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Advances in Brief

Effective Migration of Antigen-pulsed Dendritic Cells to Lymph Nodes in Melanoma Patients Is Determined by Their Maturation State[1, 2]

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Abstract

Dendritic cells are the professional antigen-presenting cells of the immune system. To induce an effective immune response, these cells should not only express high levels of MHC and costimulatory molecules but also migrate into the lymph nodes to interact with naïve T cells. Here, we demonstrate that *in vitro*-generated mature, but not immature dendritic cells, efficiently migrate into the T-cell areas of lymph nodes of melanoma patients. This difference is confirmed by *in vitro* studies, in which immature dendritic cells are strongly adherent, whereas mature dendritic cells remain highly motile. Our present findings demonstrate that the ability of dendritic cells to mount a proper immune response correlates with their ability to migrate both *in vitro* and *in vivo*.

Introduction

The DC[5] is a specialized antigen-presenting cell that can induce *de novo* antitumor responses in patients (1–3). Tissue-resident immature DCs take up antigen, followed by a complex maturation and activation process that is characterized by an up-regulation of antigen-presenting MHC molecules and costimulatory molecules, as well as a switch in their adhesion- and chemokine-receptor repertoire (4, 5). Although this process is only partially understood, it has become clear that these phenotypical changes allow DCs to migrate from peripheral tissues to the lymph nodes, in which they present processed antigens to resting T cells (6).

Recent studies in cancer patients, in which the efficacy of *in vitro*-generated DCs vaccines are evaluated, show that mature DCs, but not immature DCs, induce an effective antitumor response (7, 8). The incapacity of immature DCs to induce an immune response is at least in part because of a low expression of antigen presenting- and costimulatory- molecules. In addition, monocyte-derived immature DCs lack CCR7, required for migration into the T-cell areas of lymph nodes (9, 10). Hence, immature DCs generated *in vitro* may not colocalize and interact with naïve T cells in the lymph nodes, a prerequisite for the induction of an effective immune response.

Animal studies have provided direct evidence that DCs injected s.c., but not i.v., preferentially migrate to the draining lymph nodes to induce a measurable antitumor effect (11). Similarly, administration of radiolabeled DCs in humans demonstrates that the tissue distribution depends on the route of administration. DCs injected i.v. accumulate in the spleen and liver, whereas DCs injected s.c. or intradermally migrate to the draining lymph nodes (12, 13).

Studies in which DCs are directly injected intralymphatically show that DCs localize in the draining lymph node (13), but do not inform whether the injected DCs reach the T-cell areas of the lymphoid organs. Here we compare the migratory capacity of DC vaccines *in vitro*, by measuring morphology and speed of individual cells, with the behavior of these cells *in vivo*. By radiolabeling of the DCs, we investigate the effects not only of the maturation state but also of the route of administration on DC migration *in vivo*. Our findings demonstrate that the migration of DCs is highly dependent on their maturation state and suggest that injection of monocyte-derived mature DCs is superior to the injection of immature DC preparations.

Materials and Methods

Antibodies and Immunostaining. The following mAbs were used: anti-HLA class I (W6/32), anti-HLA DR/DP (Q5/13), and anti-CD80 (all Becton Dickinson, Mountain View, CA); anti-CD83 (Beckman Coulter, Mijdrecht, the Netherlands), anti-CD86 and anti-CCR5 (both PharMingen, San Diego, CA); and anti-CCR7, AZN-D1 against DC-SIGN, HP2/1 against $\alpha 4\beta 1$, SAM-1 against $\alpha 5\beta 1$, AZN-L19 against $\beta 2$ integrins, and AIIB2 against $\beta 1$ integrins (Developmental Studies Hybridoma Bank, Iowa City, IA).

Preparation of DCs. DC were generated from PBMCs as described previously (14, 15). After leukapheresis, PBMCs were used for the generation of monocyte-conditioned medium, and plastic-adherent monocytes were cultured in X-VIVO 15 medium (BioWhittaker, Walkersville, MD) supplemented with 2% pooled human serum (Bloodbank Rivierenland, Nijmegen, the Netherlands), interleukin 4 (500 units/ml), and granulocyte macrophage colony stimulating factor (GM-CSF, 800 units/ml; both from Schering-Plough, International, Kenilworth, NJ; Ref. 15). After the addition of KLH (10 μg/ml) on day 3–4, immature DCs were harvested on day 6–7.

Mature DCs were cultured as immature DCs. Autologous monocyte-conditioned medium with prostaglandin E_2 (PGE_2, 10 μg/ml; Pharmacia and Upjohn, Puurs, Belgium) and 10 ng/ml recombinant tumor necrosis factor α (kindly provided by Dr. Adolf Bender, Vienna, Austria) were added on day 7 (30%, v/v). Mature DCs were harvested on day 9.

Adhesion and Migration Assay. Flat-bottomed plates (96-well; Costar, Corning, NY) were coated with 20 μg/ml fibronectin (Roche, Mannheim, Germany) and blocked with 0.01% gelatin (Sigma Chemical Co., St. Louis, MO). DCs (1×10^7/ml) were labeled with Calcein-AM (25 μg/ml; Molecular Probes, Eugene, OR) for 30 min at 37°C, either untreated or preincubated (10 min at room temperature) with blocking mAb (10 μg/ml), and seeded on fibronectin-coated plates (20,000–40,000/well) for 45 min at 37°C. Nonadherent cells were removed by gentle-washing steps with warm 0.5% BSA (Boehringer Mannheim, Germany) in [150 mM NaCl, 10 mM Tris/HCL, 2 mM $MgCl_2$, 1 mM $CaCl_2$ (pH 8.0)]. Adherent cells were lysed with 100 μl of lysis buffer (50 mM Tris, 0.1% SDS), and fluorescence was quantified using the

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[5] The abbreviations used are: DC, dendritic cell; KLH, keyhole limpet hemocyanin; CCR, chemokine receptor; DTH, delayed-type hypersensitivity; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell.

cytofluorometer (PerSeptive Biosystems). Results were expressed as the mean percentage of adhesion of triplicate wells.

We used our previously established migration assay to study migration of DCs. Four thousand DCs (40 μl) per well were seeded on fibronectin-coated plates, resulting in 100 cells per image. DCs were recorded for up to 100 min, after which, migration tracks of individual DCs were analyzed. The speed is defined as the traversed path during the entire experiment divided by the imaging time.

Patients. Melanoma patients, participating in an ongoing protocol in which the *in vivo* immune responses of a DC vaccine are studied (KUN 99–150) were included in this study. Eligibility criteria included stage III/IV melanoma, planned radical lymphadenectomy for lymph node metastases, HLA-A2.1 phenotype, melanoma expressing gp100 and tyrosinase, and written informed consent. The local regulatory committee approved the study. Twenty-four to 48 h before surgery, eligible patients received a single injection of the ^{111}In-labeled DCs (10×10^6, 200 μl saline) either intradermally in the proximity, or intranodally directly into a lymph node of the lymph node region that was to be resected. Intranodal injections were performed under ultrasound guidance.

At the same time, patients also received the DC vaccine (DC pulsed with peptides gp100:154–162, gp100:280–288, and tyrosinase:369–377) intradermally or intranodally at a clinically tumor-free lymph node region. This was repeated for a total of four injections every 2 weeks.

After the DC vaccinations, a DTH reaction was performed. Briefly, DCs (5×10^5 in 0.2 ml), either loaded with KLH and/or peptide or unloaded, were injected on the back by using a 25-gauge needle and a 1 ml syringe. The DTH was characterized by swelling, erythema, and induration. The diameter (in millimeters) of edema and induration was measured after 48 h.

Proliferative Response to KLH. Cellular responses against the protein KLH were measured in a proliferation assay. Briefly, per well of a 96-well tissue culture microplate, 1×10^5 PBMCs, isolated from blood samples taken after one DC vaccination, were plated either in the presence of KLH or without. After 4 days of culture, 1 μCi/well of tritiated thymidine was added, and incorporation of tritiated thymidine was measured in a beta-counter.

^{111}Indium Oxinate Labeling, Administration, and Autoradiography. Immature and mature DCs were labeled with ^{111}indium oxinate (Mallinckrodt Medical, Petten, the Netherlands) in 0.1 M Tris-HCl (pH 7.0) for 15 min at room temperature as described previously (11, 14). Scintigraphic images of the depot and corresponding lymph node basin were acquired with a gamma camera, 24 or 48 h after injection. After the last imaging session, the lymph node basin was resected. The radioactive lymph nodes in the resected specimen were identified and counted in a gamma counter in combination with injection standards. The fraction of ^{111}In-labeled DCs that migrated from the injection depot was determined as a measure of their migratory capacity *in vivo*. Radioactive lymph nodes were embedded in paraffin. Sections (4-μm) were dipped in LM1 photographic emulsion (Amersham, Buck, United Kingdom) and exposed for 2–3 weeks at 4°C. After exposure, the sections were developed and poststained with H&E.

Online Supplementary Material. DCs were seeded on fibronectin-coated surfaces as described in the adhesion and migration assay section. Subsequent migration of both immature (Fig 2video1.mov) and mature (Fig 2video2.mov) DCs was recorded for 100 min.[2]

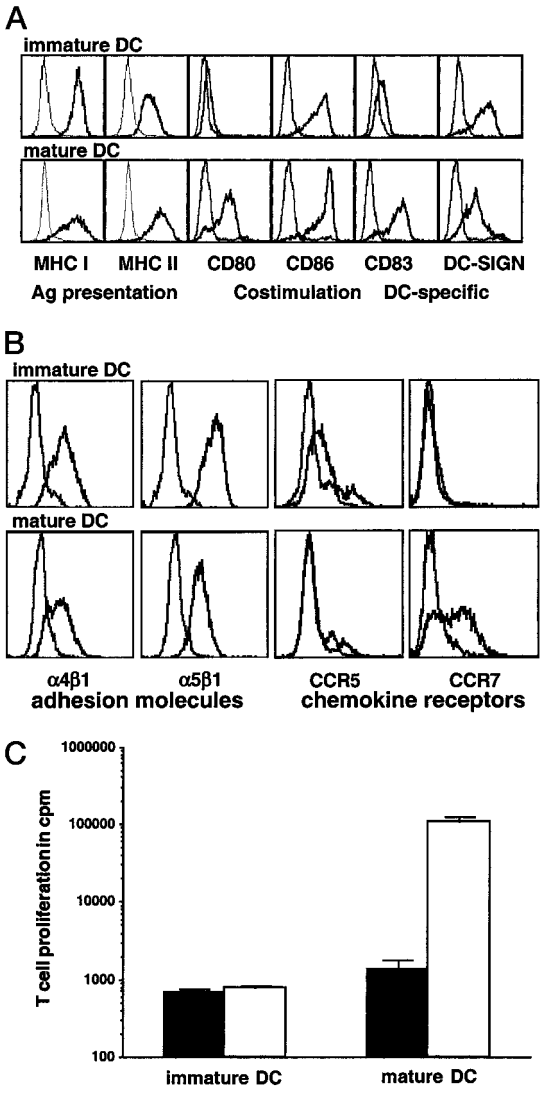

Fig. 1. Characterization of immature and mature DCs. DCs were stained with mAbs against antigen-presentation molecules, costimulatory molecules, and DC-specific molecules (*A*), and adhesion molecules and CCRs (*B*), and were measured by flow cytometry. *Gray lines*, isotype-matched controls; *dark overlays*, the markers as indicated underneath the histograms. Mature but not immature KLH-loaded DCs can induce an immune response *in vivo* after a single vaccination. In *C*, proliferative response against KLH in the PBMCs of the vaccinated patients was used as a readout: ■, without KLH; □ with KLH. The data from 1 of the 9 patients vaccinated with immature DCs and from 1 of the 10 patients vaccinated with mature DCs are shown.

Results

Characterization and Potency of Immature *versus* Mature DCs When Used as Vaccines. Immature and mature DC populations exhibited their respective characteristic phenotype and morphology (Fig. 1*A*). Expression of adhesion molecules, both β1 and β2 integrins, slightly decreased after maturation (Fig. 1*B*, and data not shown). CCR5 was expressed by ~40% of the immature DCs but was absent on mature DCs (Fig. 1*B*). Conversely, immature DCs lacked expression of the homing receptor CCR7, whereas this molecule was present on ~80% of the mature DCs (Fig. 1*B*).

We tested both immature and mature DCs for their capacity to induce a "*de novo*" immune response *in vivo*. In an ongoing study, melanoma patients received vaccinations either with immature ($n = 9$) or mature ($n = 10$) KLH-loaded DCs. We observed that only mature DCs, already after a single vaccination, induced a proliferative response against KLH in the peripheral blood of these patients (Fig. 1*C*). Moreover, DTH reactivity toward KLH-loaded DCs was observed after vaccination with mature DCs in all of the patients tested ($n = 9$), whereas none of the patients ($n = 7$) vaccinated with KLH-loaded immature DCs showed any DTH.

Adhesive and Migratory Properties of DCs to Fibronectin. To examine whether differences in adhesive and migratory behavior between immature and mature DCs contribute to their efficacy as a vaccine, we investigated their capacity to bind to the extracellular matrix protein fibronectin. Although both types of DCs bind to fibronectin, binding and, in particular, cell spreading of immature DCs was much stronger than of immature DCs (Fig. 2, *A* and *B*). DCs adhesion could be blocked completely by antibodies against β1 integrins but was only partially inhibited by β2 integrin blocking antibodies (Fig. 2*C*). Adhesion of mature DCs to fibronectin was mediated through $\alpha5\beta1$ because blocking $\alpha5\beta1$, but not $\alpha4\beta1$, antibodies inhibited this type of adhesion (Fig. 2*D*).

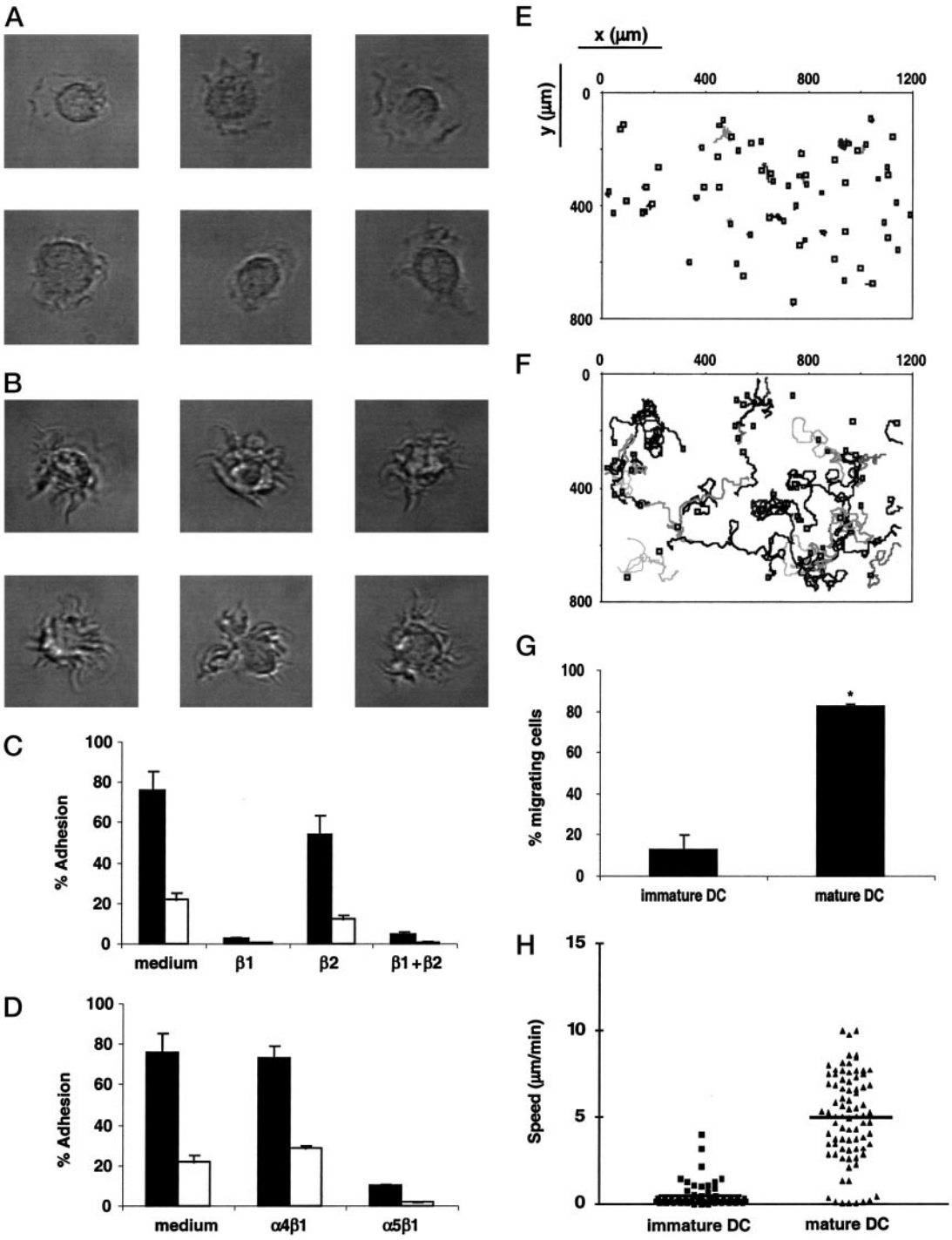

Fig. 2. Adhesion, spreading, and migration of DCs to fibronectin. Morphology of DCs on fibronectin-coated wells (*A*, immature DCs; *B*, mature DCs). Adhesion in the absence or presence of blocking antibodies against $\beta 1$, $\beta 2$ integrins (*C*), and $\alpha 4\beta 1$, $\alpha 5\beta 1$ integrins (*D*); ■ immature DCs; □ mature DCs. Results are expressed as the mean percentage of adhesion of triplicate wells. Data are representatives of three experiments. *E* and *F*, *different lines*, migration paths of DCs: *E*, immature DCs; *F*, mature DCs. *G*, percentage of migrated DCs ± SD of both immature and mature DCs (∗, Wilcoxon-rank-sum test, $P < 0.01$). *H*, speed of migrated DCs ± SD of both immature and mature DCs (Mann-Whitney U test, $P < 0.0001$); *data points*, the speed of individual cells; *horizontal bars*, the mean speed. Data are representatives of three experiments.

Consistent with the enhanced spreading of immature DCs on fibronectin (Fig. 2*A*), virtually none of these cells migrated when followed in time-lapse cinematography (Fig. 2, *E* and *G*, and Fig 2video1.mov[2]). Although cell membranes of immature DCs were highly dynamic, and expanding, contracting, and changing shape continuously, no migration was observed. In contrast, mature DCs were highly motile on fibronectin (Fig. 2, *F* and *G*, and Fig 2video2.mov[2]), using their dendrites to continuously attach and detach from the substrate. As a consequence, the speed of mature DCs was 10 times higher than of immature DC, 5.0 μm/min *versus* 0.5 μm/min (Fig. 2*H*; Mann-Whitney U test, $P < 0.0001$).

Migratory Capacity of DCs after Injection into Melanoma Patients. To examine whether the differences in migration between immature and mature DCs were similar *in vivo* as observed *in vitro*, cells were labeled with radionucleotide ^{111}In (11, 14). Previously, we showed that both immature and mature monocyte-derived DCs can be

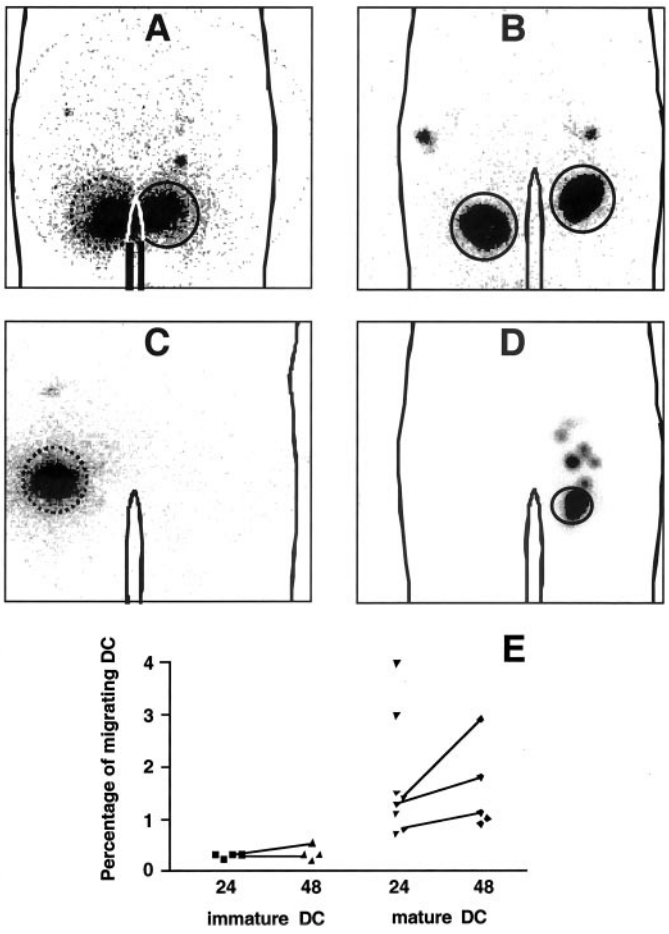

Fig. 3. Biodistribution of DCs after injection. *In vivo* localization of intradermally (*A* and *B*) and intranodally injected (*C* and *D*) ^{111}In-labeled DCs by scintigraphic imaging with a gamma camera. Body contours are indicated. The four images are windowed differently to allow identification of the lymph nodes beyond the injection depot. *A*, immature DCs 0.2%; mature DCs 0.8%. *B*, mature DCs 1.5% and 1.1%. *C*, 4%. *D*, 23%. *Dotted circles*, injection depot of immature DCs. *Closed circles*, injection depot of mature DCs. ^{111}In-labeled DCs injected intradermally were measured for their migratory capacity *in vivo*. *E*, Wilcoxon rank-sum test, $P < 0.01$; *each symbol*, one individual patient.

labeled with equal efficiency (95%), while remaining viable (~80%) for 24–48 h (14). Moreover, ^{111}In-labeling did not affect surface receptor expression by DCs (data not shown). *In vivo* migration of DCs was studied in melanoma patients, who received tumor-peptide-pulsed DCs either intradermally ($n = 8$ immature DCs; $n = 10$ mature DCs) or intranodally ($n = 7$ both immature and mature DCs), 24–48 h before radical dissection of regional lymph nodes. A time point between 24 and 48 h was chosen because the half-life of DCs, either unlabeled or ^{111}In-labeled, and of a MHC-peptide complex that they express, is limited (16).

In accordance with previous results (12), we observed that on intradermal injection, a significant percentage of both immature DCs and mature DCs remained at the site of injection (Fig. 3, *A* and *B*). Nevertheless, a distinct amount of radioactivity was observed in the draining lymph nodes after injection of the DCs. Interestingly, a significantly higher percentage of mature DCs (mean ± SD, 1.8 ± 1.1%) migrated to the lymph nodes and distributed over more lymph nodes (mean ± SD, 2.1 ± 1.6) as compared with immature DCs (mean ± SD, 0.3 ± 0.1% migrated), which never migrated to more than one lymph node (Fig. 3, *A*, *B*, and *E*). In addition, the migration of mature DCs, but not of immature, was somewhat enhanced after 48 h relative to 24 h. In one patient, we followed DC migration for up to 144 h, but no further increase in migration was observed.

Injection of DCs directly into a lymph node resulted in a large variation in the migratory capacity within the immature as well as the mature DC population. There was no difference (Wilcoxon rank-sum test) in migratory capacity of mature DCs (mean, 19.3%; range, 0.4–84%) *versus* immature DCs (mean, 10.2%; range, 0.5–30%; Fig. 3, *C* and *D*, and data not shown).

Intranodal Localization of DCs after Injection into Melanoma Patients. To investigate the capacity of DCs to migrate into lymph nodes, sections derived from resected lymph nodes distant from the node of injection were analyzed by microautoradiography. Explicit spots of radioactivity in the lymph node confirmed the migration of injected ^{111}In-labeled DCs into these nodes (Fig. 4). Intriguingly, a major difference between immature and mature DCs was observed. Immature DCs resided at the periphery of the nodes, the marginal sinus, whereas mature DCs migrated deeply into the T-cell areas, further emphasizing their superior migratory properties (Fig. 4).

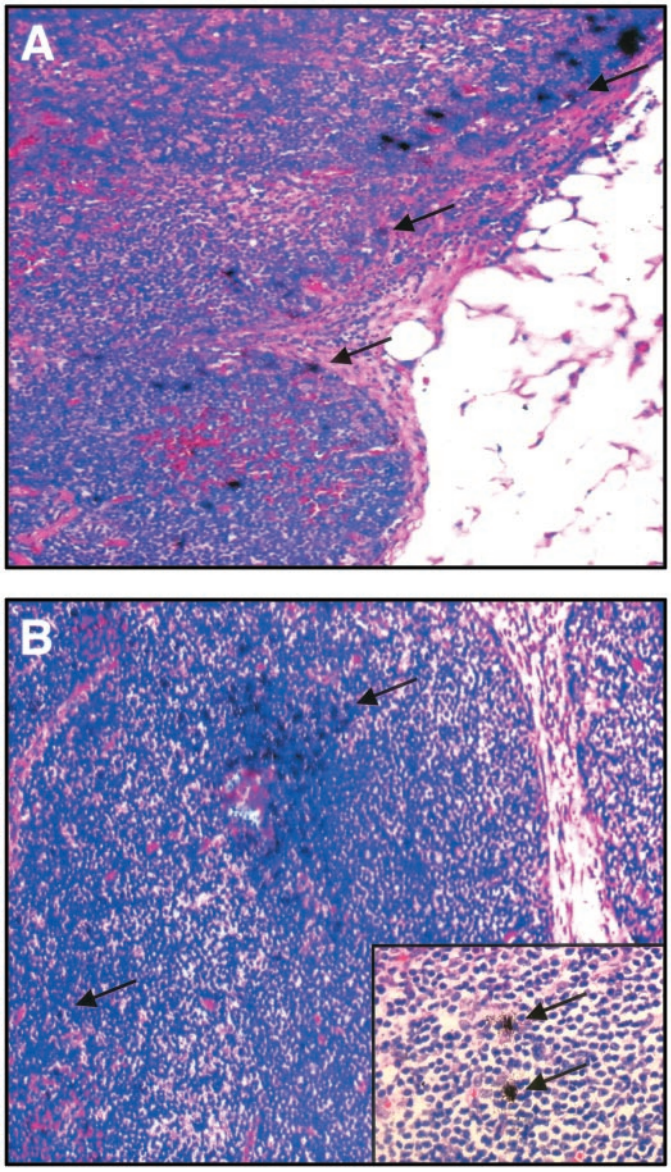

Fig. 4. Distribution of intranodally injected ^{111}In-labeled DCs in distant lymph nodes *in situ*. After autoradiography, lymph node sections were stained with H&E; *black spots*, the presence of ^{111}In-labeled DCs (*arrows*). Overview of the lymph node section with immature DCs (*A*) and mature DCs (*B*).

Discussion

Clinical studies in which tumor-antigen-loaded DCs are used to vaccinate cancer patients indicate that mature DCs are superior to immature DCs in inducing antitumor responses (7, 8). This might be attributed to their unique antigen processing and presentation machinery, which in mature DCs results in extremely high expression of antigenic peptides on MHC molecules and in high levels of costimulatory molecules (4). However, next to their superior antigen-presenting properties, it is of utmost importance that antigen-loaded DCs come in close proximity of T cells in the lymph nodes (17). Therefore, DCs injected into patients must actively migrate into the T-cell areas of lymph nodes. Here, we unequivocally demonstrate that mature DCs are migratory both *in vitro* and *in vivo*, irrespective of whether they are administered into the skin or intranodally. By contrast, *in vitro*-generated immature DCs are unable to leave the skin after intradermal injection. Moreover, when migrating to distinct lymph nodes after direct lymph node application, immature DCs do not invade the T-cell areas, which precludes effective interactions with naïve T cells.

The low migratory capacity of immature DCs relates directly to their strong adhesive properties *in vitro*, which is mediated by highly expressed $\beta 1$ integrins, in particular $\alpha 5\beta 1$ (18). Similar findings have been reported by Gunzer *et al.* (19), who show, in a collagen matrix model, that the immature state of murine DCs is characterized by low migration, whereas mature murine DCs exhibit high migratory activity. Our observations extend their findings by demonstrating that mature DCs weakly bind to extracellular matrix (fibronectin) and use their dendrites to actively migrate, whereas immature DCs stretch and flatten, thus hampering translocation.

On exposure to pathogens in peripheral tissues, resident DCs become activated through toll-like receptors (20), take up and process antigen, while migrating to the draining lymph nodes to present their antigenic load (21). To facilitate migration, CCR5 is down-regulated, whereas CCR7 is up-regulated, the latter being required for trafficking and entry into the T-cell areas of the lymph node (5, 6, 22–24). As expected, we observed that CCR7-positive mature DCs migrate into the T-cell areas of the lymph nodes, whereas immature CCR7-negative DCs do not. The inability of immature DCs to migrate into the T-cell areas could explain why vaccinations with KLH-loaded immature DCs in melanoma patients fail to induce both proliferative and DTH responses. Our observations that immature DCs fail to induce an immune response correlates with the studies of others. Immature DCs, when used as a vaccine adjuvant, might give rise to T cells that display the typical properties of regulatory T cells: nonproliferative, interleukin 10-producing T cells that can dampen pre-existing antigen-specific effector T-cell function (8, 25).

Previously, we and others showed that the majority of injected DCs reside at the injection depot (11–13, 26). Here, we demonstrate that monocyte-derived DCs that are matured *in vitro*, for the greater part, remain in the injection depot, although they express CCR7 and are highly motile *in vitro*. Gunzer *et al.* reasoned that the microenvironment largely influences emigration (19). In addition, the relative high local density of DCs at the site of injection may affect this microenvironment, thereby having a major impact on emigration. A better understanding of this lack of emigration, which can be enhanced with matrix metalloproteinases, is of importance for future DC vaccine development (27).

Although intradermally injected DCs migrate out of the skin very inefficiently, nevertheless, a 10-fold higher number of mature DCs migrated to an adjacent lymph node when compared with their immature counterparts. In addition, mature, but not immature, DCs migrate to multiple lymph nodes after intradermal injection. Intranodal application of immature or mature DCs leads to a substantial migration to several distant lymph nodes, as soon as 1 h after vaccination. Although only the mature DCs reach the T-cell areas, after intranodal injection of immature and mature DCs, little or no difference is observed between the migration to lymph nodes. Intranodal injection resulted in a rather variable migration in both cell populations. This might be because injection of DCs directly into a lymph node leads to a partial destruction of the lymph node architecture, resulting in the migration to distant lymph nodes, of DCs that would otherwise reside in the injected node. Likewise, during intranodal administration, a significant proportion of DCs may be transported by the flow of lymphatic vessels to nearby lymph nodes. A major advantage of intranodal over intradermal vaccination is, therefore, that an increased number of DCs are getting to the lymph nodes, *e.g.*, the site at which the initiation of the immune response occurs. Especially when peptide-loaded DCs are used as a vaccine, the time required to reach the T-cell areas of the lymph nodes is of great importance, because the half-life of DCs and of a MHC-peptide complex that they express is limited (16). In the first clinical study in melanoma patients, which reported on the efficacy of peptide-loaded DC vaccines, mature DCs were injected intranodally. Our results may provide a mechanistic explanation for the effectiveness of the protocol used in that study (1).

Given the previously described tolerizing capacity of immature DCs, the recent results from mature-DC vaccination studies, and the migration data presented in this study, we conclude that mature DCs are preferred over immature DCs in clinical vaccination studies in cancer patients.

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