Biodistribution and Vaccine Efficiency of Murine Dendritic Cells Are Dependent on the Route of Administration


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Abstract

Dendritic cells (DCs) are professional antigen-presenting cells, well equipped to initiate an immune response. Currently, tumor antigen-derived peptide loaded DCs are used in clinical vaccination in cancer patients. However, the optimal dose and route of administration of a DC vaccine still remain to be determined. Using indium-111-labeled DCs, we investigated whether the route of administration does affect the biodistribution of DCs in lymphoid organs and whether it influences the outcome of DC vaccination in the B16 mouse melanoma tumor model. The results demonstrate that i.v. injected DCs mainly accumulate in the spleen, whereas s.c. injected DCs preferentially home to the T-cell areas of draining lymph nodes. Using tyrosinase-related protein-2-derived peptide-loaded DC vaccination in a fully autologous B16 melanoma tumor model, we observed a delay in tumor growth, improved survival as well as increased antitumor cytotoxic T-cell reactivity after s.c. vaccination as compared to i.v. vaccination. These data demonstrate that optimal induction of antitumor reactivity against the autologous melanocyte differentiation antigen tyrosinase-related protein-2-derived peptide occurs after s.c. vaccination and correlates with the preferential accumulation of DCs in the T-cell areas of lymph nodes.

Introduction

DCs constitute a family of APCs defined by their morphology and their unique capacity to initiate a primary immune response (1, 2). They originate from bone marrow and are present as immature APCs in nonlymphoid tissues. On activation by antigen challenge and/or inflammation, the DCs mature and migrate to the secondary lymphoid tissues where they present the processed antigen to T cells. There they interact with T cells and present the processed antigen in MHC molecules (3). Because of these properties, mature DCs are thought to be ideal for generating a primary immune response against cancer, viral infections, and other diseases. In recent years, techniques have been developed to generate large numbers of functionally potent DCs by culturing bone marrow (4, 5) or peripheral blood monocytes in the presence of GM-CSF and/or other cytokines, such as IL-4 (6), tumor necrosis factor, stem cell factor, and FLT3 ligand (7, 2). The availability of class I-restricted peptides derived from tumor-associated antigens, such as tyrosinase, TRPs, gp100, and MART-1/Melan-A, provides evidence that the efficiency of DC vaccination is dependent on the route of DC administration. The implications of these findings for DC vaccination will be discussed.

Materials and Methods

Mice. Male C57BL/6 (H-2b) mice were purchased from Charles River Wiga (Sulzfeld, Germany) and held under specific pathogen-free conditions in the Central Animal Laboratory, Nijmegen University (Nijmegen, the Netherlands). For experimental purposes, mice 6–8 weeks of age were used.

Reagents. Cells are cultured in Iscove’s modified DMEM with glutamax supplemented with 10% heat-inactivated FCS (Life Technologies, Inc., Breda, the Netherlands), 50 μg/ml β-mercaptoethanol, antibiotics, and anticytokines (Life Technologies, Inc.), unless mentioned otherwise. Murine recombinant GM-CSF (rmGM-CSF) and murine recombinant IL-4 (rmIL-4) were kindly provided by Dr. G. Zurawski (DNAX Research Institute, Palo Alto, CA). The following monoclonal antibodies were used: CD45R/B220 (RA3-6B2), CD4 (MT-4), CD8 (LYT-2), I-A^k (17/227), and the activating anti-CD40 monoclonal antibody FGG-45 (kindly provided by Dr. Rolink, Basel Institute for Immunology, Basel, Switzerland; Ref. 12). The mTRP2 (VYDFFWVL) peptide and irrelevant peptide OVA (SIINFEKL) were synthesized with a free COOH terminus by F-moc peptide chemistry using ABIMED Multiple Synthesizer or by T-boc chemistry on a Biosearch SAM2 peptide synthesizer. The peptides were >90% pure, as indicated by high-performance liquid chromatography. Peptides were dissolved in DMSO and stored at −20°C.

DC Culture. DCs were generated according to Inaba et al. (4), with modifications. Briefly, femurs were dissected, placed in 70% alcohol for 1 min, and washed with PBS. Marrow was flushed and passed through nylon mesh to remove debris. After washing, lymphocytes, granulocytes, and I-A^k-positive cells were removed by immunomagnetic depletion against the CD45R, CD4, CD8, and I-A-antigens. Remaining cells were cultured overnight, and the nonadherent cells were seeded at 2 × 10^5 cells/ml and 4 ml/well in the presence of 20 ng/ml rmGM-CSF and rmIL-4 in 6-well plates (Costar, Badhoevedorp, the Netherlands). On day 4, the cultures were refreshed by adding 1 ml of culture medium supplemented with GM-CSF and IL-4 (10 ng/ml). At day 7, nonadherent and loosely adhesive proliferating DC aggregates were collected and replated in fresh medium, cytokines (1 × 10^7 cells/ml), and 500 μl of hybridoma supernatant of activating anti-CD40 antibody FGG 45.

[111In]oxinate Labeling. Gamma Camera Imaging and Biodistribution Analysis. Bone marrow DCs were labeled with [111In]oxinate (Mallinckrodt Medical, Petten, the Netherlands) in 0.1 M Tris/HCl (pH 7.0) for 20 min at room temperature. Cells were washed three times with PBS, and the labeling efficiency was calculated as the percentage of the activity that remained associated with the cell pellet. To determine the stability in vitro, DC samples were kept in medium without cytokines at 37°C. After different time periods, cells were spun down, the supernatant was transferred to other tubes, cell pellets were resuspended in the same volume, and the emitted radioactivity...
was counted in the gamma counter. The percentage of cell-associated radioactivity was calculated. For the in vivo studies, mice, five per experimental group, were given injections either s.c. (abdomen or thighs), i.p., or i.v. in the lateral tail vein with \(1 \times 10^6\) DCs (30–50 \(\mu\)Ci) in 200 \(\mu\)l of PBS/mouse. At different time points, mice were anesthetized with oxygen/nitrous oxide/ethane (ICI Farma, Rotterdam, The Netherlands), and images were recorded with a gamma camera (Siemens Orbiter; Siemens Inc., Hoffmann Estate, IL) equipped with parallel-hole medium-energy collimator. Images were obtained (minimum of 100,000 acquired counts) and were digitally stored in a 256 \(\times\) 256 matrix. For tissue biodistribution, groups of five mice were analyzed at different time points after injection of the \(^{111}\text{In}\)-labeled DCs. Blood samples, lungs, liver, kidneys, spleen, thymus, small intestine, mediastinal tissue, and lymph nodes (brachial, lumbal, inguinal, popliteal, and mesenterial) were collected, weighed, and counted in the gamma counter. To correct for radioactivity decay, injection standards were counted simultaneously. The measured activity in tissues and samples was expressed as the percentage of ID or as percentage of ID/0.1 gram tissue (%ID/0.1 g). All values are expressed as mean \(\pm\) SE.

**Microscopic Autoradiography.** Lymphoid organs were dissected 24 h after i.v. and s.c. injection of living or gluteraldehyde-fixed (0.1%; 10 min at room temperature) \(^{111}\text{In}\)-oxinate-labeled DCs (250–300 \(\mu\)Ci/\(1 \times 10^6\) DC), fixed in Unifix and embedded in paraffin. Deparaffinized sections were dipped in LM1 photographic emulsion (Amersham, Buck, United Kingdom) and poststained with hematoxylin. Parallel series of sections stained directly 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 hematoxylin. Parallel sections of sections stained directly with H&E were included for better appreciation of morphology.

**Vaccination with Peptide-pulsed DCs.** On days 8 or 9, DCs were harvested and loaded with 10–20 \(\mu\)M peptide and 5 \(\mu\)g/ml \(\beta\)2-microglobuline (Cymbus Biotechnology LTD, Canpro Scientific, Veenendaal, the Netherlands) in Optimem (Life Technologies, Inc.; 2–3 \(\times\) 10^5 cells/ml). After loading, DCs were washed in saline, and 4–10^6 DCs/0.1 ml of saline were injected. Peptide-pulsed DCs were injected twice with a 2-week interval. Two weeks after the second vaccination, mice were challenged s.c. with 1 \(\times\) 10^3 live B16 melanoma cells in 0.1 ml of saline in each flank. The size of growing tumors was measured every 2–3 days using microcalipers. The murine melanoma cell line B16 (subline F10) was grown as described by Fidler (13). The murine thymoma cell line EL-4 (American Type Culture Collection, Manassas, VA) was cultured in Iscove’s medium (Life Technologies, Inc.) supplemented with 5% FCS and 50 \(\mu\)l MTT-mercaptoethanol.

**CTL Culture and Chromium Release Assay.** After tumor challenge, spleens were isolated from tumor-bearing and protected mice, and 3 \(\times\) 10^6 single-cell splenocytes were restimulated in an upright T25 flask (10 ml; Costar) with 1 \(\times\) 10^6 irradiated (25 Gy) peptide-loaded lipopolysaccharide blasts (1 \(\times\) 10^6/ml splenocytes cultured for 3 days in the presence of 25 \(\mu\)g/ml lipopolysaccharide (Salmonella typhosa; Sigma) and 7 \(\mu\)g/ml Dextran-sulfate in a T75-flask (Costar), and loaded with 100 \(\mu\)M peptide + 5 \(\mu\)g/ml human \(\beta\)2m (Cymbus)). Bulk CTLs were isolated after a 7-day restimulation by density gradient centrifugation (Lympholite-M; Cederlane Laboratories, Sandbio, Uden, the Netherlands) and were used as effectors in a chromium release assay, performed as described previously (14). When used in a chromium release assay, B16 tumor cells were pretreated with recombinant rat IFN-\(\gamma\), 50–100 units/ml, for 48 h.

**Results**

**Bone Marrow-derived DCs can be Efficiently Labeled with \(^{111}\text{In}\)-oxinate.** To investigate the routing and tissue distribution of DCs in a vaccination setting, we generated mouse bone marrow-derived DCs using GM-CSF and IL-4 in vitro (4) and determined their labeling efficiency using different radionuclides. To obtain a homogeneous DC population with a mature phenotype, DCs were cultured for one additional day in the presence of an activating anti-CD40 antibody. Analysis of the nonadherent fraction demonstrates that \(>80\%\) of the DCs now express the characteristic DC markers MHC class II and B7 at high levels. Because initial experiments indicated that the labeling efficiency of DCs with \(^{111}\text{In}\)-oxinate was far superior to \(^{125}\text{I}\), we further optimized the DC labeling with \(^{111}\text{In}\)-oxinate and obtained a labeling efficiency of 85–90% (data not shown). As shown in Fig. 1A, the percentage of cell-associated radioactivity is still \(>75\%\) after 8 h, 50% after 20 h, and is decreased to \(25\%\) after 48 h. As shown in Fig. 1B, the observed decrease in cell-associated radioactivity in time corresponds to the loss of viable DCs. This loss of viable cells is likely due to apoptosis of the mature DC in vitro and does not occur as a consequence of the \(^{111}\text{In}\)-labeling (Fig. 1B). These findings demonstrate that in vitro-generated bone marrow-derived DCs can be efficiently labeled with \[^{111}\text{In}\]oxinate (85–90%), which is in line with the previous observation that freshly isolated spleen-derived DCs can be labeled with \[^{111}\text{In}\]chloride (75–65% efficiency; 15). The observation that the radioactivity stays associated with viable cells for up to 48 h allows usage of \[^{111}\text{In}\]-labeled DCs to study migration and homing in vivo.

**Biodistribution of i.v.-injected \(^{111}\text{In}\)-labeled DCs.** As in most animal tumor models DCs are administered by i.v. injection (16, 17, 18), we investigated the in vivo biodistribution of bone marrow-derived DCs after i.v. administration by measuring the radioactivity present in dissected organs (Fig. 2B-D), as well as by total body imaging in time (Fig. 2A).

As shown by body imaging (Fig. 2A), i.v. injection of \(^{111}\text{In}\)-labeled DCs resulted in the immediate accumulation in capillary rich, well perfused organs like the liver, lung, spleen, and kidneys. As expected, a rapid decrease in the amount of radioactivity was observed in the lungs in time. After 48 h, most of the detectable dose was located in the liver, spleen, and kidneys, resulting in a “dome-shaped” area, as shown in Fig. 2A. To determine the DC biodistribution more precisely, different organs (five mice/group) were dissected and the emitted radioactivity was measured at each time point. As shown in
Fig. 2B, 50–60% of the ID could be recovered from the dissected organs and is similar at each time point. The results further confirmed the bioimage data, revealing the decrease of radioactivity in the lungs and the accumulation in the liver, spleen, and kidneys (Fig. 2C). To correct for weight differences (liver, ~ 1 g; popliteal lymph node, ~ 0.001 g) and to visualize the accumulation of DCs in small organs, like lymph nodes, the obtained values were also adjusted to 0.1 g of tissue. As shown in Fig. 2D, the highest amount of radioactivity relative to tissue weight is present in the spleen. However, still hardly any radioactivity is contained in the small lymph nodes. Collectively, the data indicate that 111In-labeled DCs are well suited to follow DC migration in vivo and that, after i.v. injection, DCs accumulate preferentially in the spleen, whereas the amount of radioactivity recovered from lymph nodes is very low, even 48 h after injection.

The Route of Administration Affects the Accumulation of DCs in T-cell Areas of Lymph Nodes. Because of their capacity to induce immune responses, DCs loaded with tumor antigens are used to vacinate against tumors in mice, as well as in man. However, it is not clear which route of administration of DCs is preferred. Therefore, we investigated the biodistribution of DCs in relation to the route of administration (e.g., i.v., s.c., and i.p. injection of 111In-labeled DCs).

This analysis revealed (Fig. 3A), that the amount of radioactivity present in the lungs, spleen, and liver at 48 h after s.c. and i.p. injection was low in comparison to i.v. administration, whereas the amount of radioactivity present in kidneys seemed to be relatively independent of the route of administration and constant in time. The relatively low amount of radioactivity recovered from the dissected organs was due to the fact that a substantial amount of radioactivity (55–35%) is contained at the injection site after i.p. and s.c. application. Analysis of the s.c. injection site by H&E staining and by autoradiography revealed that the radioactivity containing cells were located in the s.c. fat tissue and were >90% viable (Fig. 3F and data not shown). However, in time, a marked accumulation of radioactivity (%ID) in the draining lymph nodes was observed after s.c. application (Fig. 3B), whereas intermediate amounts could be detected after i.p. application (Fig. 3B).

To investigate the localization of the anti-CD40-activated 111In-labeled DCs after s.c. administration, the draining lymph nodes were removed 24 h after injection and analyzed by histology (Fig. 3E) and autoradiography (Fig. 3, C and D). As shown in Fig. 3C, the 111In-labeled DCs specifically home to the paracortical area of the lymph node. In contrast, s.c.-injected gluteraldehyde-fixed 111In-labeled DCs (Fig. 3D) did not reach the T-cell areas, indicating that the observed migration into the T-cell areas (Fig. 3C) is specific.

Collectively, these data demonstrate that in contrast to i.v.-injected DCs, s.c.-administered DCs specifically accumulate in the draining lymph node and migrate into the T-cell area where they are known to interact with T cells to initiate an immune response.

s.c. DC Vaccination Leads to Improved Survival in the B16 Tumor Model. It has been well established that i.v. administration of antigen-pulsed DCs can protect mice from a subsequent lethal challenge with relatively immunogenic tumors, like in the B16-OVA tumor model (19). To study the effect of the route of DC administration in other models, we used the poorly immunogenic and fully autologous B16 tumor model and compared i.v. and s.c. vaccination with DCs loaded with the recently identified mouse melanocyte differentiation antigen TRP-2-derived T-cell epitope VYDFFVWL, which is endogenously presented by the B16 tumor cells (11). As shown in Fig. 4, A and C, after a lethal B16 tumor challenge, no difference was observed in tumor growth (Fig. 4A) and survival (Fig. 4C) between mice vaccinated via the i.v. route with DCs loaded with either the mTRP-2 peptide or an irrelevant peptide. However, after s.c. DC administration, a minor but reproducible delay in tumor outgrowth
(Fig. 4B) was observed. In the same experiment, one of four mice was protected against the B16 tumor challenge (Fig. 4D). In a total of three independent experiments, 5 of 15 mice remained tumor-free after s.c. DC vaccination for more than 50 days, whereas 0 of 10 mice survived after i.v. vaccination. Subsequent CTL analysis of i.v.-vaccinated tumor-bearing mice (Fig. 4E) versus s.c.-vaccinated tumor-free mice (Fig. 4F) further confirmed the presence of strong anti-TRP-2 CTL reactivity in s.c.-, but not i.v.-, vaccinated mice. Collectively, these data implicate that in the fully autologous B16/mTRP2 DC vaccination model, delivery of DCs via the s.c. route is preferred over i.v. administration and that the observed difference correlates with more efficient accumulation of DCs in lymph nodes as well as increased CTL reactivity in vitro after s.c. vaccination.

Discussion

DCs are professional APCs that are capable of initiating an immune response. The identification of tumor-derived antigens recognized by tumor-reactive CTLs (20, 21) has opened the possibility to specifically target the immune system toward the tumor by vaccination approaches. One approach, which is currently applied in mouse model systems (22, 18, 19) as well as in man (23, 24, 9), is vaccination with antigen-pulsed DCs. A number of murine tumor vaccination studies have shown that peptide-pulsed DCs induce tumor protection and, in some cases, even cure of established tumors (22). However, crucial questions concerning the optimal dose, frequency, and route of administration of a DC vaccine are still unanswered. In this study, we have used 111In-labeled DCs to investigate the effect of the route of DC administration on DC biodistribution and their capacity to induce an antitumor immune response. After s.c. administration and, to a lesser extent, after i.p. injection, DCs preferentially accumulate in the T-cell areas of the draining lymph nodes. The finding that fixed DCs do not enter the T-cell areas indicates that the observed homing is specific. Moreover, a comparison between injection of 111In-labeled living and freeze/thawed DCs demonstrated that the radioactivity is cleared from the injection side twice as fast, but the amount present in the draining lymph node is <10% of that found with living cells. In contrast, i.v. injection of DCs merely leads to...
accumulation in the spleen, and only minor amounts of DCs can be
detected in lymph nodes. Interestingly, we noted that after s.c. and
i.p. application, a relatively large amount of DCs (55–35%) re-
mained at the injection site. Microscopic analysis of the s.c.
vaccination site indicated that the remaining DCs were viable.
Similar observations have been reported recently in men using
monocyte-derived In-labeled DCs (25). Interestingly, in the
latter report, most efficient loading of the draining lymph node was
observed after intradermal injection, but also in this case 65–70%
remained at the injection site. These findings are in contrast to the
recent data obtained using a chimpanzee model in which GM-CSF/
IL-4 generated monocyte-derived DCs labeled with a fluorescent
dye rapidly leave the s.c. injection site and are virtually absent at
48 h after injection (26). Possibly, this discrepancy could be
explained by the origin of the DCs used (mouse bone marrow-
derived versus chimpanzee monocyte-derived DCs), the difference
in DC maturation stage, or the differences in the sensitivity of the
detection methods used.

Because lymph nodes are believed to be the location where DCs
encounter naive T cells to efficiently induce an immune response, we
investigated whether the observed homing patterns do influence the
outcome of vaccinations in the poorly immunogenic B16 mouse
melanoma tumor model. Whereas all i.v.-vaccinated mice developed
tumors at the same rate as control mice, a delay in tumor growth and
an enhanced survival were observed in s.c.-vaccinated mice. Analysis
of the CTL response in tumor-free, s.c.-vaccinated mice
versus
i.v.-
vaccinated mice demonstrated a highly increased reactivity of anti-
TRP-2 CTLs in s.c.-vaccinated mice. Therefore, these data suggest
that the s.c. route of DC vaccination results in the induction of a more
efficient antitumor immune response in the fully autologous B16
melanoma model.

We would like to note, however, that in more immunogenic
tumor models, the i.v. route of DC administration can also result in
tumor protection (19). Similarly, we did not observe differences
between s.c. and i.v. injection in the B16-OVA-model (data not
shown). One major difference between the aforementioned model
is usage of a highly immunogenic foreign antigen that is artificially expressed in the tumor cells versus the differentiation antigen TRP-2, which is endogenously expressed by melanocytes and the B16 tumor cells. In addition, the T-cell epitope derived from foreign antigens like OVA bind with high affinity to the presenting MHC class I molecule, whereas the self-mouse TRP-2-derived epitope has an intermediate to low affinity as has been observed previously for the peptide epitopes identified in human melanocyte differentiation antigens (11). The difference between the models might be explained by the stability of the MHC-peptide complex in combination with the time required for the DCs to migrate to the lymph nodes. As s.c. vaccination leads to a rapid accumulation of DCs in the lymph nodes, the magnitude of the immune response induced by DCs carrying low-affinity binding peptides may be higher after s.c. vaccination, relative to i.v. vaccination. As peptide-pulsed DCs are currently used in clinical trials, it may be considered, especially in vaccination studies in which peptides with moderately MHC binding are used, to administer DC s.c. Alternatively, peptide-loaded DCs may be injected directly into the lymph node, which has been demonstrated to initiate a potent antimelanoma immune response (9). Whether optimal vaccination with DCs expressing a tumor antigen for a longer period (e.g., after protein loading or transfection) is also critically dependent on the route of administration remains to be established.

In summary, our findings show that the distribution of DCs to lymphoid tissues is dependent on the route of vaccination. The efficiency of DCs administered s.c. to induce an immune response is higher when compared with i.v.-injected DCs in the autologous B16 model and correlates with the preferential accumulation of DCs in lymph nodes after s.c. injection. This tumor model, in combination with 111In-labeled DCs, will provide a valuable tool to further investigate the in vivo behavior of DCs in vaccination systems. It allows semiquantitative analysis of the amount of labeled cells that accumulate in tissues and to find ways to efficiently target DCs into secondary lymphoid tissues.

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References


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