The role of molecular imaging in the development of dendritic cell-based cancer vaccines

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Published online: 2 July 2005

Eur J Nucl Med Mol Imaging (2005) 32:725–730
DOI 10.1007/s00259-005-1858-0

Introduction

This editorial aims to highlight some of the general aspects underlying the development and use of dendritic cell (DC) vaccines against cancer and to review the main work that has been carried out in this field using molecular imaging techniques. It stems from the paper by Quillien et al. [1], published in this issue of *Eur J Nucl Med Mol Imaging*, describing a study in which, in the context of a vaccine therapy protocol for melanoma, mature DCs were labelled with ^{111}In-oxine and administered to patients via three different routes: lymphatic vessel, lymph node and intradermally. The authors showed that intralymphatic vessel injection can convey a large and precise quantity of DCs in a reproducible way to around ten nodes. In some cases, injection by the intranodal route gives a similar result, but this technique is not reproducible. Finally, they showed, for the first time in humans, that DCs with TH1 cell polarisation capacities can migrate to lymph nodes after intradermal injection.

The paper by Quillien et al. [1] is a methodologically complex study, yet straightforward and conceptually simple, which underscores three facts: first, immunology holds great potential in cancer treatment, as immunotherapy is one of the new frontiers in cancer treatment, along with surgery, chemotherapy and precision radiation therapy; second, molecular imaging techniques can make very effective and unique contributions in addressing issues related to the development of cancer treatment; third, curiosity-driven researchers in immunology and molecular imaging are working closely together to devise research strategies for the development and effective use of image-supported immunotherapy. There is indeed already a long tradition of collaboration between researchers in the fields of molecular imaging and immunology, as demonstrated by the development and use of radiolabelled monoclonal antibodies and by other studies that have assessed the immune system components and functions in vivo by means of radionuclide imaging, including previous studies with radiolabelled DCs. Thus, the paper of Quillien et al. [1] is just a further example of a study that will stimulate the interest of researchers to examine unsolved issues in the area of in vivo radio-immunology with DCs. It is worth noting that the use of DCs for cancer vaccination is only one of the numerous tracks being followed by researchers in the development of anti-cancer vaccines. Many other routes for vaccine development are being pursued by the use of cells, peptides, proteins, DNA and recombinant viral vectors. A full appreciation of the advances in the field of immunology and in particular in the field of vaccine development can be found elsewhere [2–5]; thus only a summary of the known role of DCs and their use in cancer vaccination will be provided.

Immunotherapy of cancer

Immunotherapy can be based on two main strategies: active and passive immunotherapy. Active immunotherapy relies on the activation of the patient's immune system, as in the case of vaccine development [4–6], whereas passive immunotherapy uses in vitro-stimulated immune cells or immune components. Passive immunotherapy comprises the use of anti-tumour antibodies produced outside the patient, which are administered to directly eradicate tumours by the same effector mechanisms as are used to eliminate micro-organisms [7], and immune effector adoptive therapy based on the ex vivo expansion of antigen-specific lymphocytes that are then infused back into the patient [8]. Although vaccines are currently used for prevention, be-

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fore an infectious disease is contracted, most cancer vaccines are designed and specifically developed to cause the immune system to attack existing tumours.

Certain tumours may activate the immune system and allow presentation of tumour antigens by DCs, thus determining an immune response against the tumour (Fig. 1). In particular, there is evidence that (a) chemically induced murine tumours can be rejected by an immune response, (b) immunodeficient mice and human subjects have an increased incidence of cancer, (c) the decreased function of the immune system occurring with age increases the incidence of cancer and (d) paraneoplastic syndromes (mediated by onconeuronal antibodies and CD8+ cells) are associated with tumour regression [9].

However, tumours can suppress or evade the immune response by producing potent immunosuppressive factors capable of inhibiting the function of the CD4+ and CD8+ T lymphocytes. Cytokines such as VEGF, TGF-β and IL-10, within the tumour, hamper DC maturation, CD4+ and CD8+ T lymphocyte generation and effector function. Moreover, only when the antigen presentation occurs in the presence of co-stimulatory signals are the CD4+ and CD8+ lymphocytes fully activated (Fig. 2). Because most tumour cells do not express co-stimulatory molecules, presentation of tumour antigens in the absence of stimulatory signals results in CD4+ and CD8+ lymphocyte anergy [10].

DC infiltration of tumours may correlate with survival and a reduced incidence of metastases, whereas production by the tumour of immunosuppressive factors is likely to favour cancer growth and spread. T cells infiltrating tumour deposits are commonly anergic or poorly responsive to antigenic stimulation, possibly due to an unfavourable mi-

Human DC subtypes: surface markers

Blood DC

Myeloid DCs
BDCA-1$^+$CD11c$^+$ HLA-DR$^+$
BDCA-3$^+$CD32$^-$ CD64$^-$ HLA-DR$^+$

Plasmacytoid DCs
BDCA-2/4$^+$CD123$^+$ HLA-DR$^+$

Blood precursors:
CD14$^+$ monocytes
CD34$^+$ hematopoietic stem cells

Tissue DC

Langerhans cells
Langerin$^+$ CD1a$^+$ HLA-DR$^+$

Interstitial DCs
DC-Sign $^+$ HLA-DR$^+$

Plasmacytoid DCs
BDCA-2/4$^+$CD123$^+$ HLA-DR$^+$

Fig. 1. DC subtypes. DCs were first described nearly 30 years ago. Their name derives from the presence of processes on the cell body that recall the aspect of neuronal dendrites. DCs are the most efficient antigen-presenting cells of the immune system and can be found in three main stages: precursor, immature and mature. Two major classes of DCs have been described, myeloid and plasmacytoid; these can be distinguished on the basis of their localisation and cell surface marker expression. DC progenitors in the bone marrow give rise to precursor DCs that circulate in the blood. Following such migration, DCs are found almost everywhere in the body. These precursor DCs develop into immature DCs. The various populations of immature DCs have similar morphology but diverse locations and functions; myeloid DCs can be subdivided into Langerhans cells that are located particularly in the epidermal layer of the skin and in the gut and interstitial DCs that are located in almost every other organ. Both types provide the first defence against foreign agents. Immature DCs remain as such, waiting to interact with exogenous entities entering the body. Myeloid and plasmacytoid DCs as well as their precursors are also found in the bloodstream, thymus and lymph node follicles. DCs found in the bloodstream account for less than 0.1% of circulating leucocytes. DCs in the thymus present self-antigens to developing T cells to induce self-tolerance. DCs in the lymph nodes comprise mature cells that have migrated from the periphery following an activation stimulus. These cells are very good at antigen presentation and, according to their phenotype, can perform different functions (e.g. induction of immunity or tolerance, T cell activation, induction of humoral immunity)

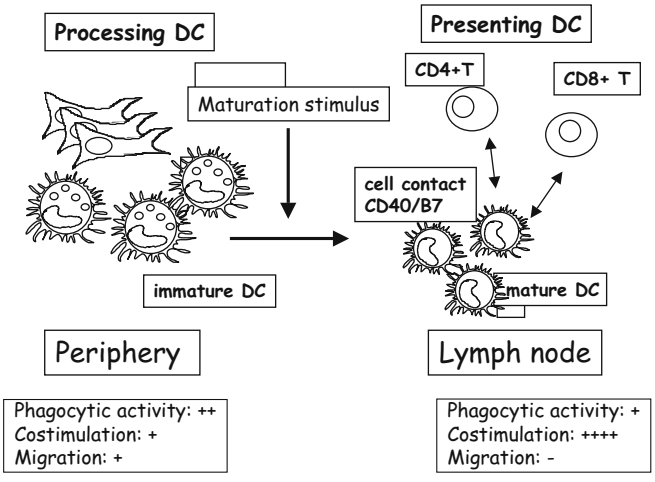

Fig. 2. Steps in DC maturation. Immature DCs capture and process antigens. Various stimuli, such as tissue damage, inflammation, viruses and bacteria, as well as cytokines, cause activation of the immature DCs, which acquire migratory properties. Immature DCs possess typical functional characteristics, including phagocytosis, macropinocytosis and endocytosis. Upon maturation, DCs express high levels of major histocompatibility complex (MHC) and co-stimulatory molecules and also migrate into the lymph nodes to interact with naive T cells. Because of these properties, the use of mature DCs is being considered for generation of vaccines against cancer. After endocytic uptake of antigens, DCs degrade the entrapped antigens and, according to the nature of the antigen, can process and channel peptides into the MHC molecules (class I or II, or both) for presentation on the cell surface as peptide–MHC complexes. Following an activation stimulus, this event is accompanied by the upregulation of co-stimulatory molecules (CD80, CD86), as well as by modifications in adhesion receptor and chemokine receptor expression. This can be regarded as a two-step sequence that is initiated by a processing stage and completed by a switch to the presenting stage of the DCs. Following activation, DCs initiate their mobilisation from the non-lymphoid tissues, where they have been activated, through the blood and lymph system towards the secondary lymphatic tissue. Whereas immature DCs are strongly adherent, as attested by the presence of philopodia, mature DCs acquire highly motility, which in turn correlates with their ability to migrate, as indicated by the presence of ruffles. When the DCs reach the spleen, lymph nodes or tonsils, they are mature DCs, no longer able to capture antigens but with increased antigen presentation capability. This increased antigen presentation capability results in a potent stimulation of T cell immunity. Under the action of adhesion molecules, DCs establish contact with T cells, while the presence of co-stimulator molecules provides the second signal for activation of antigen-stimulated CD4+ and CD8+ T lymphocytes

croenvironment [11]. Thus effective immunotherapy needs the full activity of the immune system components. It has therefore been postulated that DCs might be exploited as the basis for a form of immunotherapy, i.e. as natural adjuvants for vaccines or as a direct form of therapy to induce immunity against cancer [12, 13].

DC-based vaccines represent an attempt to initiate an individual subjective immune response to tumour antigens, with the goal of limiting and eliminating the tumour itself by a cascade of events [14]. Numerous studies have been aimed at improving the procedures for isolation, propagation, activation and antigen loading of DCs. The evidence that DCs can mediate tumour rejection in mice, along with the possibility of generating large numbers of DCs in the laboratory, has made it possible to advance the development of human vaccine trials. However, many issues are still under debate, and an optimal method of vaccination has not yet been developed. Among the most important unsolved issues are the form of the antigenic material, the administration route, the maintenance of the migratory properties of DCs and their efficacy following their manipulation outside the body [15].

The use of DCs for cancer immunotherapy was first evaluated by studies in animal models that aimed to demonstrate the possibility of using DCs as antigen-presenting cells for tumour-derived antigens. These studies showed that: (a) ex vivo-generated, antigen-loaded DCs induce antigen-specific T cell immunity, (b) ex vivo gene-loaded DCs can induce humoral immunity, (c) ex vivo-generated, antigen-loaded DCs induce tumour-specific immunity, (d) ex vivo-generated DCs are superior to other types of vaccine, (e) ex vivo-generated immature DCs induce tolerance and (f) combination therapy with ex vivo-generated DCs improves vaccine efficacy [9].

Potentially immunogenic antigens for DC-based immunotherapy include several types of antigen derived from expression of oncogenes, overexpressed genes, embryonic genes, normal differentiation genes, viral genes, tumour-suppressor genes, B cell idiotypes and other tumour-associated proteins. Furthermore, other strategies that do not require the identification of the tumour antigens for immunisation can be based on the use of multiple autologous whole cell-derived antigens, including tumour-derived RNA and apoptotic bodies, and tumour lysates [6]. Although experimentally more practical, the latter strategies have the disadvantage of potentially generating autoimmune reactions to self-antigens. The form of tumour antigens is also important to allow the presentation of tumour-derived antigenic material on both MHC class I and II presentations for the activation of cytotoxic and helper T cells, respectively.

Based on the hypothesis that tumour antigen-bearing DCs could be used as a vaccine against cancer in human subjects, clinical trials are ongoing. These trials are designed to test the possibility of using DCs as a form of vaccination to induce immunity to antigens associated with various tumours, including breast, lung, prostate and renal cell cancers and melanoma. DC vaccination has been applied in very limited series of patients, including between 4 and 50 patients, in various tumours. In these studies, the effectiveness of treatment has been monitored by assessing the anti-tumour cellular and humoral responses and the clinical outcome. Results have shown variable effectiveness of the DC vaccination approach: cellular and humoral response and reduction in tumour-associated blood markers have been observed in most cases, while to a lesser extent there has been clinical improvement and regression of metastases (the percentage of cases so affected has ranged from 10% to almost 100%) [6].

All studies with DC vaccination have shown lack of toxicity due to immunisation. This finding suggests that DC vaccines are safe and cause little or no toxicity.

Methodological issues in the development of cancer vaccines

The pursuit of effective anti-cancer vaccines by the use of DCs entails several sequential steps [9]. Technical steps include the isolation of bone marrow-derived CD34+ stem cells, the expansion of DCs from stem cells in vitro, their loading with antigens (cancer proteins) and their infusion into patients. Following these steps, it is expected that they will travel to the lymph nodes to activate and instruct the lymphocytes. Variants are possible, including the use of CD14+ peripheral blood monocytes instead of stem cells and of mature instead of immature DCs. Generation of DCs for vaccines can be pursued by different procedures. It must be remarked that these procedural differences have important effects on the efficacy of vaccination; this may in part explain the variable outcome of the vaccine trials, as they differ greatly from each other in this respect. Different antigens can be loaded onto DCs with many methods depending on the antigen, at different maturation stages of the DCs. Other methodological issues need to be addressed, including, for each cancer, the selection of the most immunogenic and appropriate set of antigens and their optimal combination. Furthermore, it is necessary to develop a series of strategies to support the activation, homing and in vivo growth of DCs. Finally, the optimal schedule, route and method of administration must be defined. DCs have been injected subcutaneously, intradermally, directly into the tumour or lymphatic system and intravenously. Several groups have combined intravenous and intradermal routes of DC administration because this might be more efficient at inducing a systemic response. Indeed, it has been shown that activation of an immune response by DCs in the lymphatic tissue is dramatically affected by the route of administration. However, one route of administration that might be adequate for one type of tumour might be inadequate for another, and the optimal route of administration of DCs may depend on the nature and route of spread of the tumour. It is conceivable that the intradermal route will be preferable in melanoma, while the intravenous route may be more adequate in other types of cancer. An attractive alternative approach, which has so far not been applied in patients, involves in situ targeting of DCs by DC-specific antibodies carrying

tumour antigens, DC-retargeted viruses or naked DNA encoding tumour antigens under the control of DC-specific promoters. As a vaccine approach, in situ targeting of DCs is the ultimate goal, and this would exploit the inherent migratory capacity of DCs in vivo [16, 17].

Is there a role for molecular imaging in cancer vaccine development?

Active research is needed to identify the most effective form of administration of DCs for each type of tumour, i.e. whether they should be administered intravenously, subcutaneously or directly into the lymphatic system, whether administration should be at one or multiple sites, whether it should be performed in a single session or over repeated sessions (weekly, at 2-week intervals or monthly), and whether constant or escalating doses are required for maximum stimulation of the immune system. In addition, it would be very useful to know the distribution pattern and viability of DCs used for therapy. An approach based on their labelling would allow the monitoring of co-injected radiolabelled and non-radiolabelled DCs and permit visualisation of their fate in vivo, in individual patients. The ability of DCs to migrate and redistribute into peripheral lymphoid organs could then be correlated with the efficiency of immune activation and clinical outcome.

Since maintenance of motility is key to the action of DCs, studies using radiolabelled DCs must be performed under conditions that do not alter the DCs' own motility. The influence of ^{111}In-oxine and ^{99m}Tc-hexamethylpropylene amine oxime (^{99m}Tc-HMPAO) labelling on the motility of antigen-loaded DCs and in vivo migration has been studied in human subjects. While no damage should result from radiolabelling of DCs in terms of maturation and motility, the results of the different studies appear very heterogeneous, and even within the same studies results are not consistent, since in some cases the motility of cells is maintained whereas in others it is lost. Such inconsistency may be due either to occasional technical problems related to the handling of cells and the labelling procedure or to damage inherent in the introduction of a radiolabelled molecule into a very delicate system; it may indicate that the practice of immunotherapy with DC vaccines is currently far from being optimised as only a few labelled cells appear to move from the site of administration to the draining lymph nodes.

The distribution of DCs in human subjects following their labelling with ^{111}In tracers depends on the route of administration. DCs injected intravenously accumulate in the spleen and liver, while DCs injected subcutaneously or intradermally migrate to the draining lymph nodes. Other studies have shown that when DCs are directly injected into the lymphatic circulation, they reach the draining lymph node. These are indeed the conclusions of Quillien et al., who, as already mentioned, found that injection of DCs into the lymphatic circulation can convey a large and precise quantity of DCs in a reproducible way to around ten nodes, that in some cases injection by the intranodal route gives a similar result and that DCs with TH1 cell polarisation capacities can migrate to lymph nodes after intradermal injection.

^{111}In is frequently chosen as the isotope for labelling of intravenously administered DCs because it is incorporated efficiently by the cells and binds tightly to cytoplasmatic proteins. In fact, the release of ^{111}In from labelled DCs over a period of 24 h has been shown to be low, indicating a good labelling stability. The efficiency of labelling has been shown to be comparable to the results reported for lymphocytes, granulocytes and macrophages. [18–22].

Mackensen et al. have shown that intravenously infused DCs undergo transient lung uptake followed by localisation in the spleen and liver for at least 7 days. DCs injected into a lymphatic vessel are rapidly detected in the draining lymph nodes, where they remain for more than 24 h [23].

Eggert et al. also investigated whether the route of administration affects the biodistribution of DCs in lymphoid organs and demonstrated that intravenously injected DCs mainly accumulate in the spleen, whereas subcutaneously injected DCs preferentially home to the T cell areas of the draining lymph nodes [24].

In a group of patients enrolled in a phase I/II vaccination trial, Blocket et al. injected antigen-loaded DCs labelled with either ^{111}In or ^{99m}Tc-HMPAO in the proximal part of the legs, one intradermally on one side, one subcutaneously on the opposite side. They did not demonstrate migration of loaded labelled DCs from intradermal or subcutaneous sites of injection to regional lymph nodes. The authors concluded that a large proportion of antigen-loaded DCs, as used in current human trials for cancer therapy, may not reach regional lymph nodes [25]. They also concluded that the outcome achieved with DCs, generated by different methods and administered by different routes, including intranodal or intratumoural injection, deserves further investigation by in vivo imaging. This indeed appears a negative but important study that highlights the need for standardised and reproducible methods if we are to succeed in achieving the goal of imaging DC mobility in vivo.

Melanoma patients participating in a protocol aimed at assessing the in vivo immune responses of a DC vaccine were studied by de Vries et al. [26]. Twenty-four to 48 h before surgery, patients received a single injection of ^{111}In-labelled DCs, either intradermally in the proximity of a lymph node or directly into a lymph node of the lymph node region to be resected. ^{111}In labelling did not affect surface receptor expression by DCs. To assess the capacity of DCs to migrate to lymph nodes, sections derived from resected lymph nodes distant from the node of injection were analysed by microautoradiography. Explicit spots of radioactivity in the lymph node confirmed the migration of injected ^{111}In-labelled DCs to these nodes. Following intradermal injection, a significant percentage of both immature DCs and mature DCs remained at the site of injection. Nevertheless, a distinct amount of radioactivity was observed in the draining lymph nodes after injection of the DCs. A significantly higher percentage of mature DCs migrated to the lymph nodes and distributed

over more lymph nodes as compared with immature DCs, which never migrated to more than one lymph node. In addition, the migration of mature DCs, but not of immature DCs, was somewhat enhanced after 48 h relative to 24 h. Ridolfi et al. [27] showed that, following DC labelling with ^{99m}Tc-HMPAO or ^{111}In-oxine, intradermal administration of DCs resulted in about a threefold higher migration to lymph nodes than was observed with subcutaneous administration, while mature DCs showed, on average, a six- to eightfold higher migration than immature DCs. The first DCs were detected in lymph nodes 20–60 min after inoculation, and the maximum concentration was reached after 48–72 h. They also showed that after 24 h, a 75% and 50% loss of activity was observed for ^{99m}Tc-HMPAO- and ^{111}In-oxine-labelled DCs, respectively.

Future outlook and conclusions

Recently, Eggert et al. [28] have used enhanced green fluorescent protein (EGFP)-transgenic mice to investigate DC trafficking. They compared the in vivo migration of EGFP-DC and ^{111}In-labelled DC after subcutaneous and intradermal administration. The fact that the EGFP protein is efficiently degraded ensures that only living EGFP-positive cells are detected after in vivo administration; in contrast, following the administration of ^{111}In-labelled DCs, radioactivity can still be detected after the cells have died or been taken up by other cells. This could explain why inconsistent results are obtained when using ^{111}In-labelled DCs without checking their viability at the target organ site after their migration. The authors conclude that the application of EGFP-DC promises to be an ideal tool for studies aimed at improving the efficiency of DC-based vaccines.

There have also been some recent attempts to label DCs with ^{18}F and to assess their distribution by planar positron imaging [29]. Labelling with ^{18}F had no significant effect on the viability or the phenotype of bone marrow-derived DCs compared with sham-treated cells, suggesting that these cells are fairly resistant to gamma and positron radiation. Moreover, the authors highlight the fact that positron emission tomography offers the possibility of studying cell migration induced by chemokines or inflammatory agents with a very high spatial resolution (2 mm) and by 3D imaging, in order to extrapolate the number of cells by measuring the exact amount of radioactivity in tissue.

Finally, the feasibility of labelling DCs with magnetic resonance imaging contrast agents and monitoring them in vivo has been explored by Ahrens et al. [30]. The results of the studies demonstrated that receptor-mediated endocytosis of magnetic nanoparticles effectively labels DCs in vitro. Furthermore, the authors showed that the labelling procedure does not harm the cell's immunological function, and that the labelled cells can be visualised in vivo in a longitudinal fashion. The authors concluded that targeting cell surface accessory molecules to stimulate endocytosis can be generalised to a wide variety of immune cell types.

In conclusion, we are witnessing the beginning of an era in which the combination of molecular immunology and molecular imaging with dedicated instruments and tracers will permit the opening of a new front to fight cancer in a targeted manner. This will be possible as long as nuclear medicine specialists appreciate the potential of molecular imaging and the value of the tools being developed and deployed by our immunologist colleagues in the pursuit of therapies that will spare normal tissues while being very effective in cancer treatment.

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