

# Migration of Human Dendritic Cells after Injection in Patients with Metastatic Malignancies<sup>1</sup>

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## Abstract

Present clinical studies of active immunotherapy for malignancies using dendritic cells (DCs) require elucidation of the sites where DCs localize after injection. We evaluated the pattern of distribution of *in vitro*-generated, antigen-loaded, human DCs labeled with indium-111 oxyquinoline after i.v., s.c., and intradermal injection. Whereas the DCs injected i.v. localized in the lungs and then redistributed to the liver, spleen, and bone marrow, they were not detected in lymph nodes or tumors. A small percentage of DCs injected intradermally migrated rapidly to the regional lymphatics in some individuals. No lymph node activity was detected after s.c. injection. Our results demonstrate that DC distribution to sites of lymphoid tissue is dramatically affected by the mode of administration.

## Introduction

Active immunotherapy strategies using tumor antigen-loaded DCs<sup>3</sup> depend on DC interactions with T cells. The best route of DC administration for ensuring migration to T cell-rich sites is uncertain, in part, because the sites where human DCs localize after injection are unknown. Although bone marrow-derived DCs or their precursors circulate in the peripheral blood to most tissues, acquire antigen, and subsequently migrate to regional lymph nodes (1, 2), it remains uncertain whether DCs (generated *in vitro* under the influence of cytokines such as GM-CSF and IL-4; Refs. 3–5) exhibit equivalent migratory capacity. Murine (6–8) and primate (9) studies have demonstrated that i.v.-administered DCs initially appear in the lungs, followed several hours later by accumulation in the liver and spleen and only minimally in mesenteric lymph nodes. s.c. injection of DCs resulted in varying degrees of migration to the regional lymphatics. Because it is important to establish the migratory pattern of the *in vitro*-generated, antigen-loaded human DCs being administered in clinical studies, we examined the tissue localization of In-111-labeled DCs, generated from PBMCs of cancer patients. We demonstrated that DCs injected i.v. initially localize in the lungs and then redistribute to the liver, spleen, and bone marrow, but apparently not to the lymph nodes or tumor masses. DCs injected intradermally or s.c. were partially cleared from the injection site, but migration to regional lymph nodes was observed only after intradermal, but not s.c., injection.

## Patients and Methods

**Patients.** The patients ( $n = 7$ ) consisted of a subset of those enrolled on a Phase I study of active immunotherapy with CEA RNA-loaded DCs. All patients were required to have a metastatic cancer-expressing CEA [breast (1), lung (4), and colon (2)] and evidence of a recall antigen response on skin testing with either

Tetanus, Mumps, Candida, or Histoplasma. Potential candidates for the study were excluded if they had received chemotherapy, radiation therapy, immunotherapy, or immunosuppressive medications within the prior 4 weeks. Candidates for intradermal injections were excluded if they had had a lymph node dissection or known lymphatic obstruction in the bed draining the injection site. Study subjects provided signed informed consent that fulfilled Institutional Review Board guidelines before completing the screening procedures.

**Generation of DCs.** The patients underwent an unmobilized peripheral blood leukapheresis, consisting of a 3–4-h processing of 8–12 liters of blood with each collection containing a minimum of  $1.5 \times 10^8$  nucleated cells/kg recipient weight. PBMCs were separated from the leukapheresis product, resuspended in AIM V media (Life Technologies, Grand Island, NY) at  $6 \times 10^6$  cells/ml, and incubated in T225 tissue culture flasks (60 ml/flask) for 2 h in 5% CO<sub>2</sub> at 37°C. The nonadherent cells were gently resuspended by rocking the flasks and removed. The adherent cells were cultured in AIM V media containing GM-CSF (800 units/ml) and IL-4 (500 units/ml; kind gifts of Mary-Ellen Ryback, Schering-Plough Research Institute, Kenilworth, NJ) for 7 days to generate a cell population enriched for DCs. After 7 days, the cultured cells were harvested by vigorous washing and the addition of cell dissociation buffer (Life Technologies). The DCs were depleted of bystander lymphocytes and natural killer cells by use of an antibody mixture containing anti-CD3, -CD16, -CD19, -CD56, magnetic colloid, and a magnetic column (Stem Cell Technologies, Vancouver, British Columbia). The cells not retained in the column were >90% DCs by morphology. The DCs were coincubated with CEA RNA (produced as described previously; Ref. 10) for 2–4 h and washed twice in normal saline.

**In-111 Labeling.** The CEA RNA-loaded DCs were labeled with In-111 as follows: for patients receiving a dose of  $100 \times 10^6$  DCs i.v., the DCs were resuspended in 500  $\mu$ Ci In-111 (Nycomed-Amersham, Chicago, IL) in a total volume of 1 ml. This was calculated to result in  $\sim 200 \mu$ Ci of radioactivity administered with the DCs, an amount felt to be safe based on calculations of radiation exposure of the lungs, liver, and spleen. Incubation was carried out at room temperature for 1 h, after which the cells were washed three times, and the labeling efficiency was determined by measuring the amount of radioactivity contained within the cellular fraction compared with the supernatant. The DCs were resuspended in a volume of 15–30 ml of normal saline for i.v. injection. For patients receiving DCs intradermally or s.c.,  $1 \times 10^7$  DCs were similarly labeled with the In-111, except that only 10  $\mu$ Ci of radioactivity was used to limit the radiation exposure of the skin. These DCs were resuspended in 1 ml of normal saline for injection.

**Immunofluorescence Staining and Fluorescence-activated Cell Sorting Analysis.** To demonstrate that the phenotype of the DC was not altered by the labeling procedure, immunofluorescence staining with the monoclonal antibodies anti-CD14-FITC, anti-CD86-PE, and anti-HLA-DR-PerCp (Becton Dickinson, San Jose, CA) and fluorescence-activated cell sorting analysis were performed on the DCs before and after labeling with the In-111, as previously described (5).

**Allogeneic MLR.** The allogeneic MLR was performed on the DC before and after labeling with the In-111 to determine whether the function of the DC was altered by the labeling. Allogeneic responder PBMCs ( $1.5 \times 10^5$ ) obtained from healthy donors were cultured in RPMI 1640 supplemented with 10% fetal bovine serum or 10% human AB serum in 96-well U-bottomed tissue culture plates. Irradiated (3500 rads) DC preparations (or the patient's PBMCs used as controls) were added in graded doses of 150–15000 cells/well in a total volume of 200  $\mu$ l. Cell proliferation after 96 h was quantified by adding 1  $\mu$ Ci (37kBq) of [methyl <sup>3</sup>H]thymidine (NEN-DuPont, Boston, MA) to each well. After 16 h, the cells were harvested onto filters, and radioactivity was meas-

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<sup>3</sup>The abbreviations used are: DC, dendritic cell; In-111, indium-111; CEA, carcinoembryonic antigen; PBMC, peripheral blood mononuclear cell; MLR, mixed lymphocyte reaction.

Table 1 Localization of i.v.-administered In-111-labeled DCs

Time	Right lung maximum activity (%)	Left lung maximum activity (%)	Liver maximum activity (%)	Spleen maximum activity (%)
1 min	100	100	4 ± 5	3 ± 0
1 hr	74 ± 19	77 ± 13	30 ± 9	36 ± 11
6 hr	25 ± 9	28 ± 5	86 ± 9	87 ± 9
24 hr	11 ± 5	13 ± 2	100	100

ured in a scintillation counter with results presented as the mean cpm for triplicate cultures.

**Injection of DCs and Imaging.** The first group of three patients received  $100 \times 10^6$  In-111-labeled DCs in 15–30 ml of normal saline as an i.v. bolus over 1 min. Immediately after the injection, imaging of the chest and abdomen with a dual-headed gamma camera was performed continuously for 1 h. Images of the chest and abdomen were then acquired at 1, 6, and 24 h. Peripheral blood samples were obtained after radiolabeled DC administration at 15, 30, and 60 min as well as at 1, 2, 3, 4, 5, 6, and 24 h to determine the kinetics of radiolabel in the circulation. Image analysis was performed by using region-of-interest analysis of the lung, liver, and spleen to obtain the decay-corrected counts and a geometric mean for the anterior and posterior views. The second group of four patients received injections of In-111-labeled DCs into the skin as follows: the first patient received intradermal injections of  $1 \times 10^6$  DCs (in a volume of 0.2 ml), each labeled with 1  $\mu$ Ci In-111, at four locations spaced approximately 1 cm apart on the upper biceps; the last three patients received four intradermal injections of  $1 \times 10^6$  DCs (in a volume of 0.2 ml), each labeled with 1  $\mu$ Ci In-111, into the left leg and four s.c. injections of a similar number of labeled DCs into the right leg. Images over the injection site and draining lymph node bed were obtained at 1 min, and 6, 24, and 48 h. Regions of interest were used on the anterior images to obtain the counts that were decay corrected. The percentage of clearance from the time of administration for each area of interest was calculated.

## Results

**DCs Demonstrate Efficient Labeling with In-111 and Are Phenotypically and Functionally Similar to Nonlabeled DCs.** In pre-clinical studies ( $n = 3$ ), we determined the ideal conditions for labeling the DCs by suspending them in In-111 oxyquinoline at various cell concentrations and for varying amounts of time. Efficiency of labeling was most dependent on cell concentration because only 10–13% In-111 uptake was observed at DC concentrations of  $1 \times 10^6$ /ml, whereas 60–78% uptake occurred at  $1 \times 10^8$  DC/ml.

Time of labeling had a lesser effect, with the greatest uptake (78%) occurring after 1 h. The viability and percentage labeling of the DCs *in vitro* remained high (60–80%) at 24 h. Neither the phenotype of the DCs (DR+, CD86+, CD14-) nor the potent allostimulatory activity in the mixed lymphocyte reaction were altered by incubation with In-111 (data not shown). We also confirmed that the passage of DCs through the small 25-gauge needles required for intradermal injections did not cause increased cell damage and In-111 release (data not shown).

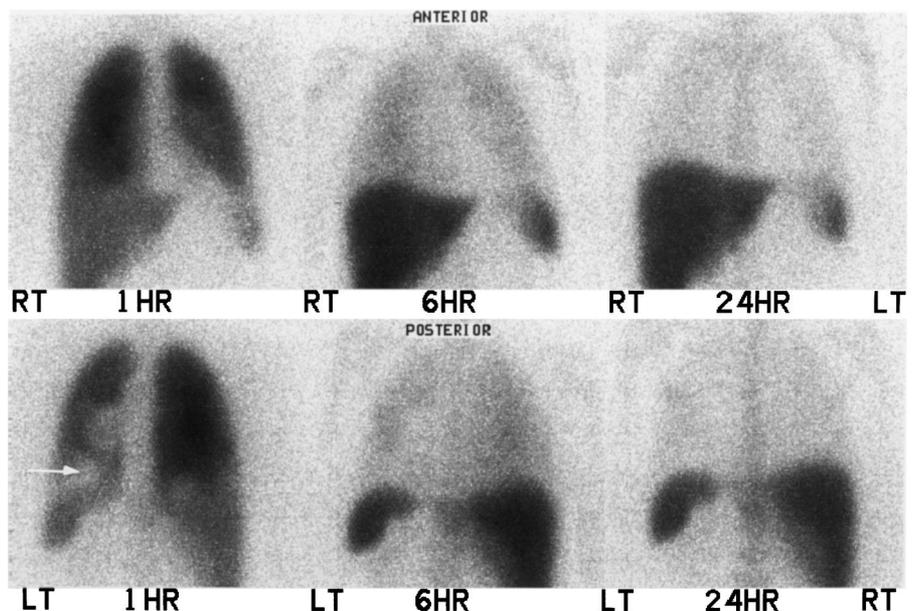
**Migration of i.v.-injected In-111-labeled DCs.** In the three patients with i.v.-administered In-111, for each time point, we calculated the percentage of the maximum activity detected in the left lung, right lung, liver, and spleen (Table 1). Fig. 1 shows the images from a representative patient demonstrating the time course of the change in activity in the lungs, liver, spleen, and bone marrow. Although in the first minute, the activity was localized to the lungs by 1 h, activity appeared in the liver and spleen. By 24 h, the activity was predominantly localized to the liver, spleen, and bone marrow, but none was observed in any lymph nodes or tumor masses (*arrow*). From the counts obtained in whole blood and plasma, minimal activity (2–7% of the injected dose) was detected in the peripheral blood.

**Localization of In-111-labeled DCs injected intradermally or s.c.** In the next four patients, In-111-labeled DCs were injected intradermally into the left anterior thigh or s.c. into the right anterior thigh. The percentage of the injected activity at each time point over 48 h in the injection site and regional lymph nodes was calculated (Table 2). Fig. 2 shows an image from a representative patient demonstrating activity in a regional lymph node and the injection site. In two of the four patients, activity, albeit low (0.1–0.4% of the injection site activity), was rapidly detected in the inguinal region of the intradermal injection site and increased slowly until 24 h, after which activity leveled off and in one case decreased slightly. No activity was ever detected in a regional lymph node draining the s.c. injection site. The activity slowly decreased in both the s.c. and intradermal injection sites, but 65–70% of the initial activity still remained after 48 h.

## Discussion

The ideal vaccination protocol for inducing protective immune responses is not established. Animal models have suggested that

Fig. 1. Sequential In-111-labeled DC images. Anterior (*top*) and posterior (*bottom*) images obtained 1, 6, and 24 h after i.v. administration of In-111 DCs. The images obtained 1 h after injection demonstrated localization in the lungs, liver, and spleen. An area of absent accumulation is noted on the posterior image at the left lung base in the region of known metastatic tumor (*arrow*). The images at 6 and 24 h demonstrate clearance of the radioactivity from the lungs, with increasing accumulation in the liver, spleen, and bone marrow.



peptide-loaded DCs administered i.v. are more effective than those administered s.c. in protecting against a tumor or infectious challenge (11, 12). Nonetheless, it is not known where the i.v. administered DC contact the T cells during induction of the immune response. Fossum (8) observed that i.v.-injected DCs localized in the liver and spleen of rats. From the liver, some of the cells migrated to celiac lymph nodes. DCs injected into the footpad remained primarily localized, though a few migrated to the popliteal nodes. Similar observations were made in mice (7) and chimpanzees (9), except that either trace (7) or no (8) lymph node migration was detected after i.v. injections. Our results are similar to the animal studies in that the patient DCs localized first to the lungs, and then the liver, spleen, and bone marrow after i.v. injection, whereas a small amount of lymph node transit (0.1–0.4% of the injected activity) was observed for intradermal injections in some patients. Although the number of DCs that must reach lymphatic tissue for an effective immune response in humans is unknown, Porgador *et al.* (13) showed in a murine model that i.v. administration of as few as  $9 \times 10^3$  DCs pulsed with tumor peptides had a measurable antitumor effect. If a similar potency of DCs applies to humans, the number of DCs we observed to migrate from intradermal sites may be adequate to induce an antitumor response. Despite the small amount of activity appearing in regional lymphatics, 20–30% of the activity was lost from the injection sites indicating that the DCs may migrate to other sites, bypassing the regional lymphatics, but we could not identify these sites. Although it is possible that the limits of resolution of the In-111 imaging technique do not permit detection of very small amounts of DC-associated activity in lymph nodes, it is clear that most of the activity in the first 24 h is localized to the liver, spleen, and bone marrow after i.v. injection, and in the local site after intradermal or s.c. injection. Because the rate of change in the level of activity at each site was leveling off by 48 h, we do not believe that further monitoring would have detected a more selective localization of DCs in tumors or lymph nodes. It is also clear that tumors, even those in the lungs, are not infiltrated with the i.v. injected DCs. We suspect that this is due to the fact that the lung tumors are supplied primarily by bronchial arteries (derived from the aorta) and not the pulmonary arteries (14). The bronchial vasculature does not possess the narrow channels found in the pulmonary vasculature, liver, and spleen in which injected cells are likely to lodge (15), and thus tumors supplied by bronchial arteries are no more likely to have a significant exposure to i.v.-injected DCs than organs such as the kidney or brain.

Our data indicate that DC trafficking is markedly dependent on their mode of delivery. s.c. administration seems to be ineffective in causing DC migration to regional lymphatics. i.v. administration results in DC migration to the spleen, whereas intradermal administration leads to regional transit in some patients. Which site of T cell contact will lead to greater antigen-specific immune responses is uncertain. Murine studies indicating that migration patterns of DCs may determine whether Th1 or Th2 responses are induced (16) underscore the need to compare immunological responses induced with each route of injection in order that the

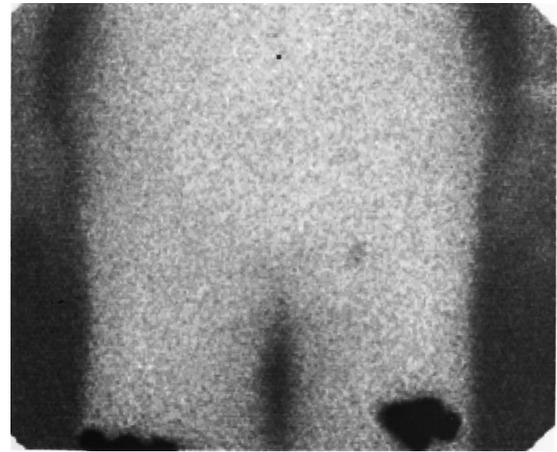


Fig. 2. Combined transmission-emission image. The In-111-labeled DCs were administered s.c. in the right leg and intradermally in the left leg. This anterior image of the upper thighs and pelvis was obtained 6 h after injection of the In-111-labeled DCs. A source of radioactivity was placed behind the patient to provide the body outline. The figure demonstrates the injection sites in both anterior thighs and a small focus of radioactivity in a left inguinal lymph node.

ideal strategy be used in clinical trials. Whether the pattern of localization can be altered by the use of other cytokines or agents to modulate adhesion molecules remains to be studied.

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Table 2. Localization of intradermally or s.c.-administered In-111-labeled DCs

Time	s.c. injection site maximum activity (%)	Intradermal injection site maximum activity (%)	Inguinal LN <sup>a</sup> draining ID site maximum activity (%)	Inguinal LN draining ID site injection site activity (%)
1 min	100	100	56 ± 5	0.08
6 hr	93 ± 3	89 ± 1	75 ± 4	0.19
24 hr	82 ± 4	77 ± 2	95 ± 7	0.37
48 hr	70 ± 4	65 ± 4	92 ± 3	0.41

<sup>a</sup> LN, lymph nodes; ID, intradermal.

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