Intradermal versus intranodal DC vaccination of melanoma patients

Route of administration modulates the induction of dendritic cell vaccine-induced antigen-specific T cells in advanced melanoma patients

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**Translation relevance**

Dendritic cell vaccination constitutes a promising novel immunotherapy in cancer. For optimal T cell induction it is crucial for the dendritic cells 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 to intradermal administration, robust clinical trials comparing the different routes of administration are lacking.

Here, we directly compared the two 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 to intranodal vaccination. Thus, the more laborious and variable intranodal route of administration does not offer an advantage over intradermal vaccination.
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 immunological responses in melanoma patients and examined if concomitant administration of interleukin-2 (IL-2) increases the efficacy of the DC-vaccine.

Experimental design

HLA-A2.1+ melanoma patients scheduled for regional lymph node-dissection were vaccinated 4 times biweekly via intradermal or intranodal injection with 12-17x10^6 mature DC 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 three 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 51Chromium-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 DC migrated to adjacent lymph nodes upon intranodal vaccination, this was also highly variable with a complete absence of migration in 7/24 intranodally vaccinated patients. Intradermal vaccinations proved superior in inducing functional tumor antigen-specific T cells. Co-administration of IL-2 did not further augment the antigen-specific T cell response, but did result in higher Treg frequencies.

Conclusion

Intradermal vaccination resulted in superior anti-tumor T cell induction when compared to intranodal vaccination. No advantage of additional IL-2 treatment could be demonstrated.
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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).

Since DC are the main antigen-presenting cells of the immune system(3), various DC vaccines have been evaluated for the induction of anti-tumor 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 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 IL-4 and GM-CSF and subsequently matured and loaded with peptides derived from tumor antigens(4-12).

We and others previously demonstrated 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 DC 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 DC 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 demonstrated 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), demonstrating 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 immunological responses(8;18-22). Although we have shown that intranodal (IN) administration results in a much higher migration of injected DC to the draining lymph nodes compared to intradermal (ID) vaccination(14;17), it is not clear whether IN administration also results in a superior immunological and clinical response. Therefore, the aim of this clinical study was to compare
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Intranodal and intradermal DC vaccine administration, to determine the effect of route of administration on the induction of immunological 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 if 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 ID or IN 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 IN without IL-2 or the ID without IL-2 arm in an alternating manner. Assignment was performed by IJMdv who had no knowledge about the clinical characteristics of the patients. IN vaccination was performed in a clinically tumor-free lymph node under ultrasound guidance. ID vaccination was performed at 5-10 cm distal from a (preferably inguinal) clinically tumor-free lymph node, by clinicians with extensive experience with the procedure (WJL, EHJGA, CJAP). Since the first vaccination was administered one 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 KLH-loaded DC on the side of the lymph node dissection, and an injection of peptide-pulsed DC on the contra-lateral side. The latter vaccine could be radionuclide-labeled or not. The DC vaccine consisted of autologous mature DC pulsed with gp100 and tyrosinase peptides and keyhole limpet hemocyanin (KLH). Patients received one cycle consisting of four DC vaccinations administered at a biweekly interval. IL-2 was administered by subcutaneous injections (at 9 MIU) once daily for one week starting three days after each DC vaccination. Twenty four to 48 hours after the first vaccination a radical lymph node dissection was performed. One to two weeks after the fourth vaccination a DTH test was performed. All patients who remained free of disease progression after the first vaccination cycle were eligible for two maintenance cycles, each at 6-
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month intervals, and each consisting of three biweekly IN vaccinations without IL-2 (supplementary figure 1). 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 DC were generated from peripheral-blood mononuclear cells (PBMC) and matured with autologous monocyte-conditioned medium containing prostaglandin E$_2$ (10 µg/ml; Pharmacia & Upjohn, Puurs, Belgium) and recombinant tumor necrosis factor alpha (10 ng/ml; provided by Dr G. Adolf, Bender Wien, Vienna, Austria), as described(29;30). This procedure gave rise to mature DC meeting the release criteria(29).

**Peptide pulsing**

DC 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 performed as described(13), and cells were resuspended in 0.1 ml for injection.

**DC migration**

DC migration node 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 dendritic cells that had migrated from the injection depot to following lymph nodes after 2 days(16). Patients simultaneously received peptide-loaded DC 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. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Paque density centrifugation. PBMC were stimulated with KLH (4 µg/2x10^5 PBMC) in medium with 10% human AB serum (Sanquin bloodbank, Nijmegen, The Netherlands). After 3 days, cells were pulsed with ^3H-thymidine for 8 hours, and incorporation was measured with a betacounter. Experiments were performed in triplicate.

KLH-specific antibody production

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, San Diego, CA). 3,3’ 5,5-tetramethylbenzidine was used as a substrate and plates were measured in a microtiter plate reader at 450nm. For quantification an isotype-specific calibration curve for the KLH response was included in each microtiter plate (Jacobs et al. in preparation).

Delayed type hypersensitivity test

Previously, we have developed a monitoring tool to assess T cell responses following vaccination, using DTH-infiltrated T cells(13). We have demonstrated that the presence of DTH-infiltrated vaccine-specific T cells correlates with clinical outcome(5). In this study, DTH skin tests were performed approximately two weeks after the fourth vaccination as described previously(5). DC pulsed with gp100 and tyrosinase peptides (2 to 10x10^6 DC each) were injected ID in the skin of the back of the patient at four 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 HS and IL-2. After 2 to 4 weeks of culturing, T cells were tested.
Outgrowth of DTH-infiltrating lymphocytes was defined as at least $1 \times 10^5$ 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 one 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-MHC complexes containing gp100\textsubscript{154-167}, gp100\textsubscript{280-288} or tyrosinase\textsubscript{369-376} peptide (Sanquin, Amsterdam, The Netherlands) as described\textsuperscript{(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 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\textsuperscript{(35)}. Cytokine production was measured in supernatants after 16 hours of coculture by cytometric bead array (Th1/Th2 Cytokine CBA\textsuperscript{1}; BD Pharmingen). The reason we used three 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\textsuperscript{(36)}.

**FOXP3 staining**
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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 performed with a FACSCalibur (Becton Dickinson).

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

Patient characteristics and treatment
Of a total of 47 patients enrolled in this study four patients were excluded from the analysis; two patients due to rapid disease progression before completing the first vaccination cycle, one patient was HLA-A2.1 negative and one patient developed brain metastases prior to initiation of treatment. The patients were assigned to four different groups (Supplementary figure 1 and 2). Thirteen patients received only one cycle consisting of four vaccinations, 7 patients received one additional maintenance cycle, consisting of 3 IN vaccinations, and 22 patients completed the full treatment schedule of two additional maintenance cycles of 3 IN 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 ID vaccinated patients not treated with IL-2, 27 months (range 6–115 months) for ID vaccinated patients treated with IL-2, 42 months (range 7–74 months) for IN vaccinated patients not treated with IL-2 and 14 months (range 4–83 months) for the IN 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 DC
Patients in the different groups received on average $12 \times 10^6$ to $17 \times 10^6$ DC per vaccination during the first cycle (Table 2). After maturation, DC 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 DC between the different groups. Furthermore, there were no significant differences in CCR7 expression between IN and ID injected DC (Figure 1A). The distribution of $^{111}$In-labeled DC was determined 24-48 h after the first ID or IN vaccination by scintigraphic imaging (Figure 1). The mean
overall redistribution of injected cells from the ID injection depot to draining lymph nodes was relatively constant, with a median migration of 1.0% (range 0.2-4.0%, Figure 1B). Although the percentage of migrating cells from the injection depot was significantly higher when the cells were injected IN, this was also much more variable (median migration 3.2%, range 0-84%): in all ID vaccinated patients at least a small fraction of the injected DC actively migrated to regional lymph nodes, whereas in 7/24 IN vaccinated patients no migration was observed at all. This may be caused by incorrect injection of intranodal vaccines during the first of four injections, as we have shown previously (16).

It is possible that upon IN injection part of the redistribution may have taken place as the result of passive lymphatic flow rather than active migration if the DC 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 LNs that were targeted upon injection and found no difference between IN (median 1, range 0-6) and ID vaccination (median 1, range 1-5).

**KLH-specific immune responses**

To investigate whether the differences in distribution after IN and ID vaccination resulted in differences in immunological 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 (Figure 2A-D). KLH-specific T cell proliferation was measured in PBMC 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 IN DC vaccination (Figure 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.

**Effect of route of administration on tumor antigen-specific responses in DTH-derived T cells**
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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, while functional tumor antigen-specific T cells were present in biopsies taken from DTH challenges(5;37). Since 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 out 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 1, Figure 4). In ID vaccinated patients, tetramer positive CD8+ T cells were detected in the DTH of 90% of the patients, compared to 70% in the IN vaccinated group (P=0.2). Although there were also no significant differences in tumor peptide recognition (90% ID vs 70% IN, P=0.2), or protein recognition (60% ID vs 37% IN, P=0.2), DTH-derived CD8+ T cells from ID vaccinated patients more frequently recognized tumor cells expressing gp100 and tyrosinase (53% ID vs 16% IN, 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 ID vaccinated patients and IN 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 ID injected DC are more potent in inducing functional antigen-specific CD8+ T cells as compared to IN injected DC.

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

In IN vaccinated patients, treatment with IL-2 resulted in tetramer positive (58% -IL-2 vs 88% +IL2) and peptide-specific (50% -IL-2 vs 86% +IL2) DTH-derived T cells in a higher percentage of patients. In addition, IL-2 treatment significantly increased the percentage of tetramer positive CD8+ T cells in IN vaccinated patients that had tetramer positive CD8+ T cells (P<0.05, Figure 4D). However, protein (42% -IL-2 vs 29% +IL2) or tumor (17% -IL-2 vs 14% +IL2) recognition was comparable irrespective of the addition of IL-2.

In ID vaccinated patients, treatment with IL-2 did not affect the numbers of tetramer positive cells (91% -IL-2 vs 89% +IL2) or IFN-γ production upon stimulation with peptide (91% -IL-2 vs 89% +IL2), protein (55% -IL-2 vs 67% +IL2) or tumor cells (55% -IL-2 vs 50% +IL2). Thus, in patients injected with a DC vaccine, concomitant treatment with IL-2 has no profound benefit on the induction of tumor-specific
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functional T cells. However, after both IN and ID vaccination, co-administration of IL-2 resulted in increased percentages of FOXP3+CD4+ regulatory T cells in peripheral blood (figure 4e), whereas percentages of total CD4+ T cells were unaffected (figure 4f).
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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 ID vaccination DC migration never exceeded 4%, whereas after IN injection up to 84% of the injected DC migrated to adjacent lymph nodes(17). Although the median percentage of migrating cells is substantially higher after IN injection, IN injection resulted in large variation in the migratory capacity of DC. Importantly, in 7 IN vaccinated patients, no redistribution of injected DC from the injection depot was found at all, whereas in all ID vaccinated patients at least a small fraction of the injected DC migrated to nearby lymph nodes. In a previous study we found that after IN injection, redistribution to adjacent lymph nodes was only observed when DC were correctly injected into the lymph node, which in that study happened in only ~50% of the cases, despite injection under ultrasound guidance by a highly experienced radiologist(16). Inadequate delivery of DC may therefore at least partly explain why also in this study in some IN vaccinated patients no DC migration was detected. However, as patients received 4 IN 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 ID and IN vaccinations induced tumor antigen-specific T cells, ID 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 et al and a study in metastatic renal cell carcinoma patients by Berntsen et al(39) in which patients were more likely to achieve immunological and clinical responses after ID administration of a DC vaccine than after IN injection. By contrast, in a small clinical study by Bedrosian et al(10) IN DC vaccination resulted in superior T cell activation compared to ID 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 demonstrated that only a fraction of the tetramer positive T cells appear to be bonafide CTL that can recognize native antigen expressed by...
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tumor cells. In conclusion, the results of our study and other studies, counter-intuitively, suggest that there is no clear advantage of IN vaccination over ID vaccination. This, together with the more technically demanding IN injections, strongly argues in favour of the ID route of administration.

Our observation that IN vaccination, despite increased redistribution of DC to draining lymph nodes, does not result in improved immunological responses compared to ID vaccination might be explained in several ways. First, injection of DC directly into a lymph node may lead to a partial destruction of the lymph node architecture(14), which is unfavourable for T cell activation. Secondly, after IN injection the distribution of DC 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 DC. Thus the percentage of actively migrating DC ending up in the T cell areas may be overestimated after IN administration. Third, and related to this previous point, active migration of DC may be related to post-administration maturation and might thereby increase the capacity of the injected DC to properly activate antigen-specific T cells. After ID injection, all DC that enter the lymph nodes are viable and have migrated. They may represent the most mature and hence most potent DC, 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, ID injected DC may activate potent antigen-specific effector and memory T cells, leading to a strong and long-lasting anti-tumor response. By contrast, as a result of IN injection, all DC, including less mature DC or nonviable DC, are directly delivered into the lymph node, where they might even activate non-specific or low affinity antigen-specific T cells, non-functional T cells, or regulatory T cells. This notion is supported by our finding that, in particular after IN injection, supplementary IL-2 treatment results in more tetramer positive CD8+ T cells but less tumor recognition when compared to ID injection. Myeloid-derived suppressor cells present in the lymph nodes may further hamper full activation of IN injected DC(41). In addition, the observation that large numbers of remaining (apoptotic) DC are cleared by CD163-expressing macrophages infiltrating the lymph nodes within 48 hours(17) may contribute to a less favourable microenvironment after IN injection. We observed only few macrophages in the draining lymph nodes upon ID injection(17). CD163 is exclusively expressed by anti-inflammatory macrophage subsets, which decrease Th1 activation and induce regulatory T cells(42;43).
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Possibly, the presence of macrophages that have phagocytosed DC may have a negative effect on the immune response, for instance by the secretion of anti-inflammatory cytokines. Lastly, the optimal cell number for DC vaccination has not been firmly established, not for ID, nor for IN vaccination(2). It is possible that the cell numbers that we used were optimal for ID vaccination but supra-optimal for IN vaccination.

In half of the patients we co-administered 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 ID 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 IN 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 non-specific 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 DC in the lymph nodes. Furthermore, IL-2 not only expands effector T cells, but likely also stimulates regulatory T cells (Treg). 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 Wiezcorek et al in melanoma patients receiving IL-2 therapy(50). Therefore, we have analysed the effect of supplemental IL-2 administration on Treg frequencies in blood of a set of randomly selected IN 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 cytometry analysis. Using this method we found that Treg frequencies were up to 3-fold increased in 5 out of 6 tested patients receiving IN vaccination plus IL-2 when compared to pre-vaccination 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 IN 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 that co-administration of IL-2 also significantly increased Treg frequencies in ID 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, ID injected DC induce significantly more potent anti-tumor responses when compared with IN injected DC. Although the percentage of DC redistributed to nearby lymph nodes is lower after ID vaccination than after IN 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 anti-tumor responses during DC-based immunotherapy.
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Tables

Table 1. Patient characteristics

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### Table 1 (continued). Patient characteristics

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<th>M status</th>
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<th>Tyrosinase</th>
<th>Nr. of cycle</th>
<th>Relapse free (stage III) or progression free (stage IV) interval</th>
<th>Overall survival</th>
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1 Gp100 and tyrosinase expression on the primary tumor was analyzed by immunohistochemistry. Intensity en percentage of positive cells were scored centrally and semi-quantitatively by a pathologist. Intensity was scored as low (+), intermediate (++), or high (+++).

2 Number of received vaccination cycles (one cycle consists of 4 (1st cycle) or 3 (2nd and 3rd cycle) vaccinations and a DTH test)

3 Ongoing progression free and overall survival after surgery of local metastasis
### Table 2. Characteristics of injected DC

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<th>Group</th>
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<th>CD80</th>
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<th>HLA-ABC</th>
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<td>IN + IL-2</td>
<td>15 (±5)</td>
<td>83 (±14)</td>
<td>82 (±14)</td>
<td>98 (±3)</td>
<td>96 (±7)</td>
<td>98 (±3)</td>
<td>91 (±11)</td>
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<tr>
<td>ID + IL-2</td>
<td>17 (±12)</td>
<td>82 (±14)</td>
<td>81 (±15)</td>
<td>97 (±2)</td>
<td>89 (±8)</td>
<td>94 (±6)</td>
<td>73 (±22)</td>
</tr>
<tr>
<td>IN – IL-2</td>
<td>12 (±3)</td>
<td>92 (±5)</td>
<td>81 (±14)</td>
<td>96 (±6)</td>
<td>96 (±7)</td>
<td>99 (±1)</td>
<td>79 (±14)</td>
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<td>ID – IL-2</td>
<td>13 (±3)</td>
<td>87 (±7)</td>
<td>78 (±12)</td>
<td>94 (±8)</td>
<td>84 (±15)</td>
<td>96 (±4)</td>
<td>68 (±24)</td>
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\(^1\)Average number of DC injected per vaccination (mean ± SD) during the first cycle. Patients received a total of four vaccinations.
Figure legends

Figure 1. DC migration after intranodal or intradermal injection.
(a) Expression of CCR7 measured by flow cytometry on mature DC for the first vaccination. Data are shown as percentage of cells expressing CCR7. One symbol represents one 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 ID and IN injection of 111In-labeled DC imaged by scintigraphy of the lymph node region. One symbol represents one injection of 15x10^6 cells; horizontal lines represent mean redistribution. (d and e) Scintigraphic images showing the redistribution of 111In-labeled cells from the injection depot (arrows) to nearby lymph nodes (arrow heads) 48h after injection are depicted from a patient after ID (d; patient II-D-01) and IN (e; patient II-C-01) vaccination.

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 IN (a) or ID (b) with concomitant IL-2 treatment, and patients vaccinated IN (c) or ID (d) without IL-2 treatment. IgM antibodies were analyzed after the first vaccination only. Per time point each dot represents one patient. Horizontal lines represent group averages per time point. Numbers in brackets indicate after which vaccination serum antibodies were analyzed.

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 during the first vaccination cycle in PBMC of patients vaccinated IN (a) or ID (b) with concomitant IL-2 treatment, and patients vaccinated IN (c) or ID (d) without IL-2 treatment. Per time point each dot represents one patient. Horizontal lines represent group averages per time point.

Figure 4. Tumor-specific responses after DC vaccination.
Intradermal versus intranodal DC vaccination of melanoma patients

The presence and functionality of antigen-specific T cells against gp100 and tyrosinase were tested in lymphocytes cultured from delayed type hypersensitivity (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. In (c) the immunological response is summarized for all patients (see also Table 4). 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 ⁵¹Chromium 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+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+ PBMC (after DC vaccinations) or CD3+ PBL isolated from leukapheresis material (before the first vaccination). Data are shown as fold increase of T cell frequencies after vaccination compared to before vaccination. The graphs represent mean ± SEM of four patients per group.
Figure 2

A. IN + IL-2

B. ID + IL-2

C. IN - IL-2

D. ID - IL-2

KLH specific Ig (mg/L)

IgG, IgA, IgM
Figure 3

A. IN + IL-2

B. ID + IL-2

C. IN - IL-2

D. ID - IL-2

T cell proliferation (cpm)

Before vacc 2 vacc 3 vacc 4
Figure 4

A box plot showing the percentage of patients with different tetramer+ T cells recognition of gp100:154, gp100:280, tyrosinase, and control tetramer.

B Graph showing IFN-γ levels in different conditions.

C Bar graph showing the percentage of patients with peptide, protein, and tumor recognition.

D Scatter plot showing the fold increase in FoxP3+ T cells with and without IL-2.

E Bar graph showing the fold increase in CD4+ T cells with and without IL-2.

F Bar graph showing the fold increase in CD4+ T cells with and without IL-2 in intradermal and intranodal sites.
Route of administration modulates the induction of dendritic cell vaccine-induced antigen-specific T cells in advanced melanoma patients


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