Immunesurveillance by dendritic cells: potential implication for immunotherapy of endocrine cancers

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Abstract

Dendritic cells (DCs) are highly efficient antigen-presenting cells in the immune system with the potential to regulate the system and induce a cytotoxic T-cell response. As a proof of principle, a multitude of animal and human studies has demonstrated that immunization with antigen-loaded DCs may lead to anti-tumour immune responses with tumour regression and rejection of cancer. The identification of tumour antigens that can be recognized by T lymphocytes has facilitated the development of new protocols and enabled immunologists to monitor immune responses. However, to date, long-term clinical effects on larger numbers of cancer patients are missing, and there is no generally accepted DC generation or activation protocol. This review will focus on the most important findings on the role of DCs within the immune system and how to generate and activate these cells in order to induce cytotoxic immunity in non-endocrine and endocrine malignancies. Recently, we and other researchers reported on DC vaccinations in patients with endocrine malignancies mainly in metastasized medullary thyroid carcinoma resulting in tumour-specific immunity and partial clinical responses in some cases. Based on these and other *in vitro* data, new DC vaccination protocols for the treatment of patients with endocrine tumours have now been conducted.

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Introduction

Therapeutic cancer vaccines are intended to activate the immune system for treating an existing tumour or preventing its recurrence. Anti-tumour immunity is coordinated by both innate and adaptive immunity, and mainly mediated by cytotoxic T cells (CTLs), natural killer (NK) cells and natural killer T (NKT) cells. The main players within this context are dendritic cells (DCs), which induce, coordinate and regulate the system (Steinman 1991, Banchereau *et al.* 2000). DCs are highly potent antigen-presenting cells (APCs) with the unique ability of taking up and processing antigens in the peripheral blood and tissues. They subsequently migrate to the draining lymph nodes, where they present antigens to naïve T lymphocytes, and thus induce a cellular immune response involving both CD4+T helper 1 (Th1) cells and cytotoxic CD8+T cells. Moreover, DCs are also important in inducing humoral immunity as explained by their

capacity to activate naïve and memory B cells (Jego *et al.* 2003). In addition, NK (Fernandez *et al.* 1999) and NKT cells (Kadowaki *et al.* 2001) may also be activated by DCs. Thus, DCs can modulate the whole immune repertoire, thereby representing an excellent tool for immunization against cancer.

Since their first description in 1973 (Steinman & Cohn 1973), DCs generated *ex vivo* have been used for vaccination in a multitude of different studies in murine models and humans. These studies provided the proof of principle that DC vaccines can work under these conditions. Despite this, the efficacy of anti-tumour immunization has recently been questioned (Rosenberg *et al.* 2004), which was chiefly due to the very limited number of cancer patients with objective (long-term) clinical responses. The question is, however, not whether DC immunizations work in humans, but rather where to direct future research focus in order to improve clinical efficacy rates.

This review article will focus not only on the recent developments in DC physiology in the context of anti-tumour immunity, but also on the vaccination trials already performed in non-endocrine and endocrine malignancies such as medullary thyroid carcinoma (MTC). Finally, future perspectives as to how vaccination protocols might be improved will be discussed.

DC pathways and functional differences

Until recently, two main pathways of differentiation of DCs have been known (Banchereau *et al.* 2000, Shortman & Liu 2002). The myeloid pathway results in two subsets – Langerhans cells found in stratified epithelia such as the skin, and interstitial DCs found in all other tissues (Caux *et al.* 1996). Plasmacytoid DCs

(pDCs) are generated in line with the lymphoid pathway. These cells are very strong inducers of a cytotoxic immunity (Cella *et al.* 1999), secreting high amounts of type I interferons (IFN- α and IFN- β) after viral infection (Cella *et al.* 1999, Siegal *et al.* 1999). Most recently, a third of the DC subtype has been independently described by two groups (Chan *et al.* 2006, Taieb *et al.* 2006). These cells are distinct from conventional DCs and pDCs with the molecular expression profile of both NK cells and DCs. They are termed IFN-producing killer DCs (IKDCs) due to their ability to produce substantial amounts of type I IFNs and kill target cells by tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) pathways (Fig. 1). Further studies, however, will be needed to characterize their function and investigate the IKDC lineage in detail.

Figure 1 Dendritic cell (DC) lineages. Two main pathways of DC differentiation were known until recently. The myeloid pathway results in two subsets: Langerhans cells found in stratified epithelia, and interstitial DCs found in all other tissues. Plasmacytoid DCs are generated in line with the lymphoid pathway. These cells are very strong inducers of a cytotoxic immunity, as they secrete high amounts of type I IFNs. Under *in vitro* conditions, immature DCs may also be generated from monocytes by culturing with granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), and are known as IL4-DCs. Maturation of these DCs might be reached by additional culturing with tumour-necrosis factor α (TNF- α). Most *in vivo* studies published so far have used these cells for vaccination. Recently, a new DC subtype has been described in the murine model by two groups independently (Chan *et al.* 2006, Taieb *et al.* 2006) termed as interferon-producing killer DCs (IKDCs) with the capacity to directly kill target cells. There are as yet no phenotypical analyses on any IKDC precursor cells in mice (*). To date, in the human system, IKDCs in general have neither been identified.

Under *in vitro* conditions, myeloid DCs may be generated from monocytes activation with granulocyte/macrophage colony-stimulating factor (GM-CSF). The monocytes which encounter interleukin-4 (IL-4) become DCs known as IL-4-DCs (Peters *et al.* 1993, Romani *et al.* 1994, Sallusto & Lanzavecchia 1994). On the other hand, the monocytes differentiate into different DC subsets after co-stimulation with other cytokines such as IFN- α , TNF or IL-15 (Luft *et al.* 1998, Paquette *et al.* 1998, Santini *et al.* 2000, Blanco *et al.* 2001, Mohamadzezh *et al.* 2001, Chomarat *et al.* 2003). IL-4-DCs represent a homologous population, whereas TNF-DCs show a heterogeneous pattern, including both CD1a+ Langerhans cells and CD14+ interstitial DCs (Chomarat *et al.* 2003). Langerhans cells seem to be efficient activators of

CD8+ CTLs, whereas interstitial DCs induce differentiation of naïve B cells into immunoglobulin-secreting plasma cells (Caux *et al.* 1996, 1997).

Different DC subtypes also reveal different functions (Fig. 2). For instance, splenic CD8 α + DCs in the murine system prime naïve CD4+ T cells to secrete Th1 cytokines in a process that involves IL-12, whereas splenic CD8 α + DCs prime naïve CD4+ T cells to produce Th2 cytokines (Maldonado-Lopez *et al.* 1999, Pulendran *et al.* 1999). This polarization also depends on the signals received by the DCs. In humans, for instance, monocyte-derived DCs activated by CD40 ligand (CD40L) prime Th1-cell responses through an IL-12-dependent mechanism, whereas pDCs activated by IL-3 and CD40L have been shown to secrete only small amounts of IL-12 and prime

Figure 2 Comparison of immature and mature dendritic cells in anti-tumour immunity. (A) Immature dendritic cells (DCs) promote immune tolerance. After recruitment to tumour sites, DCs capture antigens (from necrotic or apoptotic tumour cells) and migrate in small numbers to draining lymph nodes. In the absence of stimulation, DCs remain in an immature state. Tumour antigens are presented to T cells, however, without co-stimulation (CD80 and CD86) leading to deletion of T lymphocytes or the generation of interleukin 10 secreting inducible regulatory T cells. (B) Mature DCs induce immunity. Stimulation of immature DCs induces the maturation process and migration of large quantities of DCs to the draining lymph nodes. The mature DCs express high numbers of MHC class I and class II molecules as well as co-stimulatory molecules. Captured tumour antigens are expressed not only on MHC class II, but also on MHC class I molecules to CD4+ Th-lymphocytes and CD8+ CTLs. This phenomenon of presentation of exogenous antigens on MHC class I molecules is termed 'cross-presentation'. Interaction between DCs and Th cells further activates both cell types and increases their ability to stimulate CTLs, which are then able to lyse tumour cells that have downregulated MHC class I molecules. In addition, DCs can stimulate natural killer (NK) cells. In response to IL-12 released by DCs, NK cells upregulate both cytolytic activity and production of interferon- γ (IFN- γ). At the same time, activated DCs may recruit additional NK cells from the blood stream due to the release of IL-8. Activated NK cells are able to kill immature DCs through Nkp30, which is an activating NK-cell receptor. This might be a mechanism by which NK cells limit the recruitment and activation of immature DCs in tumour tissues. However, NK cells may also induce DC maturation. The expansion of CD4+ CD25+ regulatory T cells is another process, which downregulates the immune response.

Th2-cell responses (Rissoan *et al.* 1999). Thus, both the type of DC subset and the activation signals to which DCs are exposed are important in the polarization of T cells (Banchereau & Palucka 2005).

DCs and immune tolerance

DCs are major players not only in inducing cytotoxic immunity, but also in maintaining central and peripheral immune tolerance (Steinman *et al.* 2003). Central tolerance is mediated by mature thymic DCs by which positive and negative selections of newly generated T cells are transmitted (Brocker 1999). As many self-components might not reach the thymus, another kind of tolerance is required – peripheral tolerance – which occurs in peripheral lymphoid organs. Peripheral tolerance is mediated by immature DC and explained by the absence of appropriate co-stimulation, including CD80 and CD86, while presenting the antigen, and leads to T-cell anergy or deletion (Steinman *et al.* 2003) (Fig. 2) or the development of IL-10-secreting inducible regulatory T cells (Jonuleit *et al.* 2000, Dhodapkar *et al.* 2001). In contrast, concomitant activation of these cells with CD40-specific antibody results in a potent immune response, which is explained by the greatly increased numbers of co-stimulatory molecules on the cell surface (Dhodapkar *et al.* 2001). On the other hand, activated DCs might also contribute to the peripheral tolerance by promoting clonal expansion of the naturally occurring CD4⁺ CD25⁺ regulatory (T_{reg}) cells (Yamazaki *et al.* 2003). Therefore, the biology of DCs offers several targets for cellular immunity control (Banchereau & Palucka 2005).

In vitro generation of DCs

The research groups of Romani *et al.* and Sallusto *et al.* were the first to describe the generation of immature DCs by cultivating the monocytes with GM-CSF and IL-4 (Romani *et al.* 1994, Sallusto & Lanzavecchia 1994). Since then, there has been a multitude of vaccination trials mostly on immunogenic metastatic melanoma (Nestle *et al.* 1998, Thurner *et al.* 1999). Recent discoveries, however, point out some alternatives to the classical method of DC generation; for instance, IL-15-DCs are thought to be more efficient in inducing antigen-specific CTL differentiation *in vitro*, whereas the ability of CD4⁺ T-cell stimulation seems to be equal (Mohamadzadeh *et al.* 2001). In addition, IFN α -DCs generated in 3-day culture are efficient in inducing immunity also. This has been shown in the severe combined immuno deficiency (SCID) mouse

model (Santini *et al.* 2000) as well as in the human system (Parlato *et al.* 2001, Tosi *et al.* 2004). We have used this procedure for immunizing patients with metastasized MTC, resulting in tumour-specific immunity in some cases (unpublished data).

Apart from monocytes, other cell types have also been used as progenitors for DC vaccinations. Peripheral-blood DCs loaded with specific antigens have been administered to patients with follicular B-cell lymphoma (Hsu *et al.* 1996) and prostate cancer (Small *et al.* 2000). Furthermore, an Flt3-based DC enrichment results in immunological and clinical response in some patients (Fong *et al.* 2001c), and CD34⁺ haematopoietic progenitor cells (CD34-DCs) have also been applied in the DC immunization in cancer patients with increased rates of measurable clinical response in patients recognizing more than two antigens (Banchereau *et al.* 2001). In summary, a standardized DC generation protocol as recently proposed (Figdor *et al.* 2004) is still a matter of debate, and no consensus has been reached. Intensive work is still needed to solve this problem in the future.

Maturation of DCs

Immature DCs have long since been recognized as highly potent in capturing antigens, whereas mature DCs are highly efficient in presenting exogenous antigens on major histocompatibility complex (MHC) class I and II molecules. The maturation of DCs is closely related to the physiological migration of these cells from the periphery to the draining lymph nodes. This process is associated with various coordinated events such as the upregulation of co-stimulatory molecules (CD80 and CD86), loss of phagocytic receptors, and changes and upregulation of MHC class II compartments. The immature DCs generated over the GM-CSF/IL-4 pathway do not lead to a marked immune response, whereas the mature DCs induce a specific immune response in the same patient (Jonuleit *et al.* 2001). When generated using a 'standard method' with a cocktail of proinflammatory cytokines (IL-1 β , IFN, IL-6 and prostaglandin E₂) (Jonuleit *et al.* 1997), these cells induce functionally superior CD8⁺ T cells and polarize CD4⁺ T cells towards IFN- γ production (Schuler-Thurner *et al.* 2002). Nevertheless, this method needs to be examined in parallel with other stimuli. Indeed, the combination of IL-1 β and TNF with type I and type II IFNs (IFN- α and IFN- γ respectively) seems to yield more potent DCs in terms of secretion of IL-12 and induction of tumour-specific CTLs *in vitro* (Mailliard *et al.* 2004). An important step will be to identify the stimuli that trigger a desired DC maturation

programme leading to the induction of tumour-specific CTLs, but not regulatory T cells. Toll-like receptor ligands in general may represent such a stimulus, leading to enhanced DC function (Reis e Sousa 2001).

Loading methods in tumour antigens

There are different methods of antigen loading of the DCs. A very common method is co-culturing the purified tumour antigens or tumour lysate (TL) with DCs. This approach has been successfully used, for instance, in malignant melanoma, resulting in CTL immune and clinical responses in some patients (Nestle *et al.* 1998). In our studies on patients with neuroendocrine malignancies, we demonstrated a TL-specific immune response in one patient with parathyroid carcinoma (Schott *et al.* 1999) and another with neuroendocrine pancreas carcinoma (NEC) (Schott *et al.* 2001a). However, several other methods of antigen delivery into DCs have been investigated to facilitate and improve antigen uptake and presentation. MHC class I- and II-restricted 9–15 amino acid peptides derived from defined antigens (natural sequences or peptide analogues designed for enhanced binding to the MHC molecules or T-cell receptor) can be easily synthesized in good manufacturing practice (GMP) form and used for specific antigen loading (Gilboa 1999, Wang *et al.* 1999). Although this technique is important for proof-of-principle studies, the use of peptides has limitations, such as the limited number of well-characterized tumour-associated antigens and the relatively high turnover of exogenous peptide MHC complexes, resulting in comparatively low antigen presentation by the time the DCs arrive in the draining lymph node after injection, and the induction of a restricted repertoire of T-cell clones, thereby limiting the ability of the immune system to control the tumour antigen variation. Therefore, the use of so-called modified heteroclitic peptides needs to be discussed in this context. It is known that some peptides including those derived from the immunodominant antigens do not bind to MHC class I molecules with high affinity, possibly explaining their limited immunogenicity *in vivo* (Salgaller *et al.* 1996). The generation of peptide analogues with increased affinity to MHC class I molecules (known as heteroclitic peptides) may therefore be used to improve peptide immunogenicity (Parkhurst *et al.* 1996). In endocrine tumours, a comparable approach has already been used for patients with metastasized parathyroid carcinoma. These patients were immunized with modified parathyroid hormone (PTH) peptides together with Freund's adjuvant resulting in a marked decrease in tumour

markers and metastatic spread (Bradwell & Harvey 1999, Betea *et al.* 2004). In contrast, recent studies on patients with malignant melanoma have shown that T cells elicited *in vivo* by vaccination with heteroclitic melanoma tumour antigen (MART1 and glycoprotein 100 (gp100)) peptide show poor recognition of endogenous melanoma-derived peptide and less efficient tumour-cell lysis compared to T cells specific for the native peptides (Stuge *et al.* 2004). The most probable explanation is that these peptides differ from naturally processed epitopes. Thus, loading the DCs with total antigen preparations and allowing 'natural' processing and epitope selection is expected to improve efficacy, while allowing the generation of a diverse immune response involving many CD4+ T-cell and CTL clones. With this in mind, in our studies, we used whole calcitonin for immunization of the MTC patients and not certain peptides (Schott *et al.* 2001b).

A totally different approach of antigen delivery into DCs is to fuse the DCs with tumour cells. In this approach, the entire repertoire of tumour antigens, including those yet to be identified, is expressed with the immune-stimulating machinery of DCs. The fusion cell vaccine allows the induction of helper T and CTL responses by class II presentation of exogenous protein and class I presentation of newly synthesized endogenous protein. Vaccination with fusion cells has eradicated the established tumours in various animal models (Gong *et al.* 1997, 2002). In human MUC1 transgenic mice, vaccination with fusion cells reverses immunological unresponsiveness to MUC1 and results in the rejection of MUC1-positive tumours (Gong *et al.* 1998). Preclinical studies with patient-derived breast cancer cells and DCs have also demonstrated that fusion cells induce tumour-specific CTL responses and lysis of autologous tumour cells (Gong *et al.* 2000). Most recently, Avigan *et al.* (2004) confirmed these results by demonstrating a Th1 immunity and clinical response in patients with metastatic breast and renal cancer. Most of the works in this field have been published from the group mentioned earlier. Therefore, these data await confirmation by others. In our studies, we were able to demonstrate that DCs might be fused with the tumour cells shown to have high efficacy rates in adrenal carcinoma cells (Papewalis *et al.* 2006). Using electron microscopy, we could demonstrate viable cells indicated by intact basal membranes. So far, however, we were successful only in cell lines and not in primary cultures of endocrine tumours.

There are some additional methods of antigen delivery such as the presentation of DCs to dead tumour cells aiming the phagocytosis of tumour cell particles by DCs and to present them (Albert *et al.* 1998,

Berard *et al.* 2000). Indeed, DCs cultured with killed allogeneic melanoma, prostate- or breast-cancer cell lines prime naïve CD8+T cells against tumour antigens *in vitro* (Berard *et al.* 2000, Neidhardt-Berard *et al.* 2004). Recently, DC vaccination was reported to lead to the induction of tumour-specific T-cell immunity and ‘long-lasting’ tumour regression after co-culturing with a killed allogeneic melanoma cell line in two out of twenty patients (Banchereau & Palucka 2005).

Finally, the transfection of RNA coding for defined antigens by electroporation may represent an elegant method of antigen delivery (Van Tendeloo *et al.* 2001). RNA transfection does not require expensive production of GMP quality proteins or antibodies. In addition, RNA also appears advantageous over other transfection methods with naked DNA or viral transfection, as RNA transfection leads only to the transient antigen expression; however, this is sufficient for antigen processing and presentation.

Route of DC administration

Various studies have demonstrated that antigen-loaded DCs might prime T-cell responses regardless of the route of DC administration. However, the quality of responses might be affected. This has been demonstrated by the predominant induction of Th1-cell responses after intralymphatic and intradermal administration, albeit with non-polarized T-cell and antibody responses after i.v. injection (Fong *et al.* 2001b). Moreover, in murine studies, the induction of tissue-specific immunity was associated with the tissue origin of DCs (Mora *et al.* 2003, Mullins *et al.* 2003). In a murine melanoma model, both s.c. and i.v. immunizations with peptide-pulsed DCs induced peptide-specific memory T cells in the spleen, resulting in the control of pulmonary metastatic spread. On the other hand, s.c. immunization also induced memory T cells in the lymph nodes, allowing subsequent protection against s.c. tumour growth. Intravenous injection of DCs failed to do so (Mullins *et al.* 2003). Thus, DCs can prime T cells with different homing capacities. For clinical application, the consequence of such an instructive role of DCs is that, for example, i.v. administration of DCs in neuroendocrine tumours with disseminated metastatic spread could induce homing in effector cells, whereas DC vaccinations against melanoma would unlikely induce skin-homing in effector cells. All vaccinations were administered subcutaneously in our studies on patients with MTC, resulting in tumour-specific immune responses in some of them.

Frequency of DC administration

The frequency of DC administration has been reported to play an important role in the immune response; four vaccinations with CD34-DCs (within a 6-week period) have been reported to result in short-lived antigen-specific CD8+T-cell immunity in some patients (Paczesny *et al.* 2004). During follow-up, all patients lost specific T cells that were detectable by direct ELISPOT. There are several possible explanations for this phenomenon. One could be that T cells may migrate from blood to peripheral tissue or tumour site (Masopust *et al.* 2001). Another could be that vaccinations had been administered frequently resulting in T-cell tolerance and anergy. In contrast, mouse and human studies on vaccination against infectious agents indicated that priming should be followed by a booster vaccination after 4–6 weeks for optimal immune response (Kaeche *et al.* 2002, Zinkernagel 2003). However, whether this can be applied to cancer immunotherapy is still a matter of debate, and no consensus has been reached. In our studies on patients with MTC, we have demonstrated, for instance, that 12 DC vaccinations in one patient resulted in an antigen-specific Th1 immune response and decrease in metastatic spread (Schott *et al.* 2001b). This indicates that frequent immunizations may not be tolerogenic as recently proposed.

DC immunotherapy in non-endocrine malignancies

Within the last decade, a variety of early clinical studies with DC vaccines have been performed in different human tumour entities leading to the anti-tumour immunity in most cases. Owing to the brevity of this review, it is possible to summarize only some of these studies. Most of the research has been conducted on patients with immunogenic malignant melanoma. An update of the first study by Nestle *et al.* (1998), in which monocyte-derived DCs loaded with CD4+T-cell stimulator keyhole limpet haemocyanin (KLH) and either tumour peptides (MAGE-3A1) or TL were intranodally delivered, showed tumour regression in 6 out of 11 patients with complete remission in two cases, which lasted for several years. A reanalysis by tetramer staining of antigen-specific T cells confirmed the strong induction of MAGE-3A1-specific CTLs (Thurner *et al.* 1999). The authors reported that the treatment of skin metastases was accompanied by infiltration with CTLs, suggesting the effective activation of a cytotoxic anti-tumour immune response. Another study directly compared the immunogenicity of immature DCs and those activated in

monocyte-conditioned medium on intranodal administration into the opposite inguinal lymph nodes (Jonuleit *et al.* 2001). The mature DCs were clearly superior inducers of the effector T cells directed against two recall antigens (tetanus toxoid and purified protein derivative/tuberculin) and, more importantly, tumour peptides. Banchereau *et al.* (2001) demonstrated a correlation between clinical success after DC immunization and the number of antigen-specific reactivities – six out of seven metastatic melanoma patients who developed immunity to less than two melanoma antigens showed progressive disease, in contrast to tumour progression in only one of the ten patients with immunity to more than two antigens. A long-term follow-up on melanoma patients treated by the same research group supported this concept, as most melanoma patients with CTL reactivities towards more than two antigens survived longer than median survival time (Fay *et al.* 2005). Another study by Berger *et al.* (2004) demonstrated a broad T-cell response in a DC-treated patient towards different tumour-associated antigens. Finally, de Vries *et al.* (2005) reported a strong correlation between the presence of DC-vaccine-related T cells in delayed type hypersensitivity (DTH) biopsies and a positive clinical outcome.

Several other studies have been performed on prostate cancer using cell-associated antigens as targets for DC immunization therapies. So far, three cell-specific antigens, the prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA) and prostatic acid phosphatase (PAP) have been used. Tjoa *et al.* (1998) reported on partial tumour regressions in nine out of 33 patients suffering from an advanced disease stage after i.v. infusion of DCs pulsed with human leukocyte antigen (HLA)-A0201-specific peptides from PSMA. Later studies have confirmed the initial results of clinical response in about a third of 19 cases treated (Tjoa *et al.* 1999, Murphy *et al.* 2000). Recently, another group reported on an *in vivo* immune response (DTH skin reactivity) in 50% of 28 prostate cancer patients after vaccination with PSA peptide-pulsed DCs. An epitope-specific *in vitro* tumour cell lysis could be detected in four of them (Perambakam *et al.* 2005). An interesting xenoantigen immunization approach was taken by Fong *et al.* (2001a), who administered DCs isolated from immobilized aphaereses and pulsed with mouse PAP (two monthly vaccinations either i.v., i.d. or into a lymph node). All 21 patients with metastatic prostate cancer developed Th1 responses to mouse PAP, and 11 also developed responses to the homogenous self-antigen, and six subjects showed clinical stabilization in their previously progressing prostate cancer. Small *et al.* (2000) treated 31 patients suffering from prostate

cancer with infusions of a cellular vaccine prepared by isolating a DC precursor-enriched fraction from the aphaeresis products, which were then exposed to a recombinant fusion protein consisting of PAP linked to the GM-CSF for 2 days. The fusion protein presumably targets the cells expressing GM-CSF receptor, including DC precursors, which then undergo maturation *in vitro*. This cellular vaccine (termed APC8015 or Provenge, Dendreon Corp., Seattle, WA, USA), which contains a variable percentage of DCs (a mean of 123 million DCs at 18% purity) was infused intravenously at weeks 0, 4, 8 and 24. All patients developed immune responses to the fusion protein, and 38% developed immune responses to the PAP. An alternative approach for treating prostate cancer may be the use of RNA for transfection of DCs. The studies by Heiser *et al.* (2002) demonstrated the induction of polyclonal prostate cancer-specific CTLs in all 13 patients treated for metastatic prostate cancer following the stimulation with PSA mRNA-transfected DCs and transient clearance of the circulating tumour cells in the peripheral blood. However, no significant clinical response was reported.

Promising results were also demonstrated in several other cancer forms such as malignant lymphoma. Hsu *et al.* (1996) were the first worldwide to use DCs in the vaccination of human beings with conditions such as lymphoma. The follow-up studies demonstrated *in vitro* CTL responses as well as partial clinical responses in some patients (Timmerman *et al.* 2002). Several other studies have been performed in renal cell carcinoma (Holtl *et al.* 1998, 2002), colon carcinoma (Morse *et al.* 1999) or malignant glioma (Yu *et al.* 2001).

So far, DC vaccinations in humans have been performed in small clinical trials to demonstrate the potential efficacy and assess the safety of DC immunizations. All clinical trials reported only minor side effects, suggesting that DC-based immunotherapy is safe. The majority of the symptoms, including local erythema, indurations, pain at the site of injection, low-grade fever, bouts of sweating and chills, are part of the intended activation of an immune response. Some studies in metastasized melanoma observed localized vitiligo and induction of thyroid-stimulating hormone-receptor antibodies or antinuclear antibodies. However, no severe vaccination-associated autoimmune reactions were described (Nestle *et al.* 1998, Banchereau *et al.* 2001).

DC immunotherapy in non-thyroid endocrine malignancies

Most metastasized endocrine carcinomas are incurable by conventional radiotherapy or chemotherapy, and the

outcome is usually fatal. Therefore, new strategies are required for the treatment of these cancer types. The lack of specific tumour antigens is the major problem facing immunotherapy in endocrine tumours. This dilemma may be overcome by the use of tumour cell preparations (proteins or mRNA) or antigens specifically expressed in tumour cells (specific enzymes involved in the synthesis of hormones or polypeptide hormones (PTH)). Both approaches have been tested in pilot studies on patients at advanced stages of disease with the aim of minimizing the growth of metastatic lesions or controlling the hormone activity. One patient suffered from PTH carcinoma, which caused hypercalcaemic symptoms, including severe bone pain, profound weight loss and extreme muscle weakness. A second patient presented with a metastasized NEC, which was strongly positive for chromogranin A (CgA). After several rounds of vaccination with TL-pulsed DCs, both patients showed a dose-dependent proliferation of PBMCs towards yet unidentified antigen(s) within the TL and positive DTH reactivity, suggesting the induction of TL-specific T-lymphocytes (Schott *et al.* 1999, 2001a). Immunohistochemical analysis of a skin biopsy from the patient with the NEC revealed CD4+ and CD8+ T lymphocyte infiltration providing indirect evidence for the induction of tumour-specific cytotoxic immunity. In the latter patient, therapy was accompanied by a temporary decrease in tumour marker CgA and slight tumour regression. During the follow-up, however, this patient experienced progressive metastatic spread.

In the second patient, serum PTH and calcium levels steadily increased. Therefore, the immunization protocol was changed and a synthetic PTH peptide was used for DC pulsing in combination with T-helper antigen KLH (Bradwell & Harvey 1999). Although the tumour masses did not decrease significantly, serum PTH declined following vaccination, suggesting partial destruction of the tumour cells. During the follow-up, the patient needed repeated bisphosphonate therapy, and eventually died of pneumonia. Despite this devastating outcome, this case was the first to demonstrate the ability of using a polypeptide hormone as antigen to induce cytotoxic immunity in an endocrine carcinoma (Schott *et al.* 2000).

Recently, we also performed a DC vaccination trial in two patients with adrenocortical carcinoma (Papewalis *et al.* 2006). Within this study, the mature DCs were generated as previously described (Schuler-Thurner *et al.* 2002). Irrespective of the lethal outcome of our patients, we could demonstrate that TL-pulsed autologous DCs are able to induce a TL-specific immune

response. In one of the two patients, granzyme B-positive T cells could be detected.

DC immunotherapy in MTC

Our initial results prompted us and other researchers to design protocols for DC vaccination of patients with metastasized MTC. MTC, arising from parafollicular, calcitonin-producing C cells, represents an aggressive, usually slow-growing tumour occurring in both sporadic and familial forms such as multiple endocrine neoplasia type 2 (MEN 2) (Kebebew *et al.* 2000). Owing to its low chemosensitivity and radiosensitivity, no conventional therapy has been established for the metastatic state. In contrast, C cells of the thyroid gland are of neuroendocrine origin, and may therefore be highly susceptible to an immune attack, as are other neuroendocrine cells such as β cells of the pancreas in autoimmune type 1 diabetes mellitus or parathyroid cells in autoimmune hypoparathyroidism (Ma *et al.* 2000, Kimura *et al.* 2001, Nakayama *et al.* 2005). Thus, MTC may represent a favourable carcinoma type for testing the cancer immunotherapy approaches in general.

Potential tumour antigens in MTC

So far, different specific and non-specific MTC-associated proteins are known to be the possible target molecules for immunotherapy in MTC. The most important is C-cell-specific polypeptide hormone calcitonin, which represents a highly sensitive tumour marker for detecting and monitoring metastatic spread in MTC. In a previous study by Zhang *et al.* (1997), an epitope mapping with monoclonal antibodies was performed showing highest immunogenicity of the central region of calcitonin (amino acids 13–21). Co-culturing with specific antibodies covering this region (amino acids 10–19) led to *in vitro* inhibition of MTC cell line growth (Zhang & DeGroot 1997). Using the preprohormone of calcitonin (preprocalcitonin, PPCT), another group demonstrated an antigen-specific immune response following the cDNA vaccination in a murine MTC model (Haupt *et al.* 2001). In this study, the precursor hormone of calcitonin was used instead of hCT, since this larger molecule has a greater probability of containing suitable epitopes than the mature hCT. To enhance the immune response, the authors combined this therapy with GM-CSF and IFN- γ (Chow *et al.* 1997, 1998, Chen *et al.* 1998), demonstrating PPCT-specific proliferative cellular and antibody responses following antigen–DNA vaccine occurred in the presence of

the GM-CSF gene. In contrast, co-delivery of IFN- γ expression plasmid resulted in a decreased antibody response against hPPCT, which may however lead to enhanced Th1-like immunity (Macatonia *et al.* 1993, Chow *et al.* 1998). These data, together with the aforementioned reports on PTH-based tumour-specific immunity in vaccinated parathyroid carcinoma patients, represent the basis for using calcitonin as a specific target molecule in MTC. This idea may even be supported by the immune system's recognition of salmon calcitonin, which is being used for the treatment of osteoporosis, leading to calcitonin-specific autoantibodies (Grauer *et al.* 1993, 1995).

Carcinoembryonic antigen (CEA) may be another potential target molecule in MTC. CEA is a 180 000 glycoprotein member of the Ig supergene family. Several functions have been attributed to CEA, such as cell adhesion and inhibition of cell death induced by the loss of anchorage to the extracellular matrix (Benchimol *et al.* 1989, Screaton *et al.* 1997). CEA represents a well-established tumour marker in different human cancer forms, most importantly in gastrointestinal malignancies. Different studies reported on the identification of HLA class I and II-restricted CEA epitopes (Kawashima *et al.* 1999, Nukaya *et al.* 1999, Shen *et al.* 2004) and induction of HLA class I-restricted CEA epitope-specific CTLs (Kim *et al.* 1998). During the follow-up clinical trials, CEA-positive cancer cases treated with CEA-peptide-pulsed DCs have been reported to demonstrate partial clinical response in some patients (Morse *et al.* 1999, Nair *et al.* 1999, Itoh *et al.* 2002, Liu *et al.* 2004, Matsuda *et al.* 2004). Comparable results have also been reported in *in vitro* and *in vivo* studies following transfection of DCs with CEA-mRNA (Nair *et al.* 1999, Eppler *et al.* 2002). Induction of cytotoxic immunity in a transgenic mouse model was also recently demonstrated (Saha *et al.* 2004). In MTC, CEA is a well-established tumour marker with higher correlation to tumour mass compared with calcitonin, and may therefore represent a favourable target molecule for vaccination.

Chromogranin A (CgA) may serve as target molecules as well. Chromogranin (secretogranins) constitute a family of water-soluble acidic glycoproteins stored in secretory vesicles of neuroendocrine tumours containing proteins and peptide hormones (Taupenot *et al.* 2003). Like CEA, CgA also shows a strong correlation between serum levels and tumour mass; however, false positive values are frequently reported (Bajetta *et al.* 1999). Antigenic regions of CgA have been demonstrated using monoclonal antibodies (Corti *et al.* 1996). So far, however, they have not been used in the context of immunotherapy approaches.

Finally, cancer/testis antigens (CTAs) belong to the group of tumour-associated antigens expressed during ontogenesis in a number of solid tumours, but not in normal tissues except the testis. Examples of CTAs are MAGE, GAGE and SSX gene families, BAGE and NY-ESO-1. Most of them are highly immunogenic, eliciting spontaneous immune responses at either cellular level (Jager *et al.* 1998, 2000) or in association with a strong humoral component (Stockert *et al.* 1998) in patients with advanced cancer. An *in vitro* analysis of 23 sporadic MTC cases has revealed that 65% expressed NY-ESO-1, which significantly correlated with tumour recurrence (Maio *et al.* 2003). NY-ESO-1-specific antibodies could be detected in some cases. It may, therefore, be postulated that CTAs such as NY-ESO-1 may serve as specific target molecule for immunotherapy in MTCs as well.

DC vaccination trials in MTC

So far, three clinical trials on autologous DCs have been performed in patients with metastasized MTC; two from our group and another from an Austrian group including the follow-up analysis. The major differences between the studies were the antigens used for immunization. While we used C-cell-specific target molecules such as calcitonin and a CEA-peptide, the Austrian group applied autologous TL for antigen delivery into the DCs. The immunological and clinical results of both approaches will now be compared.

Based on our own promising data of a PTH-specific immune response in one patient with parathyroid carcinoma, polypeptide hormone calcitonin was used for the vaccination of MTC patients (Schott *et al.* 2001b). A certain CEA-peptide (YLSGANLNL) was also administered to HLA A2-positive patients in order to broaden the antigenic repertoire. This peptide has already been used before in other non-endocrine malignancies resulting in tumour-specific immunity. In our initial study, seven patients were immunized by s.c. injections of $2\text{--}5 \times 10^5$ DCs loaded either with CEA-peptide and calcitonin ($n=6$) or calcitonin only ($n=1$) (Schott *et al.* 2001b). After DC vaccination, all seven patients developed a DTH reaction that immunohistochemistry revealed to be mediated by the infiltration with CD4+ and CD8+ T lymphocytes. In addition, three patients developed significant T-cell proliferation of peripheral blood lymphocytes in response to calcitonin and CEA. Peripheral blood lymphocytes drawn after initiation of treatment responded with high-level IFN- γ secretion in some patients after stimulation with calcitonin or CEA, whereas the IL-4 production was only slightly increased

(Schott *et al.* 2002). These data indicate the induction of a Th1-dominated cellular immune response against calcitonin and CEA in the majority of patients. Clinical follow-up revealed that three of the seven patients treated developed temporary clinical response with a decrease in calcitonin and CEA serum levels, while three showed stable disease. One patient, who failed to develop T-cell response to either calcitonin or CEA showed further tumour progression. Among the responders, one subject rejected all radiologically detectable liver metastases and developed a dramatic regression of pulmonary metastases (Fig. 3) during the treatment period of 14 months (Schott *et al.* 2001b).

With the power of an improved DC generation protocol, we then performed a second vaccination trial (Schott M, unpublished observations). Autologous DCs were generated in a 3-day culturing step with GM-CSF and IFN- α . This resulted in a semi-mature DC phenotype, but potentially strong inducers of tumour-specific CD8+ T cells (Parlato *et al.* 2001, Tosi *et al.*

2004). Altogether, five MTC patients with extended metastatic spread with pulmonary and liver metastases were immunized with calcitonin-pulsed DCs only. *In vivo* delayed-type hypersensitivity skin reaction in one patient after injection of pure calcitonin revealed strong CD4+ and CD8+ T-cell infiltration as an indicator of a calcitonin-specific cytotoxic immune response. Three patients developed calcitonin-specific immune response measurable *in vitro*. Detailed epitope analyses then revealed an HLA class II-restricted immune response directed towards the central region of calcitonin. Most interestingly, all epitopes identified covered the central region of calcitonin (amino acids 13–20). This region has already been shown to be the highest immunogenic region in the context of humoral immunity (Zhang *et al.* 1997).

Parallel to our work, Stift *et al.* (2003) performed DC trials with autologous TL for antigen delivery. Based on *in vitro* data with induction of cytotoxic immunity in three MTC patients (Bachleitner- Hofmann *et al.* 2002),

Figure 3 Clinical response to dendritic cell vaccination in medullary thyroid carcinoma. Computed tomography of one patient with metastasized medullary thyroid carcinoma before (left), and 13 months after (right) initiation of dendritic cell vaccination shows complete regression of liver metastases (top) and a significant reduction of pulmonary lesions (bottom). Arrows shows sites of lesions before vaccination (from, Schott M, Seissler J, Lettmann M, Fouxon V, Scherbaum WA & Feldkamp J 2001 Immunotherapy for medullary thyroid carcinoma dendritic cell vaccination. *Journal of Clinical Endocrinology and Metabolism* **86** 4965–4969; Copyright 2001, Reprinted with permission from The Endocrine Society).

an *in vivo* trial in patients with solid cancers was initiated. Four of those patients suffered from metastatic MTC (Stift *et al.* 2003). As described by the authors, a ‘tumour marker response’ was observed. However, most importantly, two patients showed shrinkage in cervical lymph nodes by computed tomography. Then another clinical follow-up study in MTC patients was performed (Stift *et al.* 2004). In all ten immunized MTC patients, positive immunological responses could be detected as evaluated by *in vivo* delayed-type hypersensitivity reaction or *in vitro* intracytoplasmic IFN- γ detection assay. Three patients showed partial response, one presenting a minor response, and two showed stable disease, whereas the remaining four had progressive disease. Recently, the same group also reported on the establishment of human MTC cell lines, which have been generated from patients. The authors postulate that these tumours may represent a source for the generation of TL for immunization. However, the likely HLA-mismatch needs to be accounted for in future vaccinations before being administered to patients (Pfragner *et al.* 2005).

In summary, these results clearly demonstrate that autologous DCs are able to induce an antigen-specific Th1-driven immunity in MTC patients. Whether this immune response elicits tumour-specific CTLs needs to be determined in the future. This includes the question of whether the clinical response data reported will result in long-term clinical effects in these patients.

Concluding remarks and future aspects

Although the data from the above studies are encouraging, whether or not DC vaccination can provide a significant, long-term clinical benefit in cancer patients remains to be seen. A recently published meta-analysis of vaccination trials reported on an overall clinical response rate of 9.5% in DC immunizations in cancer patients; however, this was two to four times as high as with peptide vaccines or immunization with viral vectors or tumour cells (Rosenberg *et al.* 2004). To improve the clinical efficacy rate of DC tumour vaccination trials, different points need to be considered. First, standard protocol(s) for DC generation and activation should be developed to improve the reproducibility of the vaccination procedure and allow a comparison of the results from different studies. So far, a multitude of DC generation and activation protocols have been suggested. Because there are still no reports on long-term clinical effects on a larger number of patients, no single method has been generally accepted as a standard technique for DC generation. Secondly, the search for highly

immunogenic antigen/peptides should be intensified not only to possibly improve clinical benefit, but also to monitor the immunological response. Our data clearly demonstrate that calcitonin may serve as a specific target molecule in MTC in this context, and HLA class II-restricted epitopes have been identified. Our group will therefore continue to focus research interests on this molecule. Whether other methods of antigen delivery, including pulsing with TL or fusing with autologous tumour cells will result in epitope-specific immunity and in long-term clinical effects needs to be determined as well. Thirdly, future vaccination trials should also include modern techniques that allow quantification and characterization of tumour antigen-specific Th cells and CTLs, including MHC class I or II peptide tetramer staining and ELISPOT analysis from peripheral blood mononuclear cells (Klenerman *et al.* 2002). This is important, as there is still no defined threshold determining the number of T cells sufficient to induce tumour regression. Future vaccination trials should correlate clinical effects with sophisticated immunological methods in order to define these parameters. In summary, these *ex vivo* strategies will help to identify the parameters for *in vivo* targeting of DCs, which should be the next step in the development of clinically effective DC-based vaccination. This is absolutely crucial, as neither conservative nor innovative therapy approaches have yet been established in most endocrine malignancies, e.g. in metastatic MTC.

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