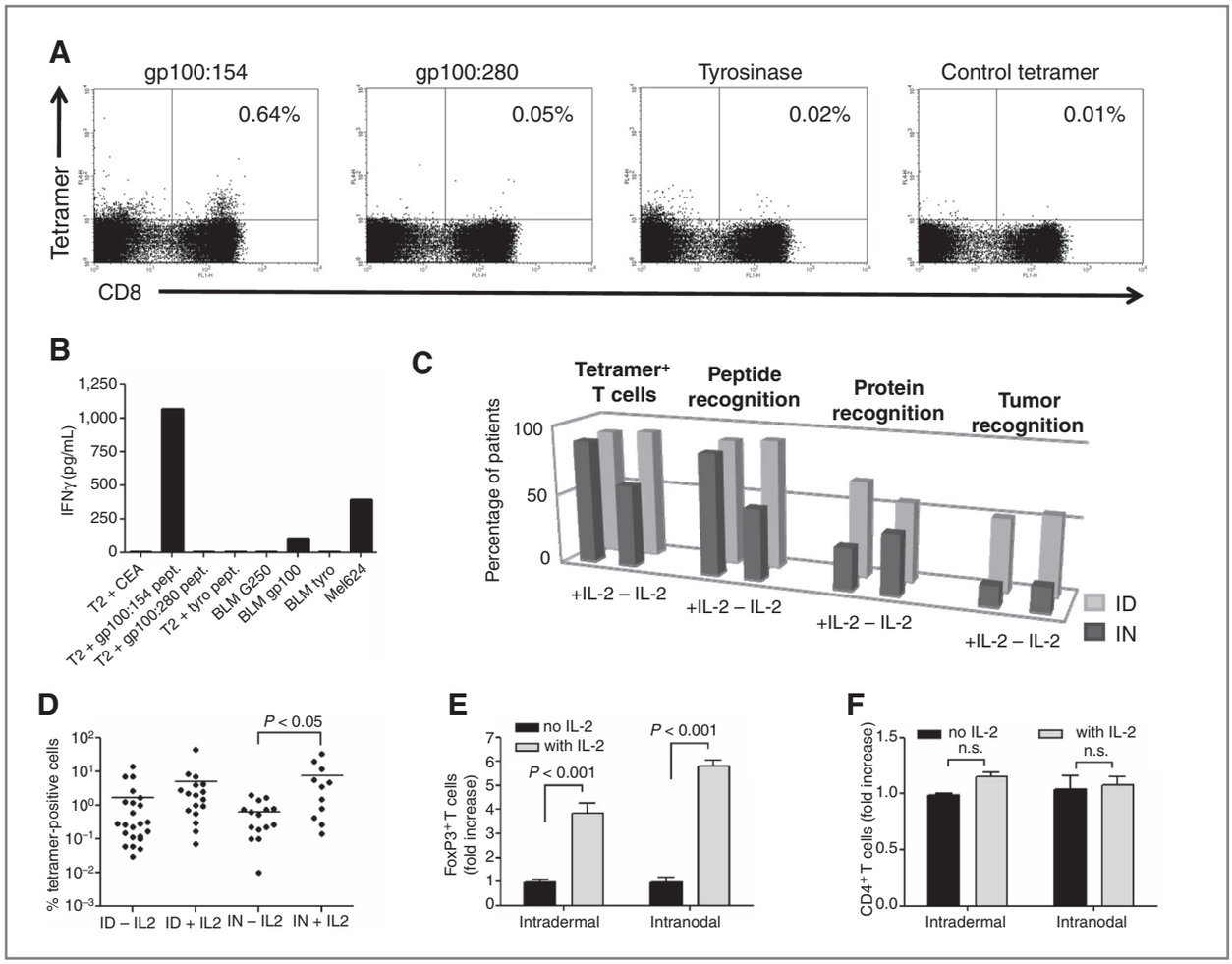

Figure 4. Tumor-specific responses after DC vaccination. The presence and functionality of antigen-specific T cells against gp100 and tyrosinase were tested in lymphocytes cultured from DTH sites. A, example of tetramer staining of T cells cultured from a DTH reaction of patient II-A-10. Cells were stained with allophycocyanin-labeled tetramers encompassing the gp100:154 peptide, gp100:280 peptide, tyrosinase peptide, or an irrelevant peptide (control) and with CD8 FITC. B, IFNγ production by the same T cells after stimulation with T2 cells loaded with tumor peptides, BLM cells expressing tumor proteins, or Mel624 cells. C, immunologic response is summarized for all patients (see also Supplementary Table S1). The plots represent percentages of patients in each group with tumor antigen–specific T cells as determined by either IFNγ release or cytolytic activity as measured by a ^{51}Cr release assay. D, percentages of gp100:154, gp100:280, or tyrosinase tetramer-positive CD8$^+$ T cells in lymphocytes cultured from DTH biopsies of tetramer-positive patients. E and F, Treg frequencies in patients vaccinated intradermally or intranodally with or without concomitant IL-2. Frequencies of CD4$^+$FOXP3$^+$ (E) and CD4$^+$ (F) T cells were determined by flow cytometry in CD3$^+$ PBMCs (after DC vaccinations) or CD3$^+$ peripheral blood leukocytes isolated from leukapheresis material (before the first vaccination). Data are shown as fold increase of T-cell frequencies after vaccination compared with before vaccination. The graphs represent mean ± SEM of 4 patients per group. CEA, carcinoembryonic antigen; n.s., not significant.

of tetramer-positive CD8$^+$ T cells in intranodally vaccinated patients that had tetramer-positive CD8$^+$ T cells ($P < 0.05$; Fig. 4D). However, protein (42% −IL-2 vs. 29% +IL-2) or tumor (17% −IL-2 vs. 14% +IL-2) recognition was comparable irrespective of the addition of IL-2.

In intradermally vaccinated patients, treatment with IL-2 did not affect the numbers of tetramer-positive cells (91% −IL-2 vs. 89% +IL-2) or IFNγ production upon stimulation with peptide (91% −IL-2 vs. 89% +IL-2), protein (55% −IL-2 vs. 67% +IL-2), or tumor cells (55% −IL-2 vs. 50% +IL-2). Thus, in patients injected with a DC vaccine, concomitant treatment with IL-2 has no profound benefit on the induction of tumor-specific functional T cells. However, after both intranodal and intradermal vaccination, coadministration of IL-2 resulted in increased percentages of FOXP3$^+$CD4$^+$ regulatory T cells (Treg) in peripheral blood (Fig. 4E), whereas percentages of total CD4$^+$ T cells were unaffected (Fig. 4F).

Discussion

In this study, we compared intradermal with intranodal injection of a DC vaccine with regard to redistribution of the vaccine to draining lymph nodes and characteristics of the antigen-specific immune responses induced. The results of this study confirm and extend our previous observation

that after intradermal vaccination, DC migration never exceeded 4%, whereas after intranodal injection, up to 84% of the injected DCs migrated to adjacent lymph nodes (17). Although the median percentage of migrating cells is substantially higher after intranodal injection, it resulted in large variation in the migratory capacity of DCs. Importantly, in 7 intranodally vaccinated patients, no redistribution of injected DCs from the injection depot was found at all, whereas in all intradermally vaccinated patients, at least a small fraction of the injected DCs migrated to nearby lymph nodes. In a previous study, we found that after intranodal injection, redistribution to adjacent lymph nodes was only observed when DCs were correctly injected into the lymph node, which in that study happened in only about 50% of the cases, despite injection under ultrasound guidance by a highly experienced radiologist (16). Inadequate delivery of DCs may therefore, at least, partly explain why also in this study no DC migration was detected in some intranodally vaccinated patients. However, as patients received 4 intranodal injections in the first vaccination cycle, it is very unlikely that none of the vaccines was delivered correctly into the lymph node, as also suggested by the induction of potent KLH-specific immune responses in all patients.

Although both intradermal and intranodal vaccinations induced tumor antigen–specific T cells, intradermal vaccination more often resulted in the induction of functional T cells recognizing full protein or tumor cells. These results are in line with previous DC vaccination trials in melanoma patients (11) and prostate cancer patients (38) by Kyte and colleagues and a study in metastatic renal cell carcinoma patients by Berntsen and colleagues (39) in which patients were more likely to achieve immunologic and clinical responses after intradermal administration of a DC vaccine than after intranodal injection. In contrast, in a small clinical study by Bedrosian and colleagues (10), intranodal DC vaccination resulted in superior T-cell activation compared with intradermal vaccination. However, in this study, only the presence of tetramer-positive cells recognizing tumor peptides was studied rather than recognition of whole tumor antigens, which need antigen processing by the proteasome. We here clearly showed that only a fraction of the tetramer-positive T cells appear to be bona fide cytotoxic T lymphocytes that can recognize native antigen expressed by tumor cells. In conclusion, the results of our study and other studies, counter intuitively, suggest that there is no clear advantage of intranodal vaccination over intradermal vaccination. This, together with the more technically demanding intranodal injections, strongly argues in favor of the intradermal route of administration.

Our observation that intranodal vaccination, despite increased redistribution of DCs to draining lymph nodes, does not result in improved immunologic responses compared with intradermal vaccination might be explained in several ways. First, injection of DCs directly into a lymph node may lead to a partial destruction of the lymph node architecture (14), which is unfavorable for T-cell activation. Second, after intranodal injection, the distribution of DCs to distant lymph nodes may partially occur passively via the flow of lymphatic vessels to nearby lymph nodes rather than via active migration of fully matured DCs. Thus, the percentage of actively migrating DCs ending up in the T-cell areas may be overestimated after intranodal administration. Third and related to this previous point, active migration of DCs may be related to postadministration maturation and might thereby increase the capacity of the injected DCs to properly activate antigen-specific T cells. After intradermal injection, all DCs that enter the lymph nodes are viable and have migrated. They may represent the most mature and hence most potent DCs that express high levels of costimulatory molecules, secrete large amounts of relevant proinflammatory cytokines, and induce the expression of tumor-relevant homing receptors on antigen-specific T cells (40). Thus, intradermally injected DCs may activate potent antigen-specific effector and memory T cells, leading to a strong and long-lasting antitumor response. In contrast, as a result of intranodal injection, all DCs, including less mature or nonviable DCs, are directly delivered into the lymph node, where they might even activate nonspecific or low-affinity antigen-specific T cells, nonfunctional T cells, or Tregs. This notion is supported by our finding that, in particular, after intranodal injection, supplementary IL-2 treatment results in more tetramer-positive $CD8^+$ T cells but less tumor recognition when compared with intradermal injection. Myeloid-derived suppressor cells present in the lymph nodes may further hamper full activation of intranodally injected DCs (41). In addition, the observation that large numbers of remaining (apoptotic) DCs are cleared by CD163-expressing macrophages infiltrating the lymph nodes within 48 hours (17) may contribute to a less favorable microenvironment after intranodal injection. We observed only few macrophages in the draining lymph nodes upon intradermal injection (17). CD163 is exclusively expressed by anti-inflammatory macrophage subsets, which decrease Th1 activation and induce Tregs (42, 43). Possibly, the presence of macrophages that have phagocytosed DCs may have a negative effect on the immune response, for instance, by the secretion of anti-inflammatory cytokines. Finally, the optimal cell number for DC vaccination has not been firmly established, not for intradermal, nor for intranodal vaccination (2). It is possible that the cell numbers that we used were optimal for intradermal vaccination but supraoptimal for intranodal vaccination.

In half of the patients, we coadministered IL-2 because of its capacity to stimulate the growth and expansion of antigen-specific cytotoxic T lymphocytes. IL-2 has been used alone or in combination with other treatments for melanoma (23–26, 44). Our data indicate that in intradermally vaccinated patients, IL-2 has no prominent effect, neither on the presence of tetramer-positive T cells nor on the occurrence of functional antigen-specific T cells. Although in intranodally vaccinated patients, simultaneous IL-2 treatment resulted in higher numbers of tetramer-positive T cells recognizing tumor peptides in a higher percentage of patients, these T cells were not capable of

recognizing native antigen on tumor cells. Most likely, addition of IL-2 causes nonspecific activation and proliferation of low-affinity antigen-specific T cells that are less potent to kill tumor cells due to their low affinity (45), especially after direct delivery of DCs in the lymph nodes. Furthermore, IL-2 not only expands effector T cells but likely also stimulates Tregs. Observations of increased FOXP3- or CD25-expressing $CD4^+$ T cells documented in several studies suggest that IL-2 therapy can increase Treg frequencies in cancer patients (46–49). Similar increases in demethylated *FOXP3i1* containing Tregs were reported recently by Wieczorek and colleagues in melanoma patients receiving IL-2 therapy (50). Therefore, we have analyzed the effect of supplemental IL-2 administration on Treg frequencies in blood of a set of randomly selected intranodally vaccinated patients in parallel to this clinical study, as described elsewhere (51). In short, we used a FOXP3 methylation–specific quantitative PCR assay (MS-qPCR) to measure Treg frequencies in PBMCs, and we validated the results by measuring CD4/Foxp3 T-cell frequencies by flow cytometric analysis. Using this method, we found that Treg frequencies were up to 3-fold increased in 5 of 6 tested patients receiving intranodal vaccination plus IL-2 when compared with prevaccination levels (mean percentage of Tregs in PBMCs from 3% to 11% as measured by MS-qPCR and from 1.1% to 7.5% by flow cytometry). No such increase was observed in the patients that had received the intranodal vaccinations alone. Increased Treg frequencies were detectable 3 weeks after the first IL-2 injection and persisted at least 3 weeks after the last injection. Here, we extend these findings: using flow cytometric analysis, we found that coadministration of IL-2 also significantly increased Treg frequencies in intradermally vaccinated patients. These findings support the notion that administration of IL-2 can increase Treg frequencies, although in this study, we did not observe major differences in the presence of functional antigen-specific T cells between patients vaccinated with or without supplemental IL-2 treatment.

In summary, intradermally injected DCs induce significantly more potent antitumor responses when compared with intranodally injected DCs. Although the percentage of DCs redistributed to nearby lymph nodes is lower after intradermal vaccination than after intranodal vaccination, the number of functional T cells is higher, which is reflected in improved tumor antigen recognition. Furthermore, our results suggest that concomitant IL-2 treatment does not enhance the induction of antitumor responses during DC-based immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The Clinical Pharmacy of the Radboud University Nijmegen Medical Centre, Christel van Riel, Linda Engelen, Mary-Lene Brouwer, and Marieke Kerkhoff are acknowledged for their assistance.

Grant Support

This work was supported by grants from the Dutch Cancer Society (KUN2006-3699), the EU (ENCITE HEALTH-F5-2008-201842, Cancer Immunotherapy LSHC-CT-2006-518234, and DC-THERA LSB-CT-2004-512074), the Netherlands Organization for Scientific Research (NWO-Vidi-917.76.363, AGIKO-92003250), the Vanderes Foundation, the NOTK foundation, and AGIKO RUNMC. C.G. Figdor received the NWO Spinoza award.

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Received May 16, 2011; revised July 8, 2011; accepted July 11, 2011; published OnlineFirst July 19, 2011.

References

1. Figdor CG, de Vries IJM, Lesterhuis WJ, Melief CJM. Dendritic cell immunotherapy: mapping the way. Nat Med 2004;10:475–80.
2. Lesterhuis WJ, Aarntzen EHJG, Vries IJM, Schuurhuis DH, Figdor CG, Adema GJ, et al. Dendritic cell vaccines in melanoma: from promise to proof? Crit Rev Oncol Hematol 2008;66:118–34.
3. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245–52.
4. Banchereau J, Palucka AK, Dhodapkar M, Burkeholder S, Taquet N, Rolland A, et al. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res 2001;61:6451–8.
5. de Vries IJM, Bernsen MR, Lesterhuis WJ, Scharenborg NM, Strijk SP, Gerritsen MJP, 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.
6. Lesterhuis WJ, de Vries IJM, Schuurhuis DH, Boullart ACI, Jacobs JFM, de Boer AJ, et al. Vaccination of colorectal cancer patients with CEA-loaded dendritic cells: antigen-specific T cell responses in DTH skin tests. Ann Oncol 2006;17:974–80.
7. Schuler-Thurner B, Schultz ES, Berger TG, Weinlich G, Ebner S, Woerl P, 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.
8. Fong L, Brockstedt D, Benike C, Wu L, Engleman EG. Dendritic cells injected via different routes induce immunity in cancer patients. J Immunol 2001;166:4254–9.
9. Fong L, Hou YF, Rivas A, Benike C, Yuen A, Fisher GA, et al. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. Proc Natl Acad Sci U S A 2001;98:8809–14.
10. Bedrosian I, Mick R, Xu SW, Nisenbaum H, Faries M, Zhang P, et al. Intranodal administration of peptide-pulsed mature dendritic cell vaccines results in superior CD8+ T-cell function in melanoma patients. J Clin Oncol 2003;21:3826–35.
11. Kyte JA, Mu L, Aamdal S, Kvalheim G, Dueland S, Hauser M, et al. Phase I/II trial of melanoma therapy with dendritic cells transfected with autologous tumor-mRNA. Cancer Gene Ther 2006;13:905–18.
12. Schadendorf D, Ugurel S, Schuler-Thurner B, Nestle FO, Enk A, Brocker EB, et al. Dacarbazine (DTIC) versus vaccination with autologous peptide-pulsed dendritic cells (DC) in first-line treatment of patients with metastatic melanoma: a randomized phase III trial of the DC study group of the DeCOG. Ann Oncol 2006;17:563–70.
13. de Vries IJM, Lesterhuis WJ, Scharenborg NM, Engelen LPH, Ruiter DJ, Gerritsen MJP, et al. Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. Clin Cancer Res 2003;9:5091–100.
14. de Vries IJM, Krooshoop DJEB, Scharenborg NM, Lesterhuis WJ, Diepstra JHS, van Muijen GNP, 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.
15. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med 2001;193:233–8.
16. de Vries IJM, Lesterhuis WJ, Barentsz JO, Verdijk P, van Krieken JH, Boerman OC, et al. Magnetic resonance tracking of dendritic cells in melanoma patients for monitoring of cellular therapy. Nat Biotechnol 2005;23:1407–13.
17. Verdijk P, Aarntzen EHJG, Lesterhuis WJ, Boullart ACI, Kok E, van Rossum MM, 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 2009;15:2531–40.
18. Dudda JC, Simon JC, Martin S. Dendritic cell immunization route determines CD8(+) T cell trafficking to inflamed skin: role for tissue microenvironment and dendritic cells in establishment of T cell-homing subsets. J Immunol 2004;172:857–63.
19. Eggert AAO, Schreurs MWJ, Boerman OC, Oyen WJC, de Boer AJ, Punt CJA, et al. Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. Cancer Res 1999;59:3340–5.
20. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature 2003;424:88–93.
21. Mullins DW, Sheasley SL, Ream RM, Bullock TNJ, Fu YX, Engelhard VH. Route of immunization with peptide-pulsed dendritic cells controls the distribution of memory and effector T cells in lymphoid tissues and determines the pattern of regional tumor control. J Exp Med 2003;198:1023–34.
22. Okada N, Tsujino M, Hagiwara Y, Tada A, Tamura Y, Mori K, et al. Administration route-dependent vaccine efficiency of murine dendritic cells pulsed with antigens. Br J Cancer 2001;84:1564–70.
23. Hersey P, Halliday GM, Farrelly ML, DeSilva C, Lett M, Menzies SW. Phase I/II study of treatment with matured dendritic cells with or without low dose IL-2 in patients with disseminated melanoma. Cancer Immunol Immunother 2008;57:1039–51.
24. Nagayama H, Sato K, Morishita M, Uchimaru K, Oyaizu N, Inazawa T, et al. Results of a phase I clinical study using autologous tumour lysate-pulsed monocyte-derived mature dendritic cell vaccinations for stage IV malignant melanoma patients combined with low dose interleukin-2. Melanoma Res 2003;13:5210–30.
25. Slingluff CL Jr, Petroni GR, Yamshchikov GV, Barnd DL, Eastham S, Galavotti H, et al. Clinical and immunologic results of a randomized phase II trial of vaccination using four melanoma peptides either administered in granulocyte-macrophage colony-stimulating factor in adjuvant or pulsed on dendritic cells. J Clin Oncol 2003;21:4016–26.
26. Schadendorf D, Algarra SM, Bastholt L, Cinat G, Dreno B, Eggermont AMM, et al. Immunotherapy of distant metastatic disease. Ann Oncol 2009;20:41–50.
27. Shimizu K, Fields RC, Giedlin M, Mule JJ. Systemic administration of interleukin 2 enhances the therapeutic efficacy of dendritic cell-based tumor vaccines. Proc Natl Acad Sci U S A 1999;96:2268–73.
28. Shrikant P, Mescher MF. Opposing effects of IL-2 in tumor immunotherapy: promoting CD8 T cell growth and inducing apoptosis. J Immunol 2002;169:1753–9.
29. de Vries IJM, Eggert AAO, Scharenborg NM, Vissers JLM, Lesterhuis WJ, Boerman OC, et al. Phenotypical and functional characterization of clinical grade dendritic cells. J Immunother 2002;25:429–38.
30. Thurner B, Roder C, Dieckmann D, Heuer H, Kruse M, Glaser A, et al. Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. J Immunol Methods 1999;223:1–15.
31. Bakker ABH, Schreurs MWJ, Tafazzul G, Deboer AJ, Kawakami Y, Adema GJ, et al. Identification of a novel peptide derived from the melanocyte-specific Gp100 antigen as the dominant epitope recognized by an Hla-A2.1-restricted antimelanoma Ctl line. Int J Cancer 1995;62:97–102.
32. Cox AL, Skipper J, Chen Y, Henderson RA, Darrow TL, Shabanowitz J, et al. Identification of a peptide recognized by 5 melanoma-specific human cytotoxic T-cell lines. Science 1994;264:716–9.
33. Wolfel T, Schneider J, Zumbuschenfelde KHM, Rammensee HG, Rotzschke O, Falk K. Isolation of naturally processed peptides recognized by cytolytic T-lymphocytes (Ctl) on human-melanoma cells in association with Hla-A2.1. Int J Cancer 1994;57:413–8.
34. Jacobs JFM, Punt CJ, Lesterhuis WJ, Sutmuller RP, Brouwer HMH, Scharenborg N, et al. Dendritic cell vaccination in combination with anti-CD25 monoclonal antibody treatment: a phase I/II study in metastatic melanoma patients. Clin Cancer Res 2010;16:5067–78.
35. Bakker ABH, Schreurs MWJ, de Boer AJ, Kawakami Y, Rosenberg SA, Adema GJ, et al. Melanocyte lineage-specific antigen Gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. J Exp Med 1994;179:1005–9.
36. Carbone FR, Moore MW, Sheil JM, Bevan MJ. Induction of cytotoxic T lymphocytes by primary *in vitro* stimulation with peptides. J Exp Med 1988;167:1767–79.
37. Lesterhuis WJ, Schreibelt G, Scharenborg NM, Brouwer HM, Gerritsen MJ, Croockewit S, et al. Wild-type and modified gp100 peptide-pulsed dendritic cell vaccination of advanced melanoma patients can lead to long-term clinical responses independent of the peptide used. Cancer Immunol Immunother 2011;60:249–60.
38. Kyte JA, Gaudernack G. Immuno-gene therapy of cancer with tumour-mRNA transfected dendritic cells. Cancer Immunol Immunother 2006;55:1432–42.
39. Berntsen A, Trepiakas R, Wenandy L, Geertsen PF, Straten PT, Andersen MH, et al. Therapeutic dendritic cell vaccination of patients with metastatic renal cell carcinoma - a clinical, phase 1/2 trial. J Immunother 2008;31:771–80.
40. Kalinski P. Dendritic cells in immunotherapy of established cancer: roles of signals 1, 2, 3 and 4. Curr Opin Investig Drugs 2009;10:526–35.
41. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol 2004;4:941–52.
42. Savage ND, de BT, Walburg KV, Joosten SA, van MK, Geluk A, et al. Human anti-inflammatory macrophages induce Foxp3+ GITR+ CD25+ regulatory T cells, which suppress via membrane-bound TGFbeta-1. J Immunol 2008;181:2220–6.
43. Verreck FA, de BT, Langenberg DM, van der ZL, Ottenhoff TH. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. J Leukoc Biol 2006;79:285–93.
44. Bjoern J, Brimnes MK, Andersen MH, Thor SP, Svane IM. Changes in peripheral blood level of regulatory T cells in patients with malignant melanoma during treatment with dendritic cell vaccination and low-dose IL-2. Scand J Immunol 2011;73:222–33.
45. Savage PA, Boniface JJ, Davis MM. A kinetic basis for T cell receptor repertoire selection during an immune response. Immunity 1999;10:485–92.
46. Zhang H, Chua KS, Guimond M, Kapoor V, Brown MV, Fleisher TA, et al. Lymphopenia and interleukin-2 therapy alter homeostasis of CD4(+) CD25(+) regulatory T cells. Nat Med 2005;11:1238–43.
47. Wei S, Kryezek I, Edwards RP, Zou LH, Szeliga W, Banerjee M, et al. Interleukin-2 administration alters the CD4(+)FOXP3(+) T-cell pool and tumor trafficking in patients with ovarian carcinoma. Cancer Res 2007;67:7487–94.
48. Cesana GC, DeRaffele G, Cohen S, Moroziewicz D, Mitcham J, Stoutenburg J, et al. Characterization of CD4(+)CD25(+) regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. J Clin Oncol 2006;24:1169–77.
49. Ahmadzadeh M, Rosenberg ST. IL-2 administration increases CD4(+) CD25(hi) Foxp3(+) regulatory T cells in cancer patients. Blood 2006;107:2409–14.
50. Wieczorek G, Asemissen A, Model F, Turbachova I, Floess S, Liebenberg V, et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. Cancer Res 2009;69:599–608.
51. de Vries IJM, Castelli C, Huygens C, Jacobs JF, Stockis J, Schuler-Thurner B, et al. Frequency of circulating Tregs with demethylated FOXP3 intron 1 in melanoma patients receiving tumor vaccines and potentially Treg-depleting agents. Clin Cancer Res 2011;17:841–8.