Dendritic cell vaccination and immune monitoring

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Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system. Following infection or inflammation, they undergo a complex process of maturation and migrate to lymph nodes where they present antigens to T cells. Their decisive role in inducing immunity formed the rationale for DC immunotherapy: DCs loaded with tumour antigens are injected into cancer patients to stimulate T cells to eradicate tumours. Effective immune responses and favourable clinical outcomes have indeed been observed, but only in a minority of patients. Therefore, it is obvious that current DC-based protocols need to be improved. For this reason, we study in small proof-of-principle trials the fate, interactions and effectiveness of the injected DC. The success of DC-based immunotherapy to induce cellular immunity against tumours is highly dependent on accurate delivery and trafficking of the DC to T cell-rich areas of secondary lymphoid tissues.

Key words: Delayed type hypersensitivity reaction, dendritic cells, immune monitoring, immunotherapy, melanoma.

For effective immunotherapy dendritic cells (DCs) need to traffic throughout the vascular and lymphatic system to reach the T cells located within lymph nodes. Although most immunotherapeutic agents are administered intravenously, DCs are predominantly administered intradermally. We observed that < 5% of intradermally administered mature DCs reach the draining lymph nodes, amounting to inefficient homing. Despite this low number, we could measure effective immune responses in some patients, but generally this may be too low.

We demonstrated that DC maturation is a prerequisite to exert their immunostimulatory capacity in vivo. Immunomonitoring revealed a remarkable difference in immune responses. In patients vaccinated with immature DC, the keyhole limpet haemocyanin (KLH) responses as well as delayed type hypersensitivity (DTH) reactivity against KLH and tumour peptides were weak and absent, respectively. In contrast, in patients vaccinated with mature DC, a pronounced T cell, as well as a B cell response (immunoglobulin) against KLH were observed. Analysis of the response against the tumour peptides demonstrated little or no reactivity in blood. However, following intradermal administration of a DTH challenge with gp100- and tyrosinase-peptide loaded DC essentially, all patients vaccinated with mature DC showed a positive induration. Moreover, we showed the predictive value of the presence or absence of antigen-specific T cells in biopsies from DTH sites. In clinically responding patients, T cells specific for the antigen preferentially accumulated in the DTH site in accordance with the applied antigen in the DTH challenge. We will provide an overview on DC immunotherapy in cancer patients and how this therapy might be improved.

Antigen-presenting dendritic cells

Dendritic cells are the professional antigen-presenting cells of the immune system that instruct and control the activation of B and T lymphocytes, the mediators of specific immunity [1]. DCs are highly mobile cells and by their sequential migration from peripheral tissues to lymphoid organs, they serve as sentinels of the immune system. Immature DCs are very efficient in antigen uptake, mediated by high endocytotic activity and expression of an array of cell surface receptors capable of capturing antigens. Inflammatory mediators and ‘danger signals’ promote maturation and re-routing of DC to the secondary lymphoid organs [1]. In the secondary
lymphoid tissues, DCs are mature and well-equipped to attract, interact and activate naive T cells to initiate a primary immune response [1,2]. DCs are also able to directly activate natural killer (NK) cells [3] and can produce large amounts of interferon upon encounter with viral pathogens, thus providing a link between the adaptive and innate immune system. In murine tumour models protective immunity as well as regression of established tumours have been observed after vaccination with DCs loaded with tumour antigens [4]. Their unique capacity to initiate and modulate immune responses is currently exploited by many groups, including ours, to fight infectious diseases and cancer.

One aspect of DC biology that is rapidly evolving is the apparent diversity of DC subsets. At least two distinct ontogenetic pathways for DC development have been reported, the myeloid progenitor- and the lymphoid progenitor-derived DC. Part of these different DC subsets may also be explained by differences in the maturation stage of DC and the local cytokine environment. The geographical localization of the DC subsets in secondary lymphoid tissues is distinct, myeloid-derived DCs mainly migrate to or reside in the marginal zone (a primary entry point for blood-born antigens), whereas the lymphoid DCs mainly reside in the T cell areas. This supports distinct functions for the DC subsets, as been shown in murine studies [1]. It is now well-appreciated that the DC subset, its maturation state and the microenvironment or type of pathogen a DC encounters in the periphery determine the type of immune response that is induced, ranging from a TH1 or TH2 response to immune tolerance.

Data are now accumulating that immature DC can induce tolerance and are able to induce regulatory T cells in vitro and in vivo [5]. Regulatory T cells are involved in the control of peripheral tolerance and the prevention of vigorous inflammatory reactions. These regulatory T cells affect immune responses at the level of antigen presentation and during the effector phase of T cells at the site of the tumour. Although the exact mechanisms by which regulatory T cells exert their suppressive functions are not yet elucidated, direct cell–cell contact and cytokines like interleukin 10 (IL-10) and transforming growth factor β (TGF-β) have demonstrated to play a role. Our data on vaccination of melanoma patients also demonstrate that mature DCs, but not immature DC, induce strong immune responses in vivo [6].

Another aspect in the evolving field of immunotherapy is the reacknowledgement of the role of the innate immune system. The eradication of a malignancy is the result of a concerted action of adaptive and innate immunity, in which NK cells and natural killer T cells are important effector cells. Next to the direct cytotoxic effect on tumour cells, NK cells produce type I interferons that contribute to a great extent to a proinflammatory microenvironment. Clinical studies on adoptive NK cell immunotherapy have shown that NK cells can target human tumours.

Dendritic cell-based cancer vaccines: current status

Over 60 different clinical studies have been carried out since 1996, applying tumour antigen-loaded DC-based vaccines. The vast majority of these studies have been performed in melanoma patients [7]. In our studies, as well as in other groups, immunological and, notably long-lasting, clinical responses have consistently been observed following cellular therapy [7,8]. In several patients, these clinical responses coincide with the induction of specific cytotoxic T cell responses.

We have explored vaccination of cancer patients with monocyte-derived DCs loaded with peptides derived from tumour-associated antigens. In our current culture protocol [9], we routinely generate large amounts of clinical grade mature and immature DCs. DC maturation is induced using monocyte conditioned medium (MCM), tumour necrosis factor α and prostaglandin E2. The first DC trial at our institution has been initiated in 1998. Herein, the safety of DC-based vaccines and the efficacy of immature DC vs. mature DC was studied. HLA-A2-1+, gp100+, tyrosinase+, metastatic melanoma patients were treated with peptide-pulsed immature or mature DCs. As peptides, we used two HLA-A2-1-restricted gp100 peptides (either native or modified peptides to improve their HLA-A2-1 binding affinity) [10,11] and a tyrosinase peptide [12]. All DC vaccines were co-loaded with the foreign protein KLH that serves as a control for immune competence and stimulation of a T-helper response. Vaccinations were given three times with 2-week intervals, followed by injections with the peptides alone. Immune monitoring consisted of DTH responses with pulsed and unpulsed DC, ELISpot-assays and tetramer assays [13,14]. In addition to peripheral blood, immune monitoring was also performed using biopsies taken from DTH sites (see below).

The results of the first clinical trial have been published [6], and unequivocally demonstrated that mature but not immature DCs are capable of inducing potent anti-KLH specific T cell and B cell responses [6]. Clinical results demonstrated that three out of 20 objective remissions were observed in stage IV melanoma patients vaccinated with mature DCs. In this group, 10 patients were vaccinated with mature DC, of these patients one was not evaluable because of deteriorating condition, and of the remaining nine patients, three showed stable disease > 4 months (4-5, 7-5 and 22, respectively), one patient showed a mixed response and one patient achieved a partial response with complete remission after surgical intervention, that now lasts > 7 years. All patients in this study were melanoma patients with metastatic disease (M1c, one patient M1a) in World Health Organization performance status 0.

Moreover, clinical results correlated with the presence of vaccine-induced immune responses against the tumour-peptides (see below) [15]. To date, > 200 melanoma patients
have been vaccinated in our ongoing DC trials, we observed no clinical benefit from vaccinations in patients with high tumour load (as judged by the clinician), elevated serum lactate dehydrogenase (LDH) brain metastases or rapid progressive disease. Therefore, we excluded these patients, with a life expectancy less than 3 months, in ongoing studies. While on the down side, the vaccine is not yet very effective, with an objective clinical response rate (i.e. > 1 year stable disease or better, stage IV melanoma patients) of approximately 10–15%, the positive message is that we clearly find T cell-mediated immunological responses: 60% in patients with regional lymph node metastasis and 30% in patients with metastatic disease. Patients exhibiting these responses show a significantly improved progression free survival.

Nevertheless, a number of variables need to be evaluated and controlled to further improve clinical outcome in cellular therapies, among these are generation of DCS, use of different DC subsets, route of administration, optimal activation stimuli for DC [16], antigen-loading of DCs, selection of tumour-derived antigens and so on [7]. These variables are in ongoing debate, but one can conclude that the full potential of DC-based cellular therapy has not yet fully been exploited. However, the current consensus is to continue cellular therapy in well-designed small trials that meet a standardized list of quality criteria. This consensus list should at least describe quality-control criteria for ex vivo generated DC, patient characteristics, trial design including the different variables that are investigated, and tests for clinical and immunological responses [7].

Significant progress in cellular therapy against cancer, including DC vaccination, is only to be expected by careful immune monitoring studies in order to obtain detailed insight of the underlying (patho-) physiological processes that determine the success or failure of treatment. Different compartments and modalities are considered to monitor induced immune responses, for example, accuracy of delivery, immune responses in peripheral blood, tumour and DTH test biopsies, and clinical evaluation. Recently, it was shown that the modality of vaccination with a tumour-specific antigen influences the differentiation pathway of the anti-vaccine CD8 T cells, which may have an effect on their capacity to trigger a tumour rejection response [17]. Palucka et al. observed that patients with a high baseline level of melanoma antigen-specific immunity more often show an immune response to the vaccine. Furthermore, they show that patients who survived longer are those who showed immune response against two melanoma antigens presented on the DC vaccine [18]. Although sometimes correlations between tumour regression and T cell responses are observed [18], the immunological studies performed so far are too diverse in their set-up to pool them in a meta-analysis [19]. However, some lessons can be drawn from these studies. For example, an intact and proper functioning immune system seems to have a higher potential to react on immune therapy. From our immune monitoring data, mentioned below, we might not only conclude that the presence of tetramer-specific T cells is correlated with an improved progression free survival. Another conclusion should be that in end-stage melanoma patients, these tetramer-specific T cells are less frequently induced (eight out of 26 patients) than in melanoma patients with regional lymph node metastasis (24 out of 31 patients).

Secondly, upon induction of tumour antigen-specific T cells, the next hurdle to take is the local immune suppressive environment created by the tumour. In end-stage melanoma patients, the misbalance is already in favour of the metastasizing tumour. In our ongoing studies, we have seen that in some patients tetramer-specific T cells are present after DC vaccination, but still experience progression. It became clear that these tetramer-specific T cells did not produce interferon-γ, nor showed cytotoxic activity upon tumour challenge. Apparently, these effector cells were not capable of breaking the local suppressive tumour environment.

We might take better advantage of the unique capacity of DCs to direct the immune response by exploiting DC-based cellular therapy earlier in the disease course. It has been demonstrated that already in sentinel nodes melanoma-specific T cells are present, together with antigen-presenting cells. In this window between primary tumour and metastasis, immunological processes can be crucial. It might be at this turning point in the development of melanoma that ex vivo generated DCs can assist the immune system.

**Dendritic cell migration in vivo**

For DCs to induce potent immune responses, their migration towards lymph nodes is essential. In mice, we have demonstrated that major differences can be found in numbers of migrating DC depending on the route of administration (subcutaneous gave the best results) and the maturation state (mature gave the best results) [20]. With respect to the latter, we were able to confirm these data in stage III melanoma patients with lymph node metastases who were scheduled for radical lymph node resection [21]. During the first vaccination, these patients received an injection of 111Indium-labelled mature or immature DCs to allow scintigraphic imaging to study in vivo migration [21]. Regardless of the route of administration (intradermal or intranodal), mature DCs were more efficient than immature DCs in reaching the draining lymph node in vivo [21].

The results described above were obtained by our developed method of radioactive labelling of DC [20, 21]. DCs have therefore been labelled with radionuclides for scintigraphic imaging of cell trafficking, which is until to date the only Food and Drug Administration-approved clinical cellular imaging modality [21]. A major drawback of scintigraphy,
however, is the lack of anatomical detail allowing only gross anatomical determination of migration between lymph nodes (LN) without the ability to assess the intranodal distribution pattern of DCs within each LN. Furthermore, due to its low spatial distribution, accurate delivery of cells that may be essential for subsequent migration into nearby lymph nodes cannot be properly evaluated using scintigraphy. In contrast, magnetic resonance (MR) imaging is well-suited to obtain three-dimensional whole body high-resolution images and is widely used in clinical practice. The currently most sensitive markers to label cells for MR detection are (ultrasmall) superparamagnetic iron oxide [(U)SPIO] particles [22]. We took advantage of the fact that DCs naturally endocytose clinically applied, Food and Drug Administration-approved SPIO labels in significant amounts, obviating these concerns. This provided us with the opportunity to label cells with high efficiency without affecting their function and use these cells in humans.

We investigated the biodistribution of these SPIO-prelabelled DCs applied as cancer vaccines in melanoma patients using MR imaging. In our DC vaccination protocols, in vitro generated DC loaded with tumour-derived antigenic peptides were administered to stage III melanoma patients as outlined in Fig. 1 [6,21]. DCs were labelled with $^{111}$In-oxine and SPIO (Endorem®, Guerbet, Aulnay-sous-bors, France) separately and co-injected in an LN in the lymph node basin to be resected. This provided the unique opportunity to not only obtain MR scans at 3 Tesla (T) before surgery, but also to generate high-resolution MR images at 7T of individual resected LNs, and to correlate the results with scintigraphy and (immuno-) histopathology. Interestingly, we found that in only approximately 50% of the cases, DCs were correctly injected into the LN, despite ultrasound guidance of the injection needle by a highly experienced radiologist. Subsequent migration could be observed only when DCs were correctly injected into the lymph node, demonstrating not only the importance of accurate delivery, but also of careful monitoring of cell tracking in cellular therapy. Inadequate delivery may be an important reason why only a limited proportion of patients respond in ongoing clinical trials using DC vaccines. We found that the accuracy of MR imaging to visualize truly DC-positive LNs was significantly better than scintigraphy. These findings illustrate the power of additional anatomical information, which can also be of value for other fields of biomedical research.

With Prussian blue staining we can visualize SPIO-labelled cells. We observed immunohistologically that the SPIO-labels DCs that do migrate enter the lymph nodes via the sinuses and reach the T cell areas where the actual DC–T cell interaction takes place. At this stage, we were able to demonstrate that intranodally injected SPIO-labelled DCs, electroporated with RNA encoding the tumour antigen gp100, express the gp100 protein. From resected lymph nodes

Fig. 1 Monitoring the accuracy of delivery of SPIO-labeled cells using magnetic resonance (MR) imaging and scintigraphy. Monocytes are obtained by cytopheresis from stage III melanoma patients (a), they are cultured and labelled with SPIO particles and $^{111}$In-Indium (b). The cells are then injected intranodally into the lymph node basin that is to be resected and their biodistribution is monitored in vivo by scintigraphy (c) and MR image (d). The lymph node basin is resected (e) and separate lymph nodes are visualized with high resolution MR image at 7 Tesla (f) and histology (g).
rosettes, containing SPIO-labelled DCs surrounded by enlarged and activated T cells, were isolated. Therefore, we disclosed the desired ‘functional unit’ within a lymph node.

Immune monitoring in tissues

Another aspect is monitoring the immune response that is thought to induce tumour regression. Immune monitoring is most straightforward after vaccination with defined antigens; however, responses have also been detected after lysate or total RNA-loaded DC vaccines. Fortunately, many novel tools are now available to detect immune responses against known and unknown tumour antigens, including major histocompatibility complex tetramers, Eli-spot- and cytokine release/catch assays [13,14]. No correlation was observed between the reactivity against KLH and the clinical outcome. We developed a novel approach to efficiently monitor DC vaccine-related T cell responses in vaccinated patients using biopsies derived from DTH sites [15]. The results of this monitoring method correlated with the clinical outcome in stage III and IV melanoma patients.

Delayed type hypersensitivity challenges consisting of peptide-loaded DC plus or minus KLH, DCs loaded with KLH, and unloaded DCs revealed that essentially all patients mounted a positive DTH response with indurations up to 33 mm. As both unloaded DCs and DCs loaded with KLH and/or peptides were positive, indurations at the DTH site were not predictive of vaccine-related T cell responses in our setting. However, as no DTH was detected after the first intradermal injection of the vaccine, the occurrence of a positive DTH reaction should be directly related to the vaccination. The reason for the DTH response to unloaded DC is not clear, but could be explained by the vast amount of chemokines produced by mature DCs [9].

Punch biopsies (6 mm) were taken from positive DTH sites and were divided in half. One part was used for histochemistry and the other part was used to isolate DTH-infiltrating leucocytes (DIL). Immune staining showed clusters of CD2+ CD3+ infiltrating cells of which 50–70% were CD4+ and 50–30% were CD8+ T cells. No clusters of infiltrating cells were observed in unchallenged control skin biopsies. DILs were generated by cutting the biopsies in pieces and culturing of the outgrowing cells for 2–3 weeks in the presence of low-dose IL-2 (100 U/ml) without re-stimulation. Interestingly, DIL specific for KLH could easily be found in biopsies from KLH-pulsed DCs, not in DIL from peptide or unpulsed DTH biopsies. Moreover, in 11 (six stage III and five stage IV) out of 22 patients tested, gp100/tyr tetramer-positive T cell populations were readily detected. In five additional patients, antigen-specific cytotoxic T cells in DIL cultures were detected after additional vaccination cycles. No tetramer-positive T cells were detected in DTH biopsies injected with unloaded DC or KLH loaded DC. Strikingly, in six of seven patients in whom no tetramer-positive cells were found in freshly isolated PBMC, significant numbers (up to 45%) of tetramer-positive T cells were present in their cultured DTH biopsies taken at the same time point. Cytokine production and cytotoxicity of DIL upon co-culture with the appropriate target cells were fully correlated with the specificity in the tetramer analysis. Moreover, DTH reactions induced with DCs pulsed with the gp100 peptides accumulated gp100-specific T cells and not tyrosinase and vice versa. Finally, in situ tetramer staining on cryo-sections revealed that gp100/tyr-specific tetramers-positive cells were specifically present in the infiltrating T cell clusters. Control tetramers against MART-1, human immunodeficiency virus or Epstein–Barr virus were negative [23]. Collectively, these data not only indicate that significant numbers of tetramer-positive T cells accumulate in the DTH site, but also demonstrated that these T cells specifically produced cytokines and/or are cytotoxic for tumour antigen expressing target cells [15].

Next, we compared the clinical and immunological data of 26 stage IV melanoma patients. Inclusion criteria of patients are described previously [6]. Patients had documented progressive disease within 2 months before study entry, serum lactate dehydrogenase ≤ 2× the upper limit of normal, no prior chemotherapy or immunotherapy within 3 months before study entry, and no residual toxicity from prior treatments. Of these patients, 15 patients had progressive disease, nine patients had stable disease with > 4 months duration, and two patients, one with multiple liver metastases at time of inclusion, are in complete remission. No tumour-reactive DILs were found in 13 of the 15 patients with PD. Of the nine patients with SD, four patients with specific T cells had a progression free survival of > 42, 22, 12 and 4.5 months (median 12 months). In the five patients with SD without tumour-reactive DIL, the median progression free survival was 6 months (range 11–4.5). Both patients in complete remission (> 60 and > 42 months) had tumour-specific T cells. Although the number of patients in this study is limited, a statistically significant correlation was observed between the presence of tumour-specific T cell reactivity and progression free survival [15]. The results were confirmed in 31 stage III melanoma patients.

Conclusions and future prospects

Dendritic cell immunotherapy has been introduced in the clinic. It has proven to be feasible, non-toxic and effective in some cancer patients, particularly if the DCs are appropriately matured and activated. However, many questions still remain. One of the concerns related to ex vivo generated DC is how to ensure effective migration to the T cell areas in the lymph node. In this context, we are pursuing the enhancement of migration of ex vivo generated DC by preconditioning the skin with inflammatory cytokines. New applications, such as
RNA technology in cellular therapy, paves the way to the next generation of DC-based therapies.

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