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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. Anti-cancer dendritic cell (DC) vaccines require the DC 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 DC to LN might increase vaccine efficacy and reduce costs. We investigated DC migration *in vivo* in humans in different conditions.

Experimental design. HLA-A*02:01 melanoma patients were vaccinated with mature DC loaded with tyrosinase and gp100 peptides together with keyhole limpet hemocyanin (KLH) (NCT00243594). For this study, patients received an additional intradermal (i.d.) vaccination with ^{111}In-labeled mature DC. The injection site was pretreated with nonloaded, activated DC, TNFα or Imiquimod; GM-CSF was co-injected or smaller numbers of DC were injected. Migration was measured by scintigraphy and compared to an intra-patient control vaccination. In an *ex vivo* tissue model, we measured CCL21-directed migration of ^{19}F-labeled DC 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 DC *in vivo*.

Conclusion. Reduction of cell density, not pretreatment of the injection site, is crucial for improved migration of DC to LNs *in vivo*.

Statement of translational relevance

To trigger an effective immune response, *ex vivo* generated tumor antigen-loaded DC need to relocate to LN. Intradermal (i.d.) delivery is commonly used, due to its feasibility; however, migration rates never exceeded 4%. Increasing the numbers of *ex vivo* generated DC reaching LN 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 TLR7/8 ligand; or co-injection with GM-CSF. 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 DC to LN *in vivo*. However, current imaging modalities for *in vivo* tracking of DC are insufficient to study migration of small numbers of ^{111}In-labeled DC in the clinic.

Introduction

Cellular therapy in cancer patients aims to activate the immune system in a highly specific response against the tumor. In most studies, autologous antigen-presenting cells, principally dendritic cells (DC), are activated and loaded with tumor antigen *ex vivo* (1). To trigger an effective immune response, the DC 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 DC to the patients, of which intradermal (i.d.) injection is the most frequently used (2). The biodistribution of DC after vaccination has been studied in humans, primarily using ^{111}In or ^{99m}Tc-labeled DC and scintigraphy (2). However, even though about 20 clinical trials have been carried out with DC 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 DC 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 DC into the draining LN could be dramatically increased by pretreating the injection site (3, 4). The skin was injected with either an extra dose of DC, pro-inflammatory cytokines (TNFα or IL-1α) or Toll-like receptor ligands before injection of the vaccine-DC. This pretreatment of the skin resulted in a five to ten-fold increase in the number of DC in the draining LN that correlated with a similar increase in T cell activation. Other parameters of DC delivery might also contribute to more efficient emigration of DC from the skin to the draining LN, e.g. 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 LN, may stimulate the emigration of DC 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 non-opaque samples and thus do not replicate clinical conditions. Furthermore, most techniques assess migration in two-dimensional 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 DC, TNFα or Imiquimod (a synthetic TLR7/8 ligand), to induce a local inflammatory microenvironment; or co-injection with GM-CSF to enhance DC survival. We tracked ^{111}In-labeled vaccine-DC over a period of 48 hours by planar scintigraphy and compared migration to the LN 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-DC 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 DC are insufficient to study migration of small numbers of DC in human studies.

Materials and Methods

DC vaccination in melanoma patients

In this study, stage III and IV melanoma patients (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 1 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, C.J.A. Punt). Because the first vaccination was administered 1-2 day 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 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 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-a (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) 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 (31–33). 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, Petten, The Netherlands) in 0.1 M Tris-HCl (pH 7.0) was added to mature DC for 15 min at room temperature as described previously (11). This results in 5 µCi per 15×10^6 cells. Cells were washed three 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 prior to scheduled RLND as mentioned above, or during the course of vaccination with increasing numbers of DC resuspended in 100 µl of injection liquid and distributed over one or multiple injection sites, as indicated. At 24 hours and 48 hours post-injection, migration of DC 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 DC that had migrated from the injection depot to draining LN.

^{19}F-labeling and MR imaging 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., Round Rock, TX, USA) and PLGA (Resomer RG 502 H, lactide: glycolide molar ratio 48:52 to 52:48; Boehringer Ingelheim, Ingelheim am Rhein, Germany) as described (12). 10 mg per 10^6 cells of particles was added to the DC culture at day 3. Upon harvesting (day 8), cells were washed three times in PBS to remove excess particles.

For the migration assay, the technique was adapted from (8) to replace the gel scaffold with a tissue sample, in this case bovine muscle. DC were injected as a bolus in the center of a 2 ml Eppendorf tube filled with a single piece of tissue, leaving about 1cm 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 consiting of 0.2µg recombinant human CCL21 (R&D Systems, Minneapolis, MN, USA).

^{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 nine ^{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=400/2.94ms was used for CSI. The sample was sealed and not moved for the duration of the imaging experiment. Temperature was maintained at 37 ^{o}C using regulated warm air flows.

Histology

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

Statistical analyses

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

Results

DC migration to LN is poor after intradermal vaccination

In current and previous studies (10, 14), migration of DC to skin-draining LN 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 DC after i.d. injection of 15×10^6 DC at 24 or 48 hrs post-injection. The average migration achieved was 1.2% after 24 hours and 1.4% after 48 hrs, indicating that most of the migration occurred within 24 hrs 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 of does not improve DC migration

Based on 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 TNFα (n=3), Imiquimod (n=4) or unloaded but activated DC 6 hours (n=3) or 24 hours (n=3) prior to vaccination (Figures 1A,B). In patients pretreated with TNFα or activated DC, the contralateral administration of 15×10^6 ^{111}In-labelled vaccine-DC served as intra-patient 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 exceeded 4% and did not significantly increase migration compared to unconditioned sites.

Our previous studies have shown that vaccine-DC rapidly lose viability at the injection site after intranodal or intradermal delivery, which might contribute to defective migration to LN (15). Addition of GM-CSF as an adjuvant during DC

vaccination might increase the survival of DC and thereby increase migration rates. Three patients were injected with 15×10^6 DC in normal saline containing 14×10^4 IU GM-SCF. Although the percentage of migrating cells after 48hours 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.

Lastly, we pretreated the injection site of 4 patients by Imiquimod application every 12 hours for 2 days prior to 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-DC to the draining LN.

Local cell density is limiting factor

We performed histological analysis of the pretreatment injection sites 48 hours post-injection in order to validate our imaging findings. Injection of DC consistently induced local inflammation, demonstrated by infiltrates of leukocytes around vessels in the dermis, mainly neutrophils and eosinophils. The number of lymphocytes in the dermis increased, compared to normal skin. Pretreatment of the injection site with either TNFα (Figure 2A,B) or unloaded DC (Figure 2C); or co-injection with GM-CSF (Figure 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 histological evidence shows that DC 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-DC were found at higher rates with larger numbers of injected cells; these data suggests 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 DC

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 (8), (Figure 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 and 9 hours after injection of 5 x 10^6 DCs. The migratory and control regions are highlighted.

Reducing the cell density improves migration rates

The following figure (Figure 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, demonstrating the sensitivity of this assay. Figure 4B summarizes the results of three individual experiments with varying cell numbers in the gel scaffold. The data indicate that indeed increasing cell number suppresses migration. Thus, the percent of migratory cells is nearly 3% with 0.5 $x10^6$ and 0% with 15 $x10^6$ DC. Finally, histological analyses on the tissue samples, taken over and around the injection site, were analysed (Figure 4C). Extensive hypoxia was observed at the injection site (CAIX stain (brown); top panel). Furthermore, at the lower cells numbers, we observed trains of migratory cells (lower panels). These trains appear to be migrating along the muscle tissue, and were completely absent with samples with 15 and 10 x 10^6 million DCs.

Planar scintigraphy 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). Due to the difficulties of testing different conditions in patients, our clinical data only reflects 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 centimeter apart. The average percentage of migratory cells was significantly increased to 5×10^6 cells compared to 1 injection with 15×10^6 cells; 1.9% ($p<0.05$), but not with 5×10^5 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 DC. So, trend in percentage of migratory cells is comparable with our *in vitro* data for 5 and 10×10^6 cells, respectively. The percentage of migratory DC 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 due to sensitivity issues.

Discussion

The interest in cellular therapy is increasing at fast pace, in particular research towards harnessing immune cells for anti-cancer 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 anti-cancer 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 DC for immunotherapy is generally the easiest approach and therefore preferred in most clinical trials involving DC-based therapy (2). Unfortunately only limited numbers of DC will reach the draining LN. Improving the efficiency of DC migration might therefore increase the immunological 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 DC after intradermal injection in melanoma patients could not be increased by pretreatment to induce a pro-inflammatory microenvironment at the injection site. Instead, by using a novel model, we demonstrate *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 *et al*. 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 DC from 0.01-0.1% to 1%. No increases in migration rates were observed when the migration rate of the untreated control was around 1-2%. Moreover, high doses of TNFα have been demonstrated, to the contrary, to inhibit migration from the skin (22). Thirdly, the timing of pretreatment is critical. Clinical studies show that intradermal administration of TNFα or IL1-β induced the emigration of resident Langerhans cells to draining LN. In this setting, application of those pro-inflammatory cytokines may act both directly on the DC and indirectly via the surrounding accessory cells. In our study, TNFα is injected to the skin 8 hours prior to DC vaccination and will therefore exert its effect on the microenvironment and not directly on the DC. Given the burden for patients in terms of injections and logistics, we chose not to titrate the dose and timing of TNFα to find the optimum. Moreover, Nair et al demonstrated that by conditioning the skin with the TLR-ligand imiquimod, the migration of *immature* DC could be stimulated (21). However, even in this trial no improvement in migration rate was achieved as the injected DC had a mature, and thus highly migratory, phenotype. Finally, note that while several factors contribute to the migration of DC, 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 DC over 10 hours, in order 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 or transparent samples (23). The ^{19}F particles used to label the DC are not toxic to the cells and do not affect their migration, when compared to 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 paper, 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 towards 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 (Figure 5A); the sensitivity in our set-up allows the detection of as little as 5000 cells/voxel, compared to 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 (SPECT, SPECT/CT) it is still far behind on MRI especially in soft tissues like LN. We previously demonstrated examination of intra-LN distribution of labeled DC 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 therefore hamper cell movement and active migration. Indeed, hypoxia is evident at the injection site (Figure 4C). Hypoxia suppresses the production of matrix metallo-proteinases and the migration of human monocyte derived-DC (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 DC 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 immune stimulating potential of DC *in vitro*, it may not be surprising that even small numbers of DC, as in clinical studies, are sufficient. Indeed, we have shown previously that even small numbers of DC are capable of effective interacting with T cells (10, 15). Hypothetically, the unfavorable conditions at the site of delivery may select the most fit DC with high

stimulatory potency, or simply those at the periphery of the bolus, which are then able to migrate to the LN 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 LN, 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 (Figure 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 LN after i.d. delivery. To the contrary, we demonstrate that reduction of cell density at the injection site is key to improved DC migration, both *in vitro* and *in vivo*. Since current imaging modalities for clinical *in vivo* tracking of DC are not sensitive enough to study migration of small numbers of DC, the *in vitro* model developed here facilitates further studies for improving migration rates.

Figure legends

Figure 1. Pretreatment of the skin does not improve DC migration.

Migration of DC to skin-draining LN (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 DC at 24 or 48 hrs post-injection, or after different pretreatments, never exceeded 4% of the total cells injected. Statistical analyses using a two-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, TNFα (n=3), GM-CSF (n=3), Imiquimod (n=4) or unloaded but activated DC 6 hours (n=3) or 24 hours (n=3) prior to 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.

Figure 2. Immunohistochemical analyses of the injection site.

We performed histological analysis of the pretreatment injection sites 48 hours post-injection in order to validate our imaging findings. **(A-C)** Pretreatment of the injection site with TNFα, GM-CSF or unloaded DC, consistently induced local inflammation, demonstrated 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-DC. **(D)** Normal skin from patient in Figure 2C, for comparison.

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)** The panels show representative data obtained at 1, 5 and 9 hrs after injection of 5×10^{6} DCs, plotted in mm. The scale bar represents the number of cells.

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 are 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 three individual experiments with varying cell numbers in the *in vitro* assay. The data indicate that increasing cell number suppresses migration. Thus, the percent of migratory cells is nearly 3% with 0.5×10^{6} and 0% with 15×10^{6} DCs. Average and standard deviation 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} DC (top; low magnification), and 1×10^{6} DC (low and high magnification respectively). Migratory cells are evident in the lower panels.

Figure 5. Reducing the cell density improves migration rates *in vivo*.

Migration of DC to skin-draining LN 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 DC at 48 hrs post-injection (as a reference), 5×10^6 (n=4), 5×10^5 DC (n=4) or TNFα pretreatment together with 5×10^6 cells (n=2). The mean percentage of migration with 5×10^6 DC was 2.6%, significantly higher than 15×10^6 DC (p=0.0496, unpaired two-tailed t-test), whereas further reduction of cell number or pretreatment with TNFα combined with reduced cell number, did not significantly improve migration rates ("n.s." denotes not significant). **(B)** For each condition with reduced number of cells, the individual images are shown. The arrows indicate the experimental site.

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 1

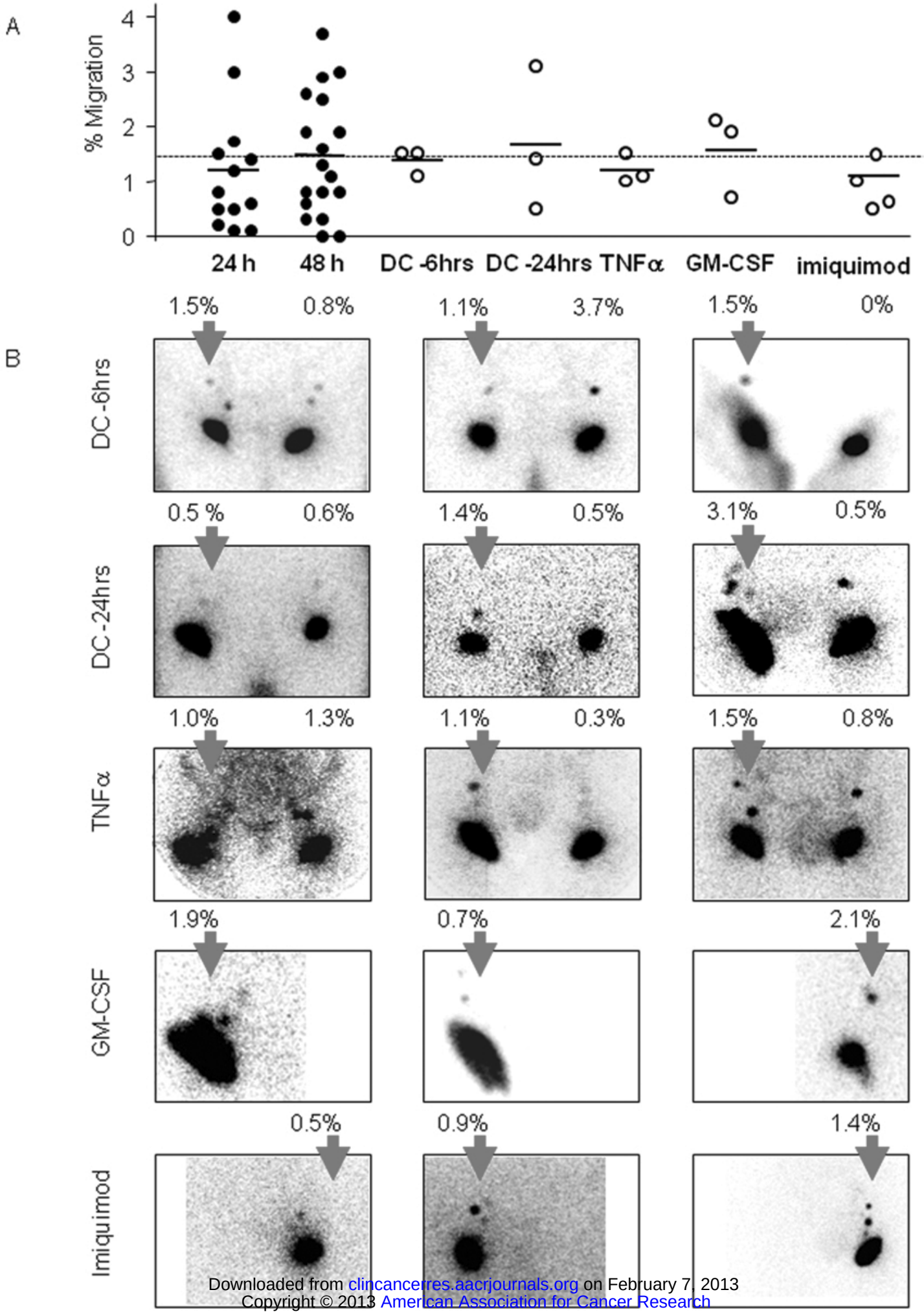


Figure 2

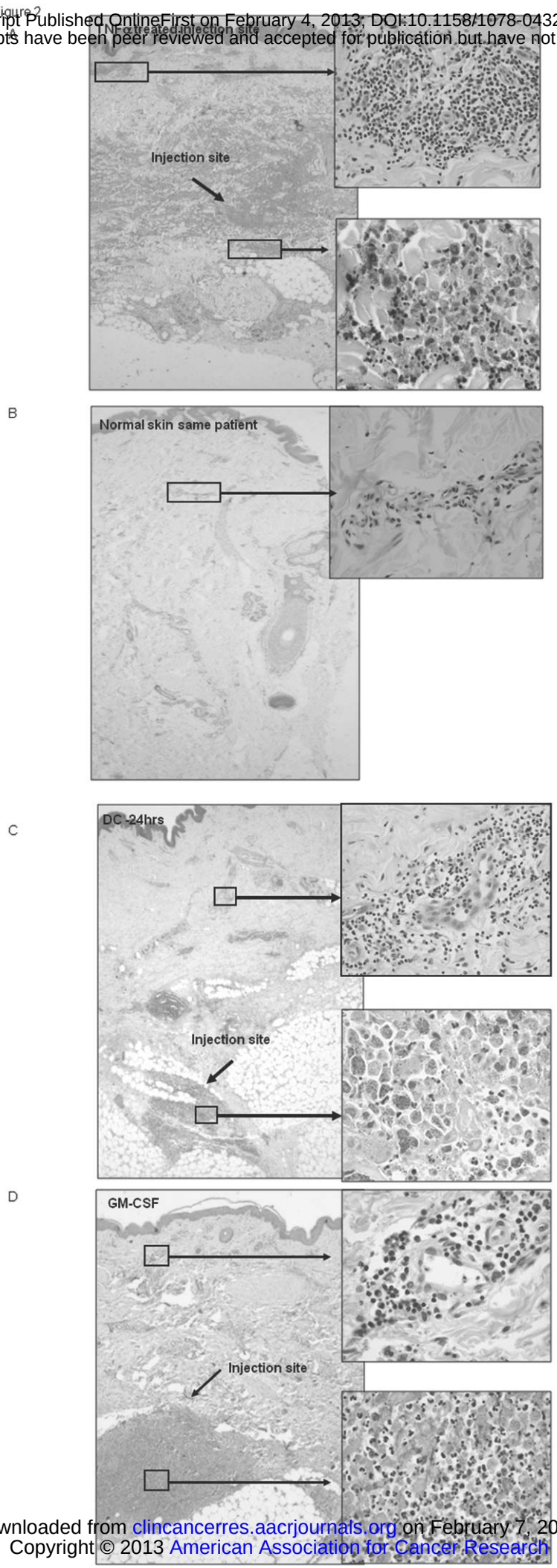
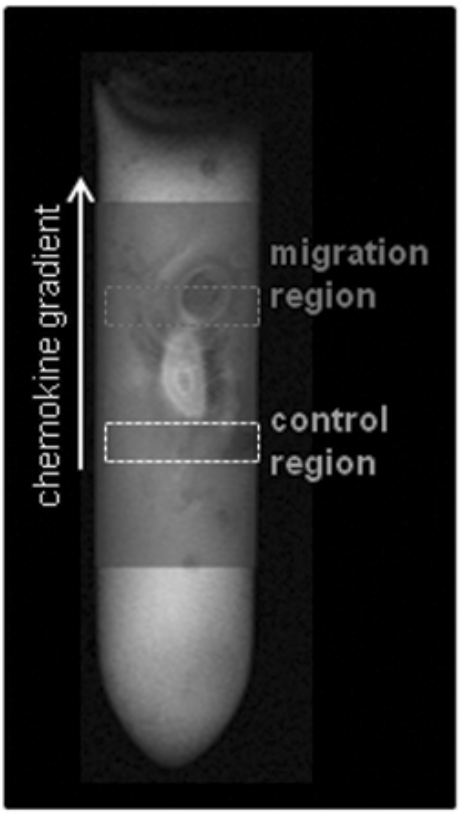


Figure 3

A



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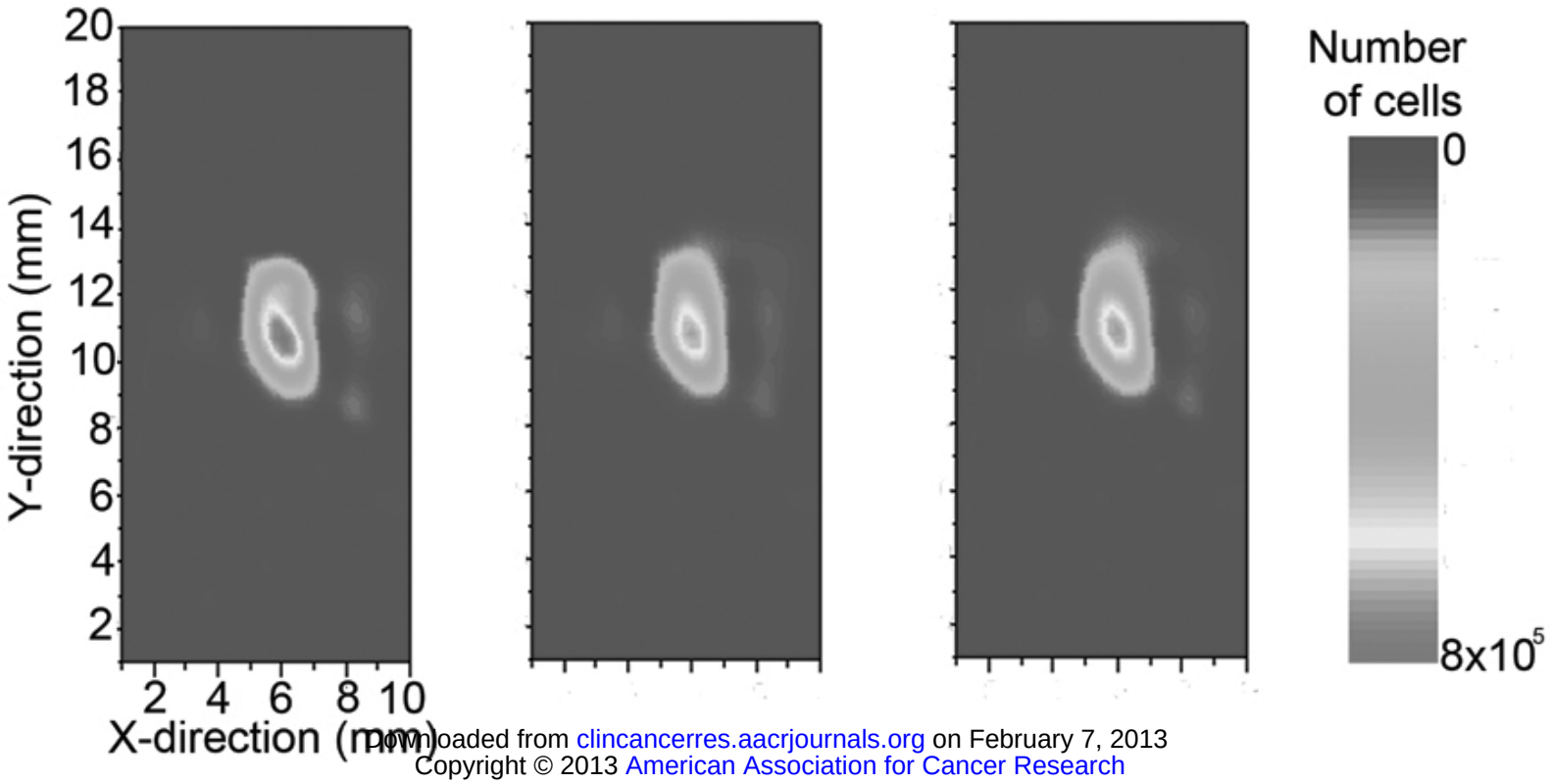

Figure 4

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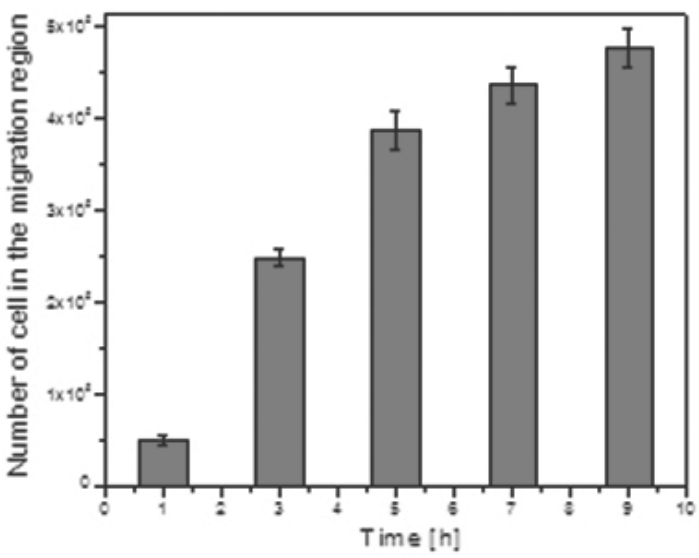

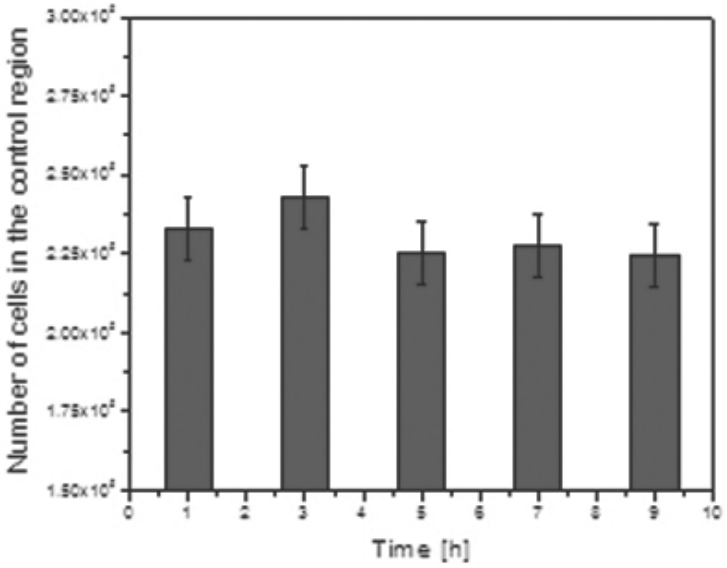

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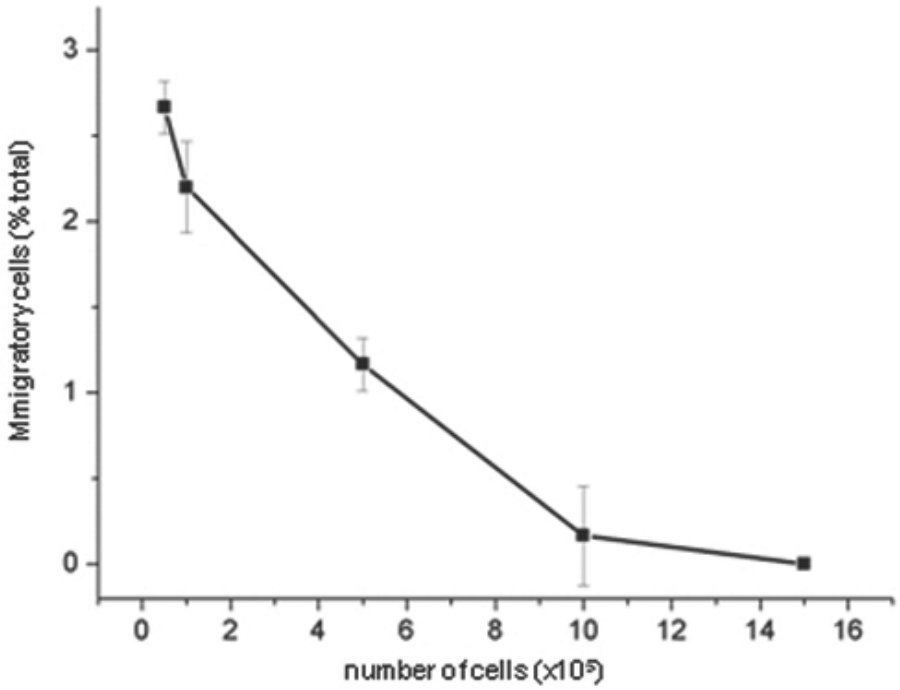

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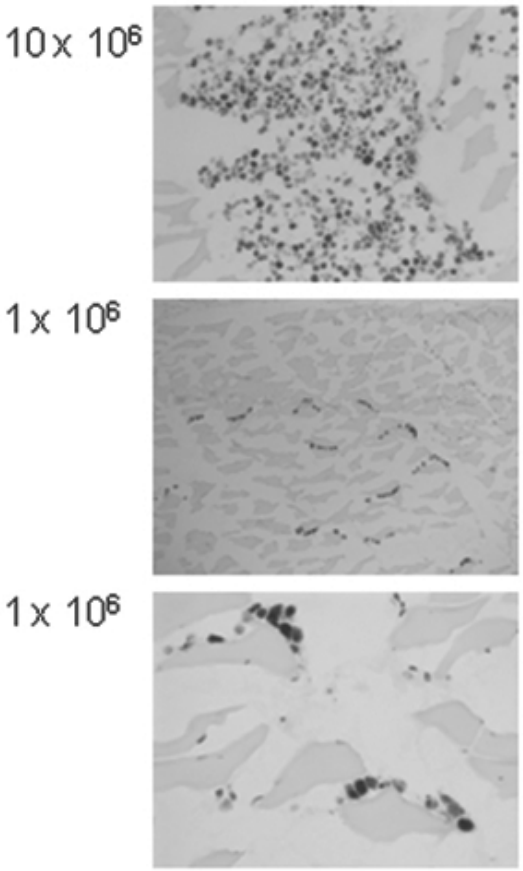

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Figure 5