Published OnlineFirst February 4, 2013; DOI: 10.1158/1078-0432.CCR-12-1879

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Clin Cancer Res 2013;19:1525-1533. Published OnlineFirst February 4, 2013.

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Cancer Therapy: Clinical

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Targeting of ^{111}In-Labeled Dendritic Cell Human Vaccines Improved by Reducing Number of Cells

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Abstract

Purpose: Anticancer dendritic cell (DC) vaccines require the DCs to relocate to lymph nodes (LN) to trigger immune responses. However, these migration rates are typically very poor. Improving the targeting of *ex vivo* generated DCs to LNs might increase vaccine efficacy and reduce costs. We investigated DC migration *in vivo* in humans under different conditions.

Experimental Design: *HLA-A*02:01* patients with melanoma were vaccinated with mature DCs loaded with tyrosinase and gp100 peptides together with keyhole limpet hemocyanin (NCT00243594). For this study, patients received an additional intradermal vaccination with ^{111}In-labeled mature DCs. The injection site was pretreated with nonloaded, activated DCs, TNFα, or Imiquimod; granulocyte macrophage colony-stimulating factor was coinjected or smaller numbers of DCs were injected. Migration was measured by scintigraphy and compared with an intrapatient control vaccination. In an *ex vivo* tissue model, we measured CCL21-directed migration of ^{19}F-labeled DCs over a period of up to 12 hours using ^{19}F MRI to supplement our patient data.

Results: Pretreatment of the injection site induced local inflammatory reactions but did not improve migration rates. Both *in vitro* and *in vivo*, reduction of cell numbers to 5×10^6 or less cells per injection improved migration. Furthermore, scintigraphy is insufficient to study migration of such small numbers of ^{111}In-labeled DCs *in vivo*.

Conclusion: Reduction of cell density, not pretreatment of the injection site, is crucial for improved migration of DCs to LNs *in vivo*. *Clin Cancer Res; 19(6); 1525–33. ©2013 AACR.*

Introduction

Cellular therapy in patients with cancer aims to activate the immune system in a highly specific response against the tumor. In most studies, autologous antigen-presenting cells (APC), principally dendritic cells (DC), are activated and loaded with tumor antigen *ex vivo* (1). To trigger an effective immune response, the DCs need to relocate to immune reactive sites, such as lymph nodes (LN) upon injection back into the patient. Different routes are used to administer the DCs to the patients, of which intradermal (i.d.) injection is the most frequently used (2). The biodistribution of DCs after vaccination has been studied in humans, primarily using ^{111}In or ^{99m}Tc-labeled DCs and scintigraphy (2). However, even though about 20 clinical trials have been carried out with DCs delivered intradermally, the number of cells that reach a LN has never reproducibly exceeded 4% of the total cells injected (2). Why migration of mature DCs from the vaccination site is so poor is still unknown. Several reasons have been suggested, for example, the lack of an inflammatory microenvironment which would promote emigration of immune cells to afferent lymphatic vessels. In a mouse model, the migration of bone marrow–derived DCs into the draining LN could be dramatically increased by pretreating the injection site (3, 4). The skin was injected with an extra dose of DCs, pro-inflammatory cytokines [TNFα or interleukin (IL)-1α], or Toll-like receptor ligands before injection of the vaccine DCs. This pretreatment of the skin resulted in a 5- to 10-fold increase in the number of DCs in the draining LNs that correlated with a similar increase in T-cell activation. Other parameters of DC delivery might also contribute to more efficient emigration of DCs from the skin to the draining LNs, for

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

E.H.J.G. Aarntzen and M. Srinivas contributed equally to this work.

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doi: 10.1158/1078-0432.CCR-12-1879

Published OnlineFirst February 4, 2013; DOI: 10.1158/1078-0432.CCR-12-1879

Translational Relevance

To trigger an effective immune response, *ex vivo* generated tumor antigen-loaded dendritic cells (DC) need to relocate to lymph nodes (LN). Intradermal delivery is commonly used, due to its feasibility; however, migration rates never exceeded 4%. Increasing the numbers of *ex vivo* generated DCs reaching LNs might therefore increase the vaccine-specific response and reduce costs.

We investigated ^{111}In-labeled DC migration *in vivo* in humans after pretreatment of the injection site, using unloaded but activated DC, TNFα, or a synthetic Toll-like receptor (TLR) 7/8 ligand, or coinjection with granulocyte macrophage colony-stimulating factor. Furthermore, we developed an *in vitro* assay to measure human DC migration in a sensitive and standardized manner.

We show that reduction of cell density, not pretreatment of the injection site, is crucial for improved migration of DCs to LNs *in vivo*. However, current imaging modalities for *in vivo* tracking of DCs are insufficient to study migration of small numbers of ^{111}In-labeled DCs in the clinic.

example, the frequency of delivery, the infrastructure in terms of vascular and lymphatic networks at site of transplant and the local availability of oxygen and nutrients (5–7). Thus, conditioning the injection site, and perhaps indirectly the draining LNs, may stimulate the emigration of DCs from the skin and directly improve the clinical efficacy of DC-based therapy. Given this multitude of parameters, *in vitro* cell migration assays are warranted, as they allow high-throughput screening of influences of single parameters or combinations of parameters. The common *in vitro* cell migration assays that do exist, such as those based on microscopy or plate-based migration assays, have some major drawbacks: these techniques typically only work with small numbers of cells or nonopaque samples and thus do not replicate clinical conditions. Furthermore, most techniques assess migration in 2-dimensional (2D) setting, whereas *in vivo* migration requires motility in 3D. Thus, studying cell migration *in vitro* in a sensitive and quantitative manner for clinical application is extremely challenging and not readily feasible with current technology (2).

In this study, we investigated DC migration *in vivo* in humans after pretreatment of the injection site, using nonloaded but activated DCs, TNFα, or Imiquimod [a synthetic Toll-like receptor (TLR) 7/8 ligand], to induce a local inflammatory microenvironment; or coinjection with granulocyte macrophage colony-stimulating factor (GM-CSF) to enhance DC survival. We tracked ^{111}In-labeled vaccine DCs over a period of 48 hours by planar scintigraphy and compared migration with the LNs to a standardized control vaccination in the same patient. Furthermore, we modified an *in vitro* assay that closely reflects *in vivo* vaccination conditions to measure human DC migration in a standardized manner in tissue samples (8). By using this model, we measured CCL21-directed migration of ^{19}F-labeled vaccine DCs over a period of up to 12 hours using ^{19}F MRI. We show that reduction of cell density, not pretreatment, at the injection site is crucial for improved DC migration *in vivo*. In particular, we found that cell numbers greater than 1 million reduced migration both *in vitro* and *in* vivo. Furthermore, current clinical imaging modalities for clinical *in vivo* tracking of DCs are insufficient to study migration of small numbers of DCs in human studies.

Materials and Methods

DC vaccination in melanoma patients

In this study, patients with stage III and IV melanoma (according to American Joint Committee on Cancer criteria) who were scheduled for regional LN dissection with either curative or palliative intention were included. Additional inclusion criteria included HLA*A02:01 phenotype, melanoma expressing the melanoma-associated antigens gp100 and tyrosinase, and WHO performance status 0 or 1. Patients with brain metastases, serious concomitant disease, or a history of a second malignancy were excluded (see Supplementary Table S1 for details). 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. Intranodal vaccination was conducted in a clinically tumor-free LN under ultrasound guidance. Intradermal vaccination was conducted at 5 to 10 cm distal from a (preferably inguinal) clinically tumor-free LN, by clinicians with extensive experience with the procedure (W.J. Lesterhuis, E.H.J.G. Aarntzen, and C.J.A. Punt). Because the first vaccination was administered 1 to 2 days before regional LN dissection, presumably a significant benefit to the patient could not be expected. For this reason, the first vaccination always consisted of an injection of ^{111}In-labeled, but not peptide-pulsed and not keyhole limpet hemocyanin (KLH)–loaded DCs on the side of the LN dissection, and an injection of peptide-pulsed DCs on the contralateral side. The latter vaccine could be ^{111}In-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 LN dissection was conducted. One to 2 weeks after the fourth vaccination, a delayed-type hypersensitivity (DTH) test was conducted (9). 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. Patients were considered evaluable when they had completed the

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first vaccination cycle. Vaccine-specific immune response was the primary endpoint, as reported in previous publication (10).

DC preparation and characterization

KLH-loaded DCs were generated from peripheral blood mononuclear cells (PBMC) and matured with autologous monocyte-conditioned medium containing prostaglandin E2 (10 mg/mL; Pharmacia & Upjohn) and recombinant TNF-α (10 ng/mL; provided by Dr. G. Adolf, Bender Wien GmbH), as described (29). This procedure gave rise to mature DCs meeting the release criteria (29), and detailed vaccine phenotype is reported in previous publication (10).

Peptide pulsing

DCs were pulsed with the HLA class I gp100-derived peptides gp100:154–162 and gp100:280–288 and the tyrosinase-derived peptide tyrosinase: 369–377. Peptide pulsing was conducted as described (13), and cells were resuspended in 0.1 mL for injection.

^{111}In labeling and scintigraphy

For ^{111}In labeling, ^{111}In-oxine (Covidien) in 0.1 mol/L Tris-HCl (pH 7.0) was added to mature DCs for 15 minutes at room temperature as described previously (11). This results in 5 μCi per 15×10^6 cells. Cells were washed 3 times with PBS. Radiolabeling efficiency was determined by measuring activity in both the cell pellet and the washing buffer.

For this study, patients received an extra injection before scheduled radical LN dissection as mentioned above, or during the course of vaccination with increasing numbers of DCs resuspended in 100 μL of injection liquid and distributed over one or multiple injection sites, as indicated. At 24 and 48 hours postinjection, migration of DCs was imaged by planar scintigraphy (256 × 256 matrix, 174 and 247 keV ^{111}In photopeaks with 15% energy window) of the injection depot and corresponding LN basin with a gamma camera (Siemens ECAM, Hoffman Estates, Ill) equipped with medium energy collimators. Migration was quantified by region of interest (ROI) analysis of the individual nodes visualized on the images and expressed as the relative fraction of ^{111}In-labeled DCs that had migrated from the injection depot to draining LNs.

^{19}F-labeling and MRI *in vitro*

For ^{19}F labeling, the label particles were prepared using perfluoro-[15]-crown-5 ether ($C_{10}F_{20}O_5$; Exfluor Research Corp.) and PLGA (Resomer RG 502 H, lactide:glycolide molar ratio 48:52 to 52:48; Boehringer Ingelheim) as described (12). Ten milligrams per 10^6 cells of particles was added to the DC culture at day 3. Upon harvesting (day 8), cells were washed 3 times in PBS to remove excess particles.

For the migration assay, the technique was adapted (8) to replace the gel scaffold with a tissue sample, in this case bovine muscle. DCs were injected as a bolus in the center of a 2-mL Eppendorf tube filled with a single piece of tissue, leaving about 1 cm above the tissue as space for medium (see Fig. 3A). The region below the cells formed the control layer to account for nonspecific cell movement or sinking due to gravity, and the region above was the migration region through the use of a chemokine gradient consisting of 0.2μg recombinant human CCL21 (R&D Systems).

^{1}H and ^{19}F images were acquired on a 7T horizontal bore MR system with a ^{1}H/^{19}F volume coil. ^{1}H 2D spin echo images were taken for localization and 9 ^{19}F chemical shift spectroscopic imaging (CSI) was done every hour for up to 9 hours to measure cell migration. Proton images were acquired with TR/TE = 1000/22 ms and 0.125 × 0.125 × 1 mm^3 resolution. A 0.94 × 0.94 × 10 mm^3 matrix size with TR/TE = 400/2.94 ms was used for CSI. The sample was sealed and not moved for the duration of the imaging experiment. Temperature was maintained at 37°C using regulated warm air flows.

Histology

Sections (5 μm) of the resected skin from the injection sites were stained with hematoxylin. The staining protocol was done as described (13). Similar sections were cut from the tissue used for the *ex vivo* migration assay. These were stained with hematoxylin and an antibody against carbonic anhydrase 9 (CAIX; Novus Biologicals).

Statistical analyses

All comparisons were conducted using a 2-tailed unpaired *t* test with the intradermal migration after 48 hours without pretreatment as comparator.

Results

DC migration to LNs is poor after intradermal vaccination

In current and previous studies (10, 14), migration of DCs to skin draining LNs after i.d. injection (n = 18) was monitored by labeling the DC vaccine with ^{111}In and subsequent scintigraphy of injected region (14, 15). Figure 1A shows the percentage of migrating DCs after i.d. injection of 15×10^6 DCs at 24 or 48 hours postinjection. The average migration achieved was 1.2% after 24 hours and 1.4% after 48 hours, indicating that most of the migration occurred within 24 hours after injection. Migration rates never exceeded 4% of the total cells injected. Figure 1B shows representative scintigraphs, with the arrow indicating a pretreated site.

The induction of local inflammation does not improve DC migration

On the basis of data from mice studies (3, 4), we investigated whether pretreatment of the injection site to create a local inflammatory microenvironment optimized DC migration. To this end, we pretreated the injection sites with TNF-α ($n = 3$), Imiquimod ($n = 4$), or unloaded but activated DCs 6 ($n = 3$) or 24 hours ($n = 3$) before vaccination (Fig. 1A and B). In patients pretreated with TNF-α or activated DCs, the contralateral administration of

Published OnlineFirst February 4, 2013; DOI: 10.1158/1078-0432.CCR-12-1879

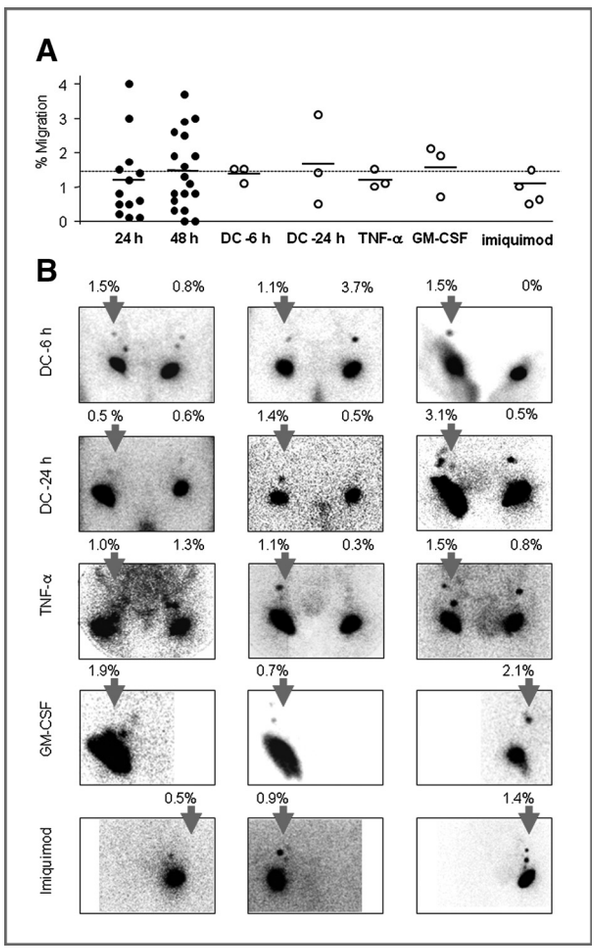

Figure 1. Pretreatment of the skin does not improve DC migration. Migration of DCs to skin draining LNs ($n = 18$) was monitored after intradermal injection with 15×10^6 ^{111}In-labeled DC vaccine and planar scintigraphy of injected region. A, the percentage of migrating DCs at 24 or 48 hours postinjection, or after different pretreatments, never exceeded 4% of the total cells injected. Statistical analyses using a 2-tailed unpaired *t* test showed no significant differences between the experimental conditions or migration at 48 hours after intradermal migration without pretreatment. B, for each pretreatment condition, TNF-α ($n = 3$), GM-CSF ($n = 3$), Imiquimod ($n = 4$), or unloaded but activated DCs 6 ($n = 3$) or 24 hours ($n = 3$) before vaccination, the individual images are shown. Upon GM-CSF addition, the induration and migration at the site of injection was markedly larger than after injection of DC alone, suggesting random migration into surrounding dermis. The arrows indicate the pretreated site.

15×10^6 ^{111}In-labeled vaccine DCs served as intrapatient control. Although the migration from the pretreated site was higher than in the control site in the majority of those patients, it still did not exceed 4% and did not significantly increase migration compared with unconditioned sites.

Our previous studies have shown that vaccine DCs rapidly lose viability at the injection site after intranodal or intradermal delivery, which might contribute to defective migration to LNs (15). Addition of GM-CSF as an adjuvant during DC vaccination might increase the survival of DCs and thereby increase migration rates. Three patients were injected with 15×10^6 DCs in normal saline containing 14×10^4 IU GM-SCF. Although the percentage of migrating cells after 48 hours was higher than average in 2 of 3 patients, migration was still within the established range. Of note, the induration at the site of injection was markedly larger than after injection of DC alone, suggesting random migration into surrounding dermis.

Finally, we pretreated the injection site of 4 patients by Imiquimod application every 12 hours for 2 days before vaccination. Again, no significant changes in migration rates were documented. Overall, the effects of pretreatment were limited and did not significantly improve subsequent migration of vaccine DCs to the draining LNs.

Local cell density is limiting factor

We conducted histologic analysis of the pretreatment injection sites 48 hours postinjection to validate our imaging findings. Injection of DCs consistently induced local inflammation, shown by infiltrates of leukocytes around vessels in the dermis, mainly neutrophils and eosinophils. The number of lymphocytes in the dermis increased, compared with normal skin. Pretreatment of the injection site with either TNF-α (Fig. 2A and B) or unloaded DCs (Fig. 2C) or coinjection with GM-CSF (Fig. 2D) induced some inflammation, as evidenced by the infiltration of leukocytes. However, this did not affect DC emigration.

Histology showed that macrophages had infiltrated the dermis and subcutis around the injection site. In areas with SPIO+, cells were enlarged and exhibited pale pink nuclei typical for necrotic cells. Thus, the histologic evidence shows that DCs die at the site of injection and invariably induce an inflammatory response consisting of macrophages and neutrophils. As no apparent differences were noted after different pretreatment regimens, together with the notion that dying vaccine DCs were found at higher rates with larger numbers of injected cells; these data suggest that it must be the local cell density itself that hampers efficient emigration of the injection site.

An *in vitro* assay to study migration of small numbers of DCs

To test this hypothesis, we adapted a novel assay we developed to mimic clinical vaccine conditions and which allows reliable quantification of reduced numbers of cells (8). The assay was modified to use a tissue sample instead of a gel scaffold, as done previously. DCs were cultured as used in patient vaccinations, with the addition of a labeling step for ^{19}F detection. This labeling has previously been shown to have no effect on the DCs, including their migration (ref. 8; Fig. 3A). The grid overlay shows the voxels used in the ^{19}F CSI scans. Imaging was carried out at hourly intervals without moving the sample, and the temperature was regulated. A control area under the cell injection was used to measure passive cell movement due to gravity. CCL21 was used to create a chemokine gradient for active CCR7-mediated migration. The percentage of total cells that migrated was calculated to represent the migratory cells. Figure 3B shows representative cell numbers at 1, 5,

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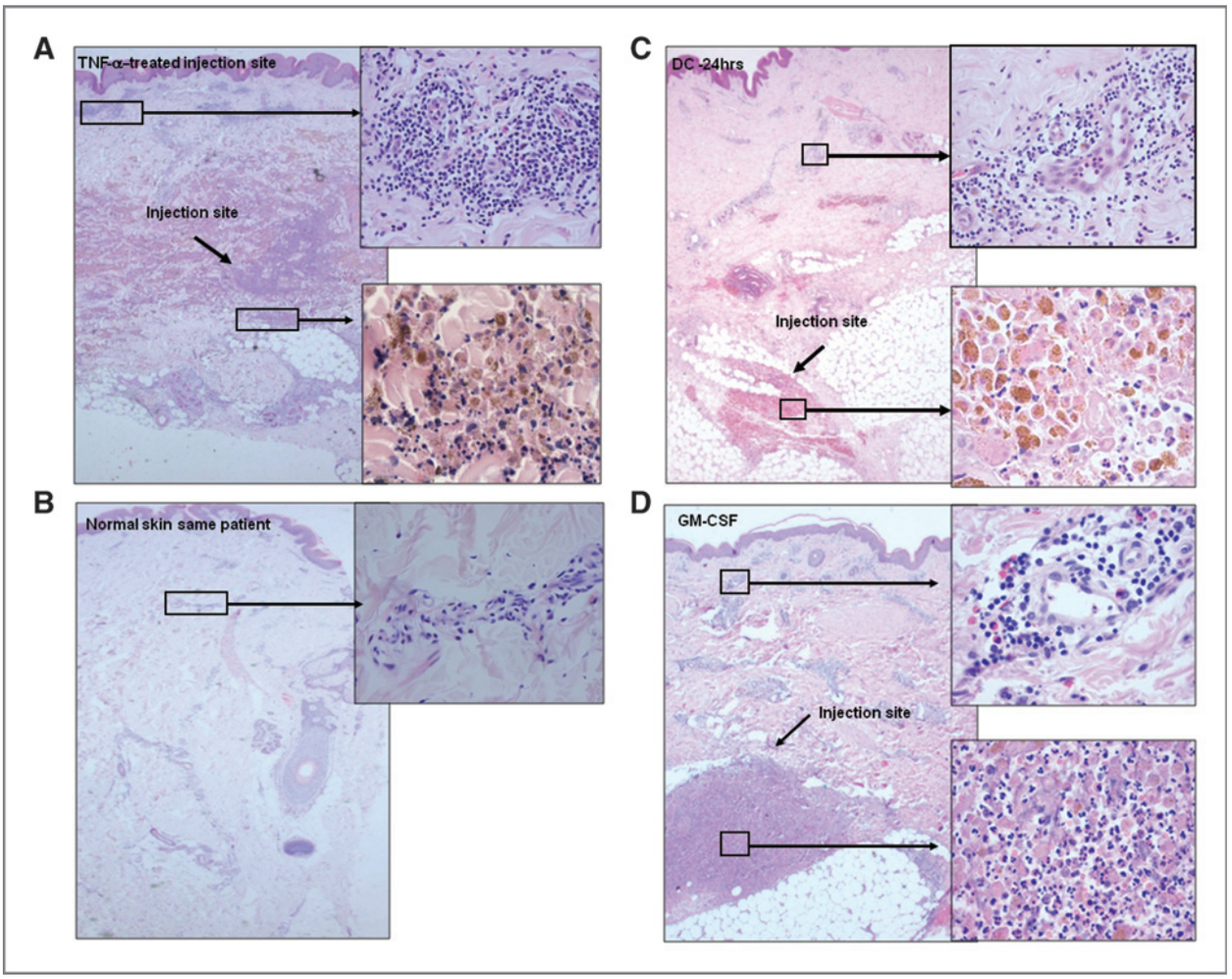

Figure 2. Immunohistochemical analyses of the injection site. We conducted histologic analysis of the pretreatment injection sites 48 hours postinjection to validate our imaging findings. A–C, pretreatment of the injection site with TNF-α, GM-CSF, or unloaded DCs consistently induced local inflammation, shown by infiltrates of leukocytes around vessels in the dermis, mainly neutrophils, and eosinophils. Within areas with high cell density, especially at the injection sites, there is a high proportion of dying cells, including vaccine DCs. D, normal skin from patient in C, for comparison.

and 9 hours after injection of 5×10^6 DCs. The migratory and control regions are highlighted.

Reducing the cell density improves migration rates

The following figure (Fig. 4A) shows the migration over time for 5×10^6 DCs, for the migratory region (red) and the control region under the cell layer (blue). These data show that clear migration which is absent in the control region. The overall numbers of cells that migrated were 3×10^4 and 4.5×10^5 with 5×10^5 and 5×10^6 cells, respectively, showing the sensitivity of this assay. Figure 4B summarizes the results of 3 individual experiments with varying cell numbers in the gel scaffold. The data indicate that indeed increasing cell number suppresses migration. Thus, the percentage of migratory cells is nearly 3% with 0.5×10^6 and 0% with 15×10^6 DCs. Finally, histologic analyses on the tissue samples, taken over and around the injection site, were analyzed (Fig. 4C). Extensive hypoxia was observed at the injection site [CAIX stain (brown); top]. Furthermore, at the lower cells numbers, we observed trains of migratory cells (bottom). These trains appear to be migrating along the muscle tissue and were completely absent with samples with 15 and 10×10^6 million DCs.

Planar scintigraphy is not sufficient for imaging of low numbers of injected cells *in vivo*

Finally, we compared the migration data obtained *in vitro* using our ^{19}F MRI assay with the clinical data obtained using scintigraphy on ^{111}In-labeled DCs (Fig. 5A). Because of the difficulties of testing different conditions in patients, our clinical data only reflect migration with 10^5 and 10^6cells per injection. Five injections of 1×10^6 or 5 injections of 1×10^5 cells were injected intradermally, at different sites 1 cm apart. The average percentage of migratory cells was significantly increased to 5×10^6 cells compared with 1 injection with 15×10^6 cells; 1.9% ($P < 0.05$), but not with 5×10^5

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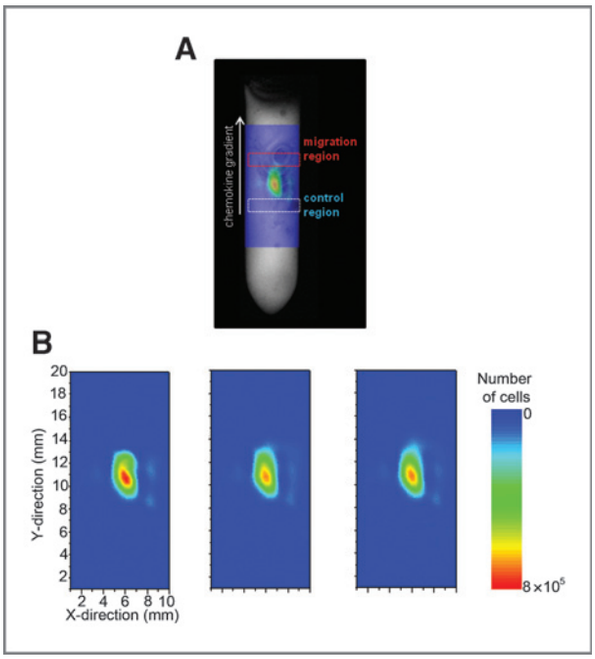

Figure 3. *In vitro* model to study migration of small numbers of cells. A, cell bolus position within the sample. Overlay of a ^{1}H MRI image and ^{19}F CSI cell map showing the position and the density of the cell bolus injection in the tissue sample. The migration region is vertically above the cell pellet and the control region below. B, representative data obtained at 1, 5, and 9 hours after injection of 5×10^6 DCs, plotted in mm. The scale bar represents the number of cells.

cells; 0.5% (not significant), respectively. Thus, lowering the number of cells to 10^6 cells per injection improved migration by about 1.5-fold, in terms of percentage, relative to a single bolus injection of 15×10^6 DCs. So, trend in percentage of migratory cells is comparable with our *in vitro* data for 5×10^6 and 10×10^6 cells, respectively. The percentage of migratory DCs in patients would be expected to be highest with 0.5×10^6 cells, as predicted by the *in vitro* results. However, this apparent discordance is due to the sensitivity limits of scintigraphy (16), where the smaller numbers of migratory cells were probably simply not detected because of sensitivity issues.

Discussion

The interest in cellular therapy is increasing at fast pace, in particular research toward harnessing immune cells for anticancer therapy, which has been revived by recent key developments (17, 18). Owing to their unique immune stimulating properties, DCs have been the "hot" target for anticancer immunotherapy in the past decades. Endowed with knowledge of the crucial steps, which underlie successful induction of tumor-specific immune responses, various vaccination parameters have been optimized in previous studies (19). At this point, the paradigm shifts from small proof-of-principle studies to large randomized controlled trials. Accordingly, attention should be paid to the feasibility of cellular therapy at large-scale. Intradermal injection of DCs for immunotherapy is generally the easiest approach and therefore preferred in most clinical trials involving DC-based therapy (2). Unfortunately only limited numbers of DCs will reach the draining LNs. Improving the efficiency of DC migration might therefore increase the immunologic response (20) and reduce costs while potentially improving patient response. In mice, immune responses were dose-dependent (3, 21) and could be increased by conditioning of the injection site. We show here that migration rates of human monocyte-derived DCs after intradermal injection in patients with melanoma could not be increased by pretreatment to induce a pro-inflammatory microenvironment at the injection site. Instead, by using a novel model, we show *in vitro* and *in vivo* that a reduction of cell numbers at the injection site is key to improved migration.

A number of factors might explain the discrepancy between our negative results and the positive effects of pretreatment of the injection site on migration in mice skin. First, closer analysis of the results in mouse models by Martin-Fontecha and colleagues revealed that migration rates were indeed dramatically increased by conditioning but even in their experiments never exceeded approximately 2% (3). Conditioning of the skin was only effective in increasing the migration of suboptimal doses of DCs from 0.01% to 0.1% to 1%. No increases in migration rates were observed when the migration rate of the untreated control was around 1% to 2%. Moreover, high doses of TNF-α have been shown, to the contrary, to inhibit migration from the skin (22). Third, the timing of pretreatment is critical. Clinical studies show that intradermal administration of TNF-α or IL1-β induced the emigration of resident Langerhans cells to draining LNs. In this setting, application of those pro-inflammatory cytokines may act both directly on the DCs and indirectly via the surrounding accessory cells. In our study, TNF-α is injected to the skin 8 hours before DC vaccination and will therefore exert its effect on the microenvironment and not directly on the DCs. Given the burden for patients in terms of injections and logistics, we chose not to titrate the dose and timing of TNF-α to find the optimum. Moreover, Nair and colleagues showed that by conditioning the skin with the TLR ligand imiquimod, the migration of *immature* DCs could be stimulated (21). However, even in this trial, no improvement in migration rate was achieved as the injected DCs had a mature, and thus highly migratory, phenotype. Finally, note that while several factors contribute to the migration of DCs, these can be very difficult to study individually and even more so when considering the costs and logistics of a clinical study. Hence, such factors may best be studied *in vitro* before final optimization *in vivo*.

The use of ^{19}F MRI for quantitative cell tracking is a relatively new technique (12). Here, we applied this technology to track migratory DCs over 10 hours, to optimize the cell numbers used in DC vaccinations in the clinic. This technique allows us to use a wide range of cell numbers, up to the millions of cells that are typical for current DC vaccination and to use opaque samples, such as tissue. Other migration assays require either small cell numbers

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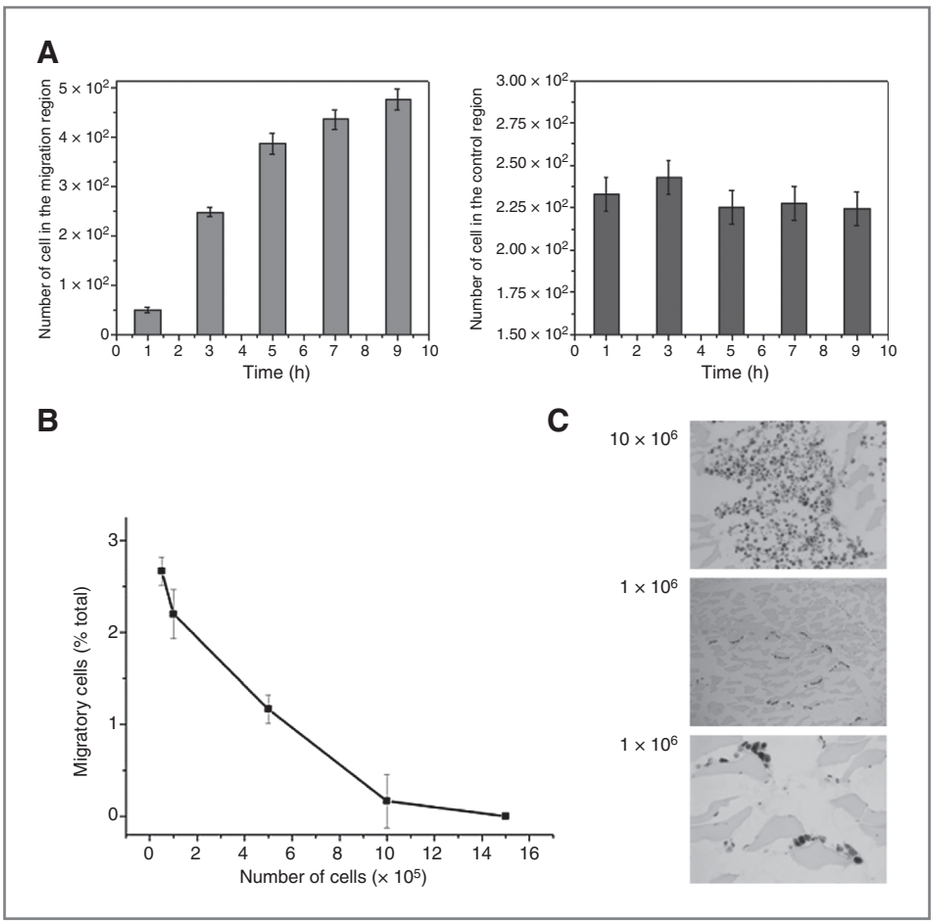

Figure 4. Reducing the cell density improves migration rates *in vitro*. The change in time in the cell numbers within the migration and the control regions is plotted for 5×10^6 DCs ($n = 3$). A, a clear trend is observed for the cell number with time dependence in the migration region (left, in red); improved migration is observed for 5×10^6 and 1×10^6 cells (not shown). No pattern is observed for the control region (right, in blue). B, summary of 3 individual experiments with varying cell numbers in the *in vitro* assay. The data indicate that increasing cell number suppresses migration. Thus, the percentage of migratory cells is nearly 3% with 0.5×10^6 and 0% with 15×10^6 DCs. Average and SDs are indicated. C, sections from the tissue used for the migration assay were cut and stained for a hypoxia marker (dark stain); cell nuclei are stained lighter. Nuclei are sparse in the surrounding muscle tissue. Representative images are shown for 10×10^6 DCs (top; low magnification), and 1×10^6 DCs (low and high magnification, respectively). Migratory cells are evident in the bottom.

or transparent samples (23). The ^{19}F particles used to label the DCs are not toxic to the cells and do not affect their migration, when compared with unlabeled cells (24). The expression of typical cell surface markers, used in chemotaxis and migration, is also unaffected. Although ^{19}F MRI has not currently been applied to clinical cell tracking, the technique will be available for the clinic in the near future. The label we used here can also be adapted for clinical use. Furthermore, the assay conditions can easily be modified to include different cytokines or combinations of cytokines to study their effect on migration. Different cell types can also be used, as long as the cells can be labeled with a ^{19}F agent. Some ^{19}F labels may also be sensitive to oxygen partial pressures, and thus these can be measured during the experiments to indicate hypoxic regions. In this article, we used bovine tissue instead of human tissue to avoid differences due to mismatched immune cells and the inflammatory condition of the tissue. However, the assay can readily be adapted to use other tissue types. Here, we used CCL21 to provide a chemotactic gradient for DC migration. CCL21 is known to induce the active migration of DCs toward lymphatic vessels *in vivo* (25).

One of the main issues with ^{19}F MRI is its sensitivity in terms of detectable cells/voxel/unit imaging time. Under our conditions, we found the sensitivity to be higher than that of clinical scintigraphy (Fig. 5A); the sensitivity in our setup allows the detection of as little as 5,000 cells/voxel, compared with 2×10^4 with scintigraphy (16). This is 4-fold higher than the sensitivity of scintigraphy *in vivo*. Note that the MRI was carried out on a clinical scanner using sequences that can be applied to humans; using a higher field research magnet and faster imaging sequences would improve sensitivity further. In our experiments, we chose to measure only migration in the vertical plane (i.e., along the chemokine gradient), as we felt this was the most relevant direction. However, it would certainly be feasible to measure migration in both planes or even in 3D in this setup. Furthermore, the relatively poor sensitivity of scintigraphy might be one reason why smaller numbers of migratory cells are not detected, and thus the percentage of migratory cells calculated is artificially low, especially when smaller cell numbers were injected initially. Although the spatial resolution has improved with more recent nuclear imaging modalities [single-photon emission computed tomography (SPECT), SPECT/CT], it is still far behind on MRI especially in soft tissues such as LNs. We previously showed examination of intra-LN distribution of labeled DCs using MRI with iron-loaded cells (16).

The high density of cells at the injection site may prevent the access to sufficient oxygen and nutrients and may

Published OnlineFirst February 4, 2013; DOI: 10.1158/1078-0432.CCR-12-1879

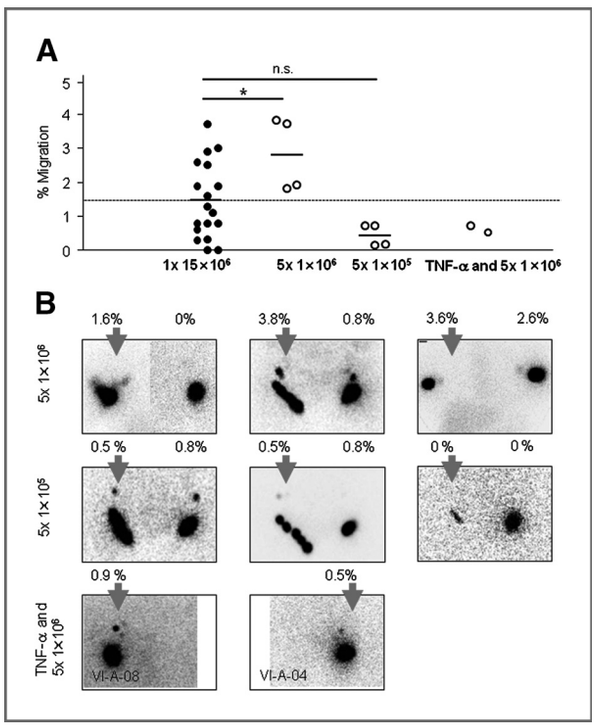

Figure 5. Reducing the cell density improves migration rates *in vivo*. Migration of DCs to skin draining LNs was monitored after intradermal injection with reduced numbers of ^{111}In-labeled DC vaccine and planar scintigraphy of injected region. A, the percentage of migration with 15×10^6 DCs at 48 hours postinjection (as a reference), 5×10^6 ($n = 4$), 5×10^5 DCs ($n = 4$), or TNF-α pretreatment together with 5×10^6 cells ($n = 2$). The mean percentage of migration with 5×10^6 DCs was 2.6%, significantly higher than 15×10^6 DCs ($P = 0.0496$, unpaired 2-tailed *t* test), whereas further reduction of cell number or pretreatment with TNF-α combined with reduced cell number did not significantly improve migration rates (n.s., not significant). B, for each condition with reduced number of cells, the individual images are shown. The arrows indicate the experimental site.

therefore hamper cell movement and active migration. Indeed, hypoxia is evident at the injection site (Fig. 4C). Hypoxia suppresses the production of matrix metalloproteinases and the migration of human monocyte derived–DCs (3). After injection of a cell suspension in the dermis, the fluid will be readily drained into the afferent lymphatic vessels, due to elasticity of the skin. The cells, however, will be caught in the extracellular matrix and will be packed together, devoid of nutrients and oxygen. The cells on the border of the injection depot may have the opportunity to move away from the depot.

The optimal numbers of DCs per LN for adequate immune induction in clinical studies has not been established. Some studies report a dose-dependent relation with immune responses in human (26, 27). Given the high immunostimulating potential of DCs *in vitro*, it may not be surprising that even small numbers of DCs, as in clinical studies, are sufficient. Indeed, we have shown previously that even small numbers of DCs are capable of effective interacting with T cells (10, 15). Hypothetically, the unfavorable conditions at the site of delivery may select the most fit DCs with high stimulatory potency or simply those at the periphery of the bolus, which are then able to migrate to the LNs and adequately induce immune responses. Combining this information with the current data, we suggest that multiple intradermal injections with small numbers of cells to target multiple LN basins would increase DC migration to LNs, for example, using a "tattoo" delivery device (28). In particular, cells that appear to be actively migratory were only evident with the lower cell numbers (Fig. 4C). It is possible that DC aggregation or chemotaxis could also be involved in reducing emigration with larger cell numbers, although these factors were not studied here.

In conclusion, we have shown that pretreatment of the skin to create an inflammatory microenvironment at the injection site does not improve DC migration to LNs after i.d. delivery. On the contrary, we show that reduction of cell density at the injection site is key to improved DC migration, both *in vitro* and *in vivo*. Because current imaging modalities for clinical *in vivo* tracking of DCs are not sensitive enough to study migration of small numbers of DCs, the *in vitro* model developed here facilitates further studies for improving migration rates.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

The authors thank Andor Veltien, Sandra Croockewit, Emile Koenders, Eddy Mijnheere, Martin Engels, Cathelijne Frielink, and Wenny Peeters for their assistance, and Han Bonenkamp for providing TNF-α.

Grant Support

This work was supported by grants KUN 1999/1950, 2004/3126, and 2008/035 from the Dutch Cancer Society, Netherlands Organization for Scientific Research (NWO), grants 920-03-250 and NWO-Veni 700.10.409, NWO-Vidi-917.76.363, AGIKO-92003250, The Radboud University Nijmegen Medical Centre AGIKO-2008-2-4, the ATK foundation, ENCITE, NWO Spinoza award, NIH R01 NS045062, and an European Research Council (ERC) grant ERC-2010-AdG-269019-PATHFINDER and KWF2009-4402.

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Received June 7, 2012; revised January 17, 2013; accepted January 18, 2013; published OnlineFirst February 4, 2013.

Published OnlineFirst February 4, 2013; DOI: 10.1158/1078-0432.CCR-12-1879

References

1. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nat Rev Cancer 2012;12:265–77.
2. Srinivas M, Aarntzen EH, Bulte JW, Oyen WJ, Heerschap A, de Vries IJ, et al. Imaging of cellular therapies. Adv Drug Deliv Rev 2010;62:1080–93.
3. MartIn-Fontecha A, Sebastiani S, Hopken UE, Uguccioni M, Lipp M, Lanzavecchia A, et al. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. J Exp Med 2003;198:615–21.
4. Tripp CH, Ebner S, Ratzinger G, Romani N, Stoitzner P. Conditioning of the injection site with CpG enhances the migration of adoptively transferred dendritic cells and endogenous CD8+ T-cell responses. J Immunother 2010;33:115–25.
5. Verdijk P, Aarntzen EH, Punt CJ, de Vries IJ, Figdor CG. Maximizing dendritic cell migration in cancer immunotherapy. Expert Opin Biol Ther 2008;8:865–74.
6. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. Nat Rev Immunol 2005;5:617–28.
7. Angeli V, Randolph GJ. Inflammation, lymphatic function, and dendritic cell migration. Lymphat Res Biol 2006;4:217–28.
8. Bonetto F, Srinivas M, Weigelin B, Cruz LJ, Heerschap A, Friedl P, et al. A large-scale (19) F MRI-based cell migration assay to optimize cell therapy. NMR Biomed 2012;25:1095–103.
9. Aarntzen EH, Bol K, Schreibelt G, Jacobs JF, Lesterhuis WJ, van Rossum MM, et al. Skin-test infiltrating lymphocytes early predict clinical outcome of dendritic cell based vaccination in metastatic melanoma. Cancer Res 2012;72:6102–10.
10. Lesterhuis WJ, de Vries IJ, Schreibelt G, Lambeck AJ, Aarntzen EH, Jacobs JF, et al. Route of administration modulates the induction of dendritic cell vaccine-induced antigen-specific T cells in advanced melanoma patients. Clin Cancer Res 2011;17:5725–35.
11. de Vries IJ, Lesterhuis WJ, Scharenborg NM, Engelen LP, Ruiter DJ, Gerritsen MJ, et al. Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. Clin Cancer Res 2003;9:5091–100.
12. Srinivas M, Heerschap A, Ahrens ET, Figdor CG, de Vries IJ. (19)F MRI for quantitative *in vivo* cell tracking. Trends Biotechnol 2010;28: 363–70.
13. Aarntzen EH, Srinivas M, De Wilt JH, Jacobs JF, Lesterhuis WJ, Windhorst AD, et al. Early identification of antigen-specific immune responses *in vivo* by [18F]-labeled 3′-fluoro-3′-deoxy-thymidine ([18F]FLT) PET imaging. Proc Natl Acad Sci U S A 2011;108:18396–9.
14. DeVries IJ, Krooshoop DJ, Scharenborg NM, Lesterhuis WJ, Diepstra JH, Van Muijen GN, 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. Verdijk P, Aarntzen EH, Lesterhuis WJ, Boullart AC, 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.
16. Verdijk P, Scheenen TW, Lesterhuis WJ, Gambarota G, Veltien AA, Walczak P, et al. Sensitivity of magnetic resonance imaging of dendritic cells for *in vivo* tracking of cellular cancer vaccines. Int J Cancer 2007;120:978–84.
17. Mellman I, Nussenzweig M. Retrospective. Ralph M. Steinman (1943–2011). Science 2011;334:466.
18. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 2012;363:411–22.
19. Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nat Med 2004;10:475–80.
20. Celli S, Day M, Muller AJ, Molina-Paris C, Lythe G, Bousso P. How many dendritic cells are required to initiate a T cell response? Blood 2012;120:3945–8.
21. Nair S, McLaughlin C, Weizer A, Su Z, Boczkowski D, Dannull J, et al. Injection of immature dendritic cells into adjuvant-treated skin obviates the need for *ex vivo* maturation. J Immunol 2003;171: 6275–82.
22. Geissmann F, Dieu-Nosjean MC, Dezutter C, Valladeau J, Kayal S, Leborgne M, et al. Accumulation of immature Langerhans cells in human lymph nodes draining chronically inflamed skin. J Exp Med 2002;196:417–30.
23. Toetsch S, Olwell P, Prina-Mello A, Volkov Y. The evolution of chemotaxis assays from static models to physiologically relevant platforms. Integr Biol (Camb) 2009;1:170–81.
24. Srinivas M, Cruz LJ, Bonetto F, Heerschap A, Figdor CG, de Vries IJ. Customizable, multi-functional fluorocarbon nanoparticles for quantitative *in vivo* imaging using 19F MRI and optical imaging. Biomaterials 2010;31:7070–7.
25. Tal O, Lim HY, Gurevich I, Milo I, Shipony Z, Ng LG, et al. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. J Exp Med 2011;208:2141–53.
26. Dillman RO, Fogel GB, Cornforth AN, Selvan SR, Schiltz PM, DePriest C. Features associated with survival in metastatic melanoma patients treated with patient-specific dendritic cell vaccines. Cancer Biother Radiopharm 2011;26:407–15.
27. Engell-Noerregaard L, Hansen TH, Andersen MH, thor SP, Svane IM. Review of clinical studies on dendritic cell-based vaccination of patients with malignant melanoma: assessment of correlation between clinical response and vaccine parameters. Cancer Immunol Immunother 2009;58:1–14.
28. van den Berg JH, Nujien B, Beijnen JH, Vincent A, van Tinteren H, Kluge J, et al. Optimization of intradermal vaccination by DNA tattooing in human skin. Hum Gene Ther 2009;20:181–9.
29. Tel J, Aarntzen EH, Baba T, Schreibelt G, Schulte BM, Benitez-Ribas D, et al. Natural human plasmacytoid dendritic cells induce antigen-specific T-cell responses in melanoma patients. Cancer Res 2013;73: 1063–75.