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Dendritic cells in cancer immunotherapy

Een wetenschappelijke proeve op het gebied van
de Medische Wetenschappen

Proefschrift

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aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het college van decanen
in het openbaar te verdedigen op maandag 6 december 2010
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door

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“In my occasional attempts to work with clinical colleagues, I encountered a kind of ‘two cultures’ problem [...]. Most clinicians, even academic ones, and basic scientists, even those trained originally as physicians, do not necessarily converse easily. They know different things, seem to speak different dialects, have somewhat different goals and standards, and even organize their days differently – clinicians arriving early, scientists arriving late - hampering opportunities to meet. Clinicians often lack the laboratory support that would be required to store and keep track of patient materials for research, they are extremely busy, and their primary responsibilities lie outside of the laboratory. Furthermore, the time from biological discovery to clinical advance can be extraordinarily long, and the process may be encumbered with administrative and regulatory hurdles. These things can try the patience of those accustomed to frequent gratification in the laboratory.”

Harold Varmus, _Nobel Laureate in Medicine - The Art and Politics of Science._
Outline of the thesis

Classically, in the last century there have been three treatment modalities in cancer: surgery, radiotherapy and medical therapy (which concerns chemotherapeutics, hormones, immunotherapy, and more recently targeted therapy). Immunotherapy concerns the use of the patients’ own immune system to fight cancer.

There are several possibilities to reinforce anti-cancer immunity: make tumor cells more susceptible for immune-attack, strengthen the effector cells that kill tumor cells or alarm and activate the directors of the immune system. The latter approach is used in dendritic cell (DC) vaccination.

DCs are the orchestrators of immune responses, they activate and direct the effector arm of the immune system towards the cancer cells. Several years ago techniques were developed to culture large quantities of DCs from peripheral blood, providing the opportunity to manufacture a cancer-specific vaccine, consisting of the patients’ own DCs that are activated and loaded with immunogenic particles of tumor cells (antigens) for clinical administration. After administration, the DCs migrate to lymph nodes and activate T cells, which subsequently proliferate and spread throughout the body to kill the cancer cells that express the same antigen.

At least, that is how it works in theory... Reality unfortunately is less straightforward. DC vaccination includes many intrinsic variables, such as the type of DC, the type of antigen, the method of antigen-loading, the route of administration, the treatment schedule, the monitoring of immune responses and the type and stage of cancer. In addition, there are several hurdles to overcome, especially the many levels of immune suppression that may be present in the cancer patient.

This thesis focuses on clinical studies in which patients with melanoma or colorectal cancer were vaccinated with DCs. Several variables were tested, often with vaccine-induced immune response as endpoint. In addition, *in vitro* studies were performed focusing on DC antigen loading strategy and the effect of cytotoxic anticancer drugs on DC function and tumor cell immunogenicity.
Chapter 1

General Introduction

Adapted from:


General Introduction

The immunogenicity of tumors has been established. Non-specific stimulation of immunity, such as by cytokine therapy, has been shown to induce durable clinical remissions in metastatic disease\(^1\). The identification and molecular characterization of tumor-associated antigens in the last 10-15 years has allowed the development of immunotherapy targeted specifically at the tumor. Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system, with the potential to either stimulate or inhibit immune responses. As such, they are currently applied in clinical studies in cancer patients with the goal of inducing a tumor-specific T cell response.

IMMUNOBIOLOGY OF DENDRITIC CELLS

DCs reside in peripheral tissues where they act as the sentinels of the immune system, continuously patrolling the environment in search of antigen (figure 1a). At this stage they possess an ‘immature’ phenotype, which is mainly characterized by a low surface expression of MHC class I and II molecules and costimulatory molecules\(^2\). Immature DCs are specialized in the recognition and uptake of antigen. Exogenous antigens are internalized and processed by DCs, and the antigenic peptides are presented in the MHC class II complexes on the cell surface. Endogenous antigens, such as unstable self-proteins or viral proteins, are cleaved into peptides by proteasomes and assembled into stable MHC class I-peptide complexes in the endoplasmatic reticulum which are subsequently transported to the cell surface. Of importance for DC-based vaccines in cancer immunotherapy is the finding that internalized antigens from exogenous sources, such as apoptotic or necrotic tumor cells, may also be presented in MHC class I\(^3\). Thus, DCs may present tumor antigens to both CD4+ and CD8+ T cells.

In the presence of a ‘danger’ signal, derived from tissue damage or microbial products, DCs undergo an activation process called maturation\(^4\). This maturation process involves a cascade of events that convert the DC into a cell that is exceptionally well equipped for antigen presentation and T cell activation. During maturation DCs upregulate chemokine receptors CCR7 and CD62L which leads to migration to secondary lymphoid organs. Furthermore, surface expression of MHC class I and II, co-stimulatory molecules CD80, CD86 and CD40, and CD83 are upregulated, the latter functioning as a maturation marker for DCs\(^2\). In the lymph nodes DCs interact with high numbers of T cells. Two signals are essential for T cell activation: the interaction between the MHC-peptide complex and the T cell receptor (‘signal 1’), and a co-stimulatory signal from the DC to the T cell (‘signal 2’). A third signal determines the differentiation pathway of the T cell. This ‘signal 3’ is induced by several environmental factors that may skew the differentiation of CD4+ T cells into Th1, Th2 and possibly regulatory T cells\(^5\). The activated T cells subsequently leave the lymph nodes and circulate through the body in search of antigen.
Chapter 1

FACTORS THAT DETERMINE DENDRITIC CELL VACCINE EFFICACY

DC Subsets
Since DCs constitute only about 0.2% percent of peripheral blood leukocytes, several ways to generate DCs from precursors have been investigated. Immature DCs cultured from monocytes by adding GM-CSF and IL-4 to the culture medium allow the generation of large quantities of clinical grade DCs. Further maturation may be achieved by adding cytokines such as TNFα, prostaglandin E2, IL-1β, IL-6 or monocyte conditioned medium. Most clinical studies to date have used this method to generate DCs (figure 1b). The main disadvantage of culturing DCs from monocytes lies in the laborious and costly nature of the culture protocol. DCs derived from CD34+ precursors in the blood are also used in vaccination protocols. They consist of two distinct populations, one with Langerhans cell-like properties and the other termed interstitial/dermal DCs with properties resembling monocyte-derived DCs.

Also, blood DCs can be obtained by several enrichment steps after leukapheresis. However this usually requires repeated leukaphereses due to the low numbers of blood DCs.

Lastly, DCs may be induced in vivo by the administration of DC growth factors. For this purpose Flt3L has been investigated in patients with resectable metastases of colorectal cancer. Increased numbers of DCs were observed in both resected tumor specimens as well as in peripheral blood. These findings demonstrate that in vivo expansion of the blood DC pool in cancer patients is feasible and may be an alternative to the laborious ex vivo culturing procedures.

Antigen loading of DCs
In order to induce an immune response in cancer patients, DCs should present the relevant tumor antigens. For this purpose most clinical studies so far have used DCs pulsed with autologous tumor lysate or MHC class I peptides. Tumor lysate has the advantage that the antigen does not have to be identified, but has the disadvantages that sufficient tumor material is needed for preparation of the lysate and that it is impossible to monitor immune responses as the antigens are not known.

Tumor antigen-derived peptides have the advantage that many peptides are commercially available. However, the half-life of MHC-peptide complexes is relatively short and the immune response, if any, is restricted to the epitope(s) used. It has been described however that antigen-spreading may occur: killing of tumor cells after vaccination against a single epitope results in release of other tumor antigens. These antigens are subsequently taken up by DCs and presented to T cells, resulting in T cell responses against antigens which were not included in the vaccine.

MHC class II peptides also allow the activation of CD4+ T helper cells. A more recent method of antigen-loading concerns the use of transfection of DCs with either whole tumor RNA or RNA encoding a specific cancer antigen. Also fusion of tumor cells with DCs has shown to be feasible, resulting in effective antigen-presentation.

It remains to be established which type of antigens (i.e. for example tumor differentiation antigens, cancer-germline genes, broadly expressed tumor antigens such as TERT or unique tumor antigens) are most suited in terms of the induction of anti-tumor immune responses.
Maturation
In the majority of clinical studies immature or semi-mature DCs have been used. Maturation can be achieved by coculturing the DCs with several stimuli such as cytokines, pathogen associated triggers or endogenous ‘danger signals’ such as heat shock proteins. It is as yet not known whether full-blown DC activation enhances the immunogenicity of a DC vaccine. Fully activated DCs may on the one hand be superior Th1 inducers, but on the other hand may quickly loose their capacity to produce IL-12, which could be a potential draw-back in a vaccination setting. The optimal mode of DC maturation has therefore not been established. In this respect it should be noted that currently used markers to identify mature DCs do not cover all functional properties.

Route of administration
DCs interact with T cells in the peripheral lymphoid organs. Therefore the migration of antigen loaded DCs to those areas is essential. Mice models have shown that after intravenous injection most DCs end up in highly vascularized organs, such as the spleen and the lungs. DCs that are injected subcutaneously or intradermally remain for the greater part at the site of injection, although intradermal injection leads to a higher DC yield in draining lymph nodes in mice. We found that intranodal injection results in a much higher accumulation of DCs in lymph nodes compared to intradermal vaccination. This concerned not only the injected node, but also in subsequent draining nodes. A study in melanoma patients found a small increase in peptide-specific T cell

Figure 1. The induction of antigen-specific T cells in vivo. (a) Antigen is taken up in peripheral tissues by immature DCs. In the presence of a danger signal the DCs mature and migrate to draining lymph nodes, where they present the antigen in combination with a co-stimulatory signal, thereby activating T cells. The activated antigen-specific T cells proliferate and migrate out of the lymph node towards the site of the antigen. (b) Current strategies in DC vaccination. DCs cultured directly from blood, from monocytes or CD34+ progenitor cells can be loaded with antigen ex vivo and administered to cancer patients after a culture in the presence of maturation stimuli such as pro-inflammatory cytokines.
responses after intranodal injection as compared to intradermal or intravenous injection\textsuperscript{27}. Others compared vaccination with blood DCs administered via three different routes in advanced prostate cancer patients: intradermal, intravenous and intralymphatic injection\textsuperscript{28}. T cell responses occurred regardless of the route of delivery. There was however a difference in T and B cell responses, with an absent IFN\gamma-secretion in the intravenous group, and less antibody production in the intralymphatic and intradermal groups. This suggests that the type of immune response may depend on the route of DC administration. The same authors showed in mice that intravenous injection of DCs is essential for immune responses against lung melanoma metastases, whereas subcutaneous vaccination is essential for the response against non-visceral metastases\textsuperscript{29}. These observations may relate to the interactions between DCs and different types of T cells in lymph nodes and spleen that are differentially involved in controlling tumors at different sites, and/or to different homing patterns of the activated T cells. These results provide a rationale to combine different routes of DC administration.

**DC VACCINATION TRIALS IN CANCER PATIENTS**

**Melanoma**

Melanoma is the most studied cancer type in DC immunotherapy. This is mainly due to the fact that in melanoma a large variety of tumor-associated antigens have been characterized, consisting of tumor differentiation antigens such as gp100 and tyrosinase and tumor-specific antigens such as MAGE-3\textsuperscript{30}.

In the first reported study in advanced melanoma patients monocyte-derived DCs loaded with peptides or tumor lysate were injected intranodally\textsuperscript{31}. Out of 16 patients 2 complete remissions and two partial remissions were observed. The results of further trials with DCs cultured in the presence of maturation stimuli are summarized in table 1.

Several studies have used mature monocyte-derived peptide-pulsed DCs injected via different routes in advanced stage IV melanoma patients, using different culture protocols\textsuperscript{15,20,27,32,33} (table 1). In all these studies antigen-specific T cells were detected in peripheral blood or in biopsies of delayed type hypersensitivity (DTH) reactions, and clinical responses were observed. A correlation between clinical outcome and antigen-specific T cell response was found in a minority of these studies. This may relate to the fact that most studies monitor T cell responses in peripheral blood. We have focused on the detection of T cells in DTH biopsies and found a correlation between the presence of antigen-specific DTH-infiltrating T cells and clinical response\textsuperscript{35}.

An interesting observation was made in advanced melanoma patients vaccinated with monocyte-derived DCs that were pulsed with a single melanoma peptide: the only clinically responding patient showed evidence of spreading of T cell reactivity against other antigens as well, indicating that determinant spreading is of importance for the induction of clinical responses\textsuperscript{13}.

An alternative to monocyte-derived DCs are CD34+ progenitor-derived DCs, consisting of CD1a+ CD14- Langerhans cells and CD1a\pm CD14+ interstitial DCs\textsuperscript{10,34}. Banchereau and colleagues vaccinated stage IV melanoma patients with multiple MHC class I peptides loaded on CD34+ progenitor-derived DCs\textsuperscript{10}. Clinical responses were observed, which were sig-
significantly correlated with T cell responses against the used melanoma antigens (summarized in table 1).

**Box 1- Minimum quality criteria for designing clinical trials of DC vaccines**

**Description of vaccine preparation according to GMP guidelines**

**Quality control for ex vivo-generated DCs (see Box 2)**

**Description of patient characteristics**
- Clinical stage at time of inclusion
- Prior treatment
- Documented progressive disease

**Description of trial design**
- Start/end dates of trial
- Patient selection
- Route of administration
- Number of DCs
- Vaccination schedule
- Sampling time points

**Clear documentation and definition of clinical response,** for example by World Health Organization or RECIST criteria (to provide insight into the biological activity of a DC vaccine, it can be appropriate to deviate from these criteria, provided that the new criteria are predefined and clearly described)

**Description of clinical outcome of all patients** (to evaluate the outcome of a study, patients must be followed for a sufficiently long period of time)

**Description of immunological measures before and after vaccination**
- Presence of antigen-specific T cells
- Antibody titers (if relevant)
- Antigen expression on tumor cells

**Proposed assays**
- Antigen-specific CD4+ and CD8+ T-cell counts in peripheral blood
- Delayed-type hypersensitivity biopsies
- Phenotypic analysis (including tetramer analysis)
- Functional analysis (including ELISpot, cytokine production or cytotoxicity)
- Analysis of T-cell receptor repertoire
- Measurement of antigen-specific antibody titers in serum
- Measurement of antigen loss variants (if tumor is available)
- Immunohistochemistry

**Hematologic malignancies**

Malignancies of B cell origin express a monoclonal immunoglobin, carrying unique tumor-specific antigenic determinants in the variable regions, called idio-type. These idiotypes can be isolated from B cell malignancies and subsequently be used as an antigen for the induction of CD4+ and CD8+ T cells. In contrast to the antigens used in solid tumors, this antigen is thus not only tumor-specific but also patient-specific.

The first clinical results using autologous antigen-loaded DCs concerned 4 patients with follicular non-Hodgkin lymphoma. Blood DCs were enriched from peripheral blood mononuclear cells and loaded with idiotype after several enrichment steps. A follow-up of this trial was published in which also patients in first remission after cytoreductive chemotherapy were included. Some patients received DCs that were loaded with idiotype immunoglobulin coupled to keyhole limpet haemocyanin (KLH), a protein providing a-specific CD4+ T cell help. Most patients developed anti-idiotype T cell responses and some objective clinical responses were observed. Interestingly, booster injections of idiotype coupled to KLH induced clinical responses in patients that initially progressed on DC therapy.

Several studies have focussed on the application of idiotype-pulsed monocyte-derived DCs in multiple myeloma patients. Clinical and immunological responses were infrequent, which may relate to the use of immature DCs in most studies. An interesting observation was made in two patients with cutaneous T cell lymphoma: after disease progression upon discontinuation of vaccination, a second clinical remission was achieved after revaccination.

**Gastrointestinal malignancies**

In colorectal cancer the most widely used antigen for loading on DCs is carcinoembryonic antigen (CEA).
Early clinical trials clearly demonstrate the potency of DC therapy, but there are still too many variables preventing its introduction as a standard cancer treatment. There is a strong need for standardization and quality control of this technique. Additional research driven (pre)clinical studies must be carried to provide to evaluate the most effective DC subtypes, the optimal conditioning and activation stimuli, the optimal antigen-loading strategy and route of administration and the optimal dose and frequency of DC vaccinations.

Patients with CEA-expressing malignancies have been treated with Flt3L expanded, in vitro-enriched blood DCs, pulsed with a modified CEA-peptide. Antigen-specific immune responses and objective clinical responses were observed. Interestingly, a correlation between clinical response and the magnitude of the expansion of tetramer positive cytotoxic T cells in peripheral blood was found.

Monocyte-derived DCs transfected with RNA encoding tumor antigens is an alternative method of antigen loading in DC-based immunotherapy. Specific T cell reactivity was demonstrated in a patient with advanced CEA-expressing cancer of unknown origin upon vaccination with monocyte-derived TNFα-maturated DCs transfected with CEA-RNA. In a study using immature DCs pulsed with MAGE-3, an antigen which is expressed in a variety of gastro-intestinal tumors, antigen-specific T cell reactivity in peripheral blood and minor tumor regressions were observed.

**Urological malignancies**

In renal cell carcinoma studies with immature tumor-lysate pulsed DCs have not resulted in either immunological or clinical responses. One study used TNFα/PGE2 maturated monocyte-derived DCs that were either loaded with autologous tumor lysate or tumor lysate from an allogeneic tumor cell line. Clinical responses were restricted to patients that were vaccinated with autologous lysate-pulsed DCs. Patients with metastatic prostate cancer have been vaccinated with recombinant prostatic acid phosphatase-pulsed mature blood DCs resulting in proliferative T cell responses against the protein, however no clinical responses occurred.
The immunogenicity of DCs transfected with whole tumor RNA or RNA encoding prostate-specific antigen has been investigated in patients with metastatic renal cancer and prostate cancer. Due to the PSA-transfection the DCs underwent phenotypical changes with upregulation of CD83 and CD86. In most patients antigen-specific IFNγ-producing T cell responses were detected, which support the immunogenicity of this mode of antigen loading.

Other malignancies
MUC-1 and HER-2/neu peptide-pulsed monocyte-derived DCs have been used in breast and ovarian cancer patients resulting in peptide-specific cytotoxic T cell responses in peripheral blood and some long-term clinical responses. Epstein Barr Virus peptide-pulsed monocyte-derived DCs in nasopharyngeal carcinoma have resulted in antigen-specific T cell responses in peripheral blood up to 3 months after vaccination, and partial responses were noted.

Other tumor types that have been investigated using DC vaccines include hepatocellular carcinoma, pancreatic tumors, adrenal carcinoma, cholangiocarcinoma, parathyroid carcinoma, non-small cell lung cancer, head and neck cancer, sarcoma, bladder cancer, glioma and pediatric malignancies. In all these studies DCs were cultured without maturating stimuli. Although some specific T cell responses and clinical responses were seen in these studies, results may be improved using mature DCs.

BOX 2- Quality criteria for DC vaccines

There is a strong need for standardization and quality control of DC vaccines. The release criteria define the proper description of the vaccine preparation. Criteria describing the DC vaccine, including quality control, should be described in publications regarding DC vaccination trials. DCs should be grown without fetal calf serum, preferably in serum-free media. If frozen DCs are used, quality control should be carried out after samples are thawed. The optional criteria are important in validating the antigen-presenting capacity of the cultured DCs. These tests are not required for every vaccine preparation.

<table>
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<th>Release criteria:</th>
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<tr>
<td><strong>Microbiological controls</strong></td>
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<tr>
<td>Negative for bacterial and fungal contamination</td>
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<tr>
<td><strong>Purity</strong></td>
</tr>
<tr>
<td>&gt;80% as determined by flow cytometry, light scatter or staining with non-DC lineage markers.</td>
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<tr>
<td><strong>Morphology</strong></td>
</tr>
<tr>
<td>Immature: loosely adherent, floating, roundish cells with some extensions</td>
</tr>
<tr>
<td>Mature: loosely attached, veiled and clustered cells</td>
</tr>
<tr>
<td><strong>Phenotype</strong></td>
</tr>
<tr>
<td>Monocyte-derived:</td>
</tr>
<tr>
<td>Immature: CD14+/low, CD83−, CD80−/low, CD86−/low, MHC class I+, MHC class II+, CCR5+</td>
</tr>
<tr>
<td>Mature: CD83+, CD80+, CD86+, MHC class I+, MHC class II+, CCR7+</td>
</tr>
<tr>
<td>CD34+ cell-derived:</td>
</tr>
<tr>
<td>Interstitial: CD14+, CD1a+, CD83+, CD80+, CD86+, MHC class I−, MHC class II−</td>
</tr>
<tr>
<td>Langerhans cells: CD14+, CD1a+, CD83+, CD80+, CD86+, MHC class I−, MHC class II−</td>
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<tr>
<td><strong>Viability</strong></td>
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<td>&gt;70% as determined by Trypan blue exclusion</td>
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<table>
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<th>Optional validation criteria:</th>
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<tr>
<td><strong>Stability of DC phenotype</strong></td>
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<tr>
<td>Determined after one and two days of subsequent culture in medium either without or with cytokines</td>
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<tr>
<td>Washout test: DCs must remain viable and maintain their characteristic morphology and phenotype over two days in medium without cytokines (characteristic of fully mature and stable DCs)</td>
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<td><strong>Induction of immune response:</strong></td>
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<tr>
<td>Mixed lymphocyte reaction: T-cell proliferation at DC/PBMC (peripheral blood mononuclear cell) ratio of 1:20 in at least one donor</td>
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<tr>
<td>Recognition of loaded antigen by T cells, as determined by cytotoxicity assay or cytokine production (especially important when antigen is loaded before freezing)</td>
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<tr>
<td>Antigen-loaded state</td>
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<tr>
<td>(Only possible when DCs are loaded with well-defined antigens, such as peptides, proteins or RNA)</td>
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<tr>
<td>Antigen-specific stimulation assay: tests ability of antigen-loaded DCs to stimulate antigen-specific T cells (from T-cell lines or reporter cell lines transfected with T-cell receptor and reporter constructs)</td>
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<tr>
<td>Cancer Type</td>
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<tr>
<td>Melanoma</td>
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<td>NHL after CT</td>
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<td>Cutaneous T Cell Lymphoma</td>
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<td>Prostate cancer</td>
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<tr>
<td>RCC</td>
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<tr>
<td>Breast and Ovarian cancer</td>
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<td>Nasopharyngeal carcinoma</td>
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Table 1. Results of dendritic cell-based vaccines. Included are studies in which DCs were used that were cultured in the presence of maturing stimuli. Number of patients: the number of patients that have received at least one vaccination. T cell responses: number of patients in which peptide/tumor specific T cell responses were detected. Abbreviations: NHL, Non-Hodgkin lymphoma; CT, chemotherapy; MM, multiple myeloma; SCT stem cell transplantation; RCC, renal cell carcinoma; moDCs, monocyte-derived DCs; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; TNFα, tumor necrosis factor alpha; PGE2, prostaglandin E2; MCM, monocyte-conditioned medium; CD34+, CD34+ progenitor derived DCs; Id, idiotype; i.n., intranodal; s.c., subcutaneous; i.d. intradermal; i.v. intravenous; i.l., intralymphatic; n.d. not documented; CR, complete response; PR partial response; SD, stable disease; molR, molecular response; PF, progression-free; RD, residual disease; MxR mixed response.
RECENT DEVELOPMENTS

New developments in DC therapy soon to be tested in clinical studies include the targeting of DCs in vivo via DC-specific molecules which may circumvent the problem of laborious ex vivo culturing and antigen-loading protocols\textsuperscript{59}. Furthermore, the depletion of CD4+/CD25+ regulatory T cells which have the ability to suppress T cell mediated immunity may be a way of enhancing the potency of the DC vaccine-induced immune responses\textsuperscript{60}. Lastly, plasmacytoid DCs may be an attractive DC-subtype in vaccination of cancer patients as they have the capacity to infiltrate tumors, induce primary T cell responses and secrete large amounts of type I interferons\textsuperscript{61}.

CONCLUSIONS

Generation of large quantities of DCs for clinical use is feasible, and this applies to monocyte-derived DCs, CD34 progenitor-derived DCs and blood DCs. Vaccination with DCs appears to be safe, and immunological responses as well as durable clinical remissions have been observed. More importantly, in several trials a correlation between immunological and clinical outcome has been demonstrated. These studies have greatly enhanced our knowledge of the induction of anti-tumor immunity in cancer patients. For reasons of comparison details on culture methods as well as phenotypical data of the administered DCs should be provided in scientific publications (Box 1)\textsuperscript{19}. Release criteria of the vaccine should at least contain purity, viability and mature phenotype (Box 2). Current questions regarding the optimal DC subset, culture method, mode of maturation, route of administration, antigen loading and role of adjuvants should be addressed in small-scale studies with extensive immunomonitoring as primary parameter for efficacy (figure 2). Given these questions, as well as the theoretical options to further improve DC vaccines it is too early to put DC vaccines to the test of randomized comparison in phase III trials.

REFERENCES


Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients


Clinical Cancer Research 2003 Nov 1;9(14):5091-100
Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients

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We have investigated the capacity of immature and mature monocyte-derived dendritic cells (DCs) pulsed with melanoma-associated peptides (gp100 and tyrosinase) to induce a primary cytotoxic T lymphocyte response in vivo.

Advanced HLA-A2.1+ melanoma patients were vaccinated with peptide- and keyhole limpet hemocyanin (KLH) -pulsed DCs, either immature (9 patients) or matured by monocyte-conditioned medium/TNF-alpha/PGE₂ (10 patients). All patients vaccinated with mature DCs showed a pronounced proliferative T cell and humoral response against KLH. By contrast, KLH responses were absent in most of the patients vaccinated with immature DCs. Delayed type hypersensitivity (DTH) reactions against antigen-pulsed DCs were only observed in patients vaccinated with mature DCs and not in patients vaccinated with immature DCs. MHC-peptide tetramer staining of DTH-derived T cells revealed the presence of specific T cells recognizing the melanoma-associated peptides in one patient. In a second patient, DTH-derived T cells showed specific lysis of tumor cells expressing the antigens used for DC pulsing. Only patients vaccinated with mature DCs showed objective clinical responses. Interestingly, both patients with long-term progression-free survival (22 and > 40 months), were both vaccinated with mature DCs and demonstrated antigen-specific T cell reactivity of DTH-derived T cells. We conclude that mature DCs are superior to immature DCs in the induction of immunological responses in melanoma patients, which may translate into improved clinical results.

INTRODUCTION

Dendritic cells (DCs) constitute a family of antigen-presenting cells defined by their morphology and their unique capacity to initiate a primary immune response¹. It has been shown that DCs pulsed with MHC class I peptides are potent inducers of a cytotoxic T lymphocyte (CTL) response in vitro². Furthermore, the presence of DCs in tumor tissue has been correlated with a favorable clinical prognosis³⁴. Therefore DCs are thought to play a pivotal role in the induction of T cell-mediated antitumor responses in vivo⁵.⁶ The use of peptide-pulsed DCs in antitumor vaccination trials has been facilitated by the availability of class I-restricted peptides derived from tumor-associated
antigens such as gp100, tyrosinase, MAGE, and NY-ESO-1, the ability to grow large numbers of DCs from monocytes, and the finding that antigen-pulsed DCs are able to induce specific CTL reactivity in vitro\(^2\),\(^7\),\(^8\). Recent studies have demonstrated the safety and the ability of antigen-loaded DCs to induce an immune response in humans\(^9\),\(^10\),\(^11\),\(^12\). Evidence is accumulating that the type of DCs and the route of administration play a critical role in determining the quality and quantity of the immune response\(^13\),\(^14\). We recently showed that migration of DCs is highly dependent on their maturation status\(^15\). To further optimize clinical efficacy, DC vaccination strategies need more investigation. Immature DCs are characterized by the presence of MHC class I and class II molecules and low expression of costimulatory molecules CD80, CD86 and CD40. Upon inflammation or infection, immature DCs take up antigen, followed by a complex maturation and activation process that is characterized by an up-regulation of antigen-presenting MHC molecules and costimulatory molecules, as well as a switch in their adhesion- and chemokine-receptor repertoire\(^16\),\(^17\).

In this study we compare the efficacy of immature and mature DCs in inducing an immune response in advanced stage IV melanoma patients. Melanoma is well suited to explore vaccination strategies because it is one of the more immunogenic tumors in which melanoma-associated antigens, like gp100 and tyrosinase, and specific T cell responses towards these antigens have been identified\(^18\),\(^19\). DCs used for vaccination were pulsed with HLA-A2.1-binding peptides derived from gp100 and tyrosinase, and keyhole limpet hemocyanin (KLH)\(^9\). The latter was used to provide T cell help and to verify the immunogenicity of the two DC populations as well as competence of the patients’ immune system to mount an immune response.

The results of this study implicate that maturation of DCs is a crucial step for the induction of T- and B cell responses in melanoma patients and a correlation with a favorable clinical outcome is suggested.

**MATERIALS AND METHODS**

**Patient criteria**

Inclusion criteria were: histologic evidence of metastatic melanoma, progressive disease, measurable disease parameters, focal or diffuse expression of gp100 and/or tyrosinase in at least one metastasis as determined by immunohistochemistry, HLA-A2.1 phenotype, WHO performance status 0 or 1, and written informed consent. Patients were staged according to the 2001 AJCC staging system: patients with distant non-visceral metastases were categorized as stage M1a, patients with metastasis to the lung were categorized as stage M1b, and patients with metastases to any other visceral site or with an elevated serum LDH were categorized as stage M1c\(^20\). Patients with clinical signs of brain metastases, serious concomitant disease or a history of second malignancy were excluded. Prior treatment was allowed, provided a treatment-free period of at least four weeks was observed and all related toxicity had resolved. Approval from the local regulatory committee was obtained.

**Clinical protocol and immunization schedule**

In eligible patients a leukapheresis was performed from which DCs were generated. The protocol consisted of two parts. In the first part, antigen-pulsed DCs were administered 3 times at biweekly intervals, either intravenously (i.v.) and subcutaneously (s.c.) or i.v. and intradermally (i.d.). In the second part, patients without tumor progression received 3 monthly vaccinations with peptides alone (100 g) and KLH (2 g), either s.c. or i.d. as in the first part of the study. Prior to each vaccination 80 ml of blood was collected for immunological monitoring.

A clinical response was defined as stable disease for more than 4 months or any partial or complete response. Stable disease and partial response were defined according to WHO criteria\(^21\). Toxicity was assessed according to NCI common toxicity criteria. Progression free survival was calcu-
lated from the day of the first vaccination. Patients were evaluated for response after completing the first and second part of the protocol and every three months thereafter. Patients who remained free of disease progression were eligible for 2 maintenance cycles at six months interval, each consisting of 3 bi-weekly intranodal vaccinations in a clinically tumor-free, usually inguinal lymph node region under ultrasound guidance with mature DCs, pulsed with modified gp100-, and tyrosinase peptides and KLH.22,23. Intranodal administration was chosen for maintenance treatment because in a related study we observed a more pronounced migration of DCs to distant lymph nodes after intranodal injection in comparison with intradermal injection.22

Antibodies and immunostaining
To characterize and compare the phenotype of the DC populations, flow cytometry was performed using either FITC-conjugated or PE-conjugated mAbs. The following FITC-conjugated mAbs were used: anti-HLA class I (W6/32), and anti-HLA DR/DP (Q5/13); and PE-conjugated mAbs: anti-CD80 (Becton Dickinson, Mountain View, CA), anti-CD14, anti-CD83 (both Beckman Coulter, Mijdrecht, The Netherlands), and anti-CD86 (Pharmingen, San Diego, CA). For immunohistochemistry the following mAb were used: HMB-45 (Dako, Glostrup, Denmark) against gp100 and T311 (Novocastra, Newcastle, UK) against tyrosinase24.

DCs: Preparation, characterization, and route of administration
DCs were generated as described previously.25 Briefly, peripheral blood mononuclear cells (PBMC) were isolated by Percoll (1.073) density gradient centrifugation (Pharmacia, 30 min, 4°C, 2100 rpm) after leukapheresis. PBMC were washed and 1x10^10 cells were used for the generation of monocyte-conditioned medium (MCM).26 From the remaining PBMC, monocytes were isolated by either counterflow centrifugation (the first 3 patients) or by adherence to plastic (all other patients). Monocytes isolated by elutriation were cultured at 15x10^6 per 75 cm^2 tissue culture flask (Costar, Badhoevedorp, The Netherlands) in 20 ml of interleukin (IL)-4 (500 U/ml Schering-Plough International, Kenilworth, USA), granulocyte monocyte-colony stimulating factor (GM-CSF) 800 U/ml, Schering-Plough International, Kenilworth, USA) and 2% human serum (HS) bloodbank Rivierenland, Nijmegen, The Netherlands)-containing X-VIVO 15 medium (BioWhittaker, Walkersville, MD). The cells were harvested on day 9. In case adherence to plastic was used for isolation of the monocytes, immature DCs were cultured from adherent PBMC in 10 ml of X-VIVO 15 medium of which half was replaced on day 1 with X-VIVO 15 medium supplemented with IL-4 and GM-CSF and cells were harvested on day six.25 The first 4 patients were vaccinated i.v. and s.c. with these immature DCs, but analysis of the s.c. injection site revealed that most DCs did not migrate from the injection site (data not shown). Based on this finding and our data from murine experiments, we switched from s.c. to i.d. together with i.v. vaccinations. The injected immature DCs were characterized by the presence of MHC class I and class II, a moderate expression level of CD86 and a low expression of CD14 and CD83 (figure 1). Mature DCs were cultured as immature DCs from adherent PBMC with addition of autologous MCM enriched with 10 g/ml PGE2 (Pharmacia & Upjohn, Puurs, Belgium) and 10 ng/ml tumor necrosis factor (TNF)-α (kindly provided by Dr. Adolf, Bender, Vienna, Austria), on day 7 (30%, v/v).29 This procedure gave rise to mature DCs on day 9 as demonstrated by high expression levels of MHC class I and II, CD80, CD83 and CD86 and absence of CD14 (figure 1). Both immature as well mature DC preparations were endotoxin free.

Cryopreservation of PBMC and DCs
All DCs were cultured directly after leukapheresis and frozen as immature or mature DCs for multiple vaccinations. DCs and PBMC were frozen using a cryo 1°C freezing container (Nalgene, Rochester, NY, USA) which was put in -80°C for 24 hours, in freezing medium consisting of 50% X-VIVO-15 (5% HS), 40% human serum albumin and 10% DMSO (final concentration, Sigma). Cells were frozen in 1 ml per vial containing a maximum of 40x10^6 cells. Cells were thawed in a 37°C water bath after which the cells were washed once in cold medium and once in medium of room temperature before further use. We previously showed that the recovery of viable immature DCs (62±15%) did not differ from that of mature DCs (65±10%)25.

Peptide pulsing and KLH loading
DCs were pulsed with wild type peptides, gp100:154-167, gp100:280-288 or modified pep-
tides (gp100:154-167 QA, gp100: 280-288 AV) and wild type tyrosinase 369-376 and other peptides were added directly after harvesting or after thawing. On the day of vaccination, we added peptides (50 μg/ml) for 90 min and kept DCs at 37°C /5% CO2. Thereafter, fresh peptides (25 μg/ml) were added and DCs were kept at room temperature for 60 min. After peptide loading, DCs were washed once in 0.9% sodium chloride and resuspended in 0.2 ml or 1 ml for i.d/s.c. or i.v. injections, respectively. At day 3 of the cell culture 10 μg/ml KLH (Calbiochem, USA) was added to both immature and mature DCs.

Delayed Type Hypersensitivity
One to two weeks after the three DC vaccinations a delayed type hypersensitivity (DTH) skin test was performed. Briefly, unpulsed DCs, DCs pulsed with peptides, DCs pulsed with KLH, and DCs pulsed with peptides plus KLH (2x10⁵ DCs each) were injected i.d. in the skin of the back of the patients at four different sites. The diameter (in millimeters) of induration was measured after 48 hours, each time by the same investigator (M.J.P.G.), and an induration of more than 2 mm was considered positive. From positive DTH sites, punch biopsies (6 mm) were obtained under local anesthesia. Biopsies were cut in half, one part for immunohistochemistry and the other part was cut in small pieces and cultured in RPMI/7%HS supplemented with IL-2 (100 U/ml). Every 7 days, half of the medium was replaced by fresh IL-2-containing RPMI/7%HS. After 2 to 4 weeks of culturing, T cells were tested for antigen recognition in a cytotoxicity assay or tested for tetramer binding.

Humoral response to KLH
Antibodies against KLH were measured in the serum of vaccinated patients by enzyme-linked immunosorbent assays (ELISA) as described by Holst et al. Briefly, 96-well plates were coated overnight at 4°C with the protein KLH (25 μg/ml) in phosphate buffered saline (0.1 ml/well). After washing the plates, different concentrations of patient serum (range 1 in 100 to 1 in 500,000) were added for 1 hour at room temperature. After extensive washing, specific Ab (total IgG, IgG1, IgG2, and IgG4) labeled with horseradish peroxidase were allowed to bind for 1 hour at room temperature. Peroxidase activity was revealed using 3,3’,5,5’-tetramethyl-benzidine as substrate and measured in a microtiter plate reader at 450 nm. A positive signal at a 1 in 400 dilution of the patients’ serum was considered positive.

Proliferative response and cytokine production to KLH
Cellular responses against KLH were measured in a proliferation assay. Briefly, 1x10⁵ PBMC, isolated from blood samples taken before each vaccination, were plated per well of a 96-well tissue culture micro plate either in the presence of KLH or without. After 16 hours of culture, supernatants (50 μl) were taken and IL-2, IL-4, IL-5, IL-10, TNF-α, and interferon (IFN)-γ were measured by a cytometric bead array (Th1/Th2 Cytokine CBA 1; BD Pharmingen, San Diego, CA) according to the manufacturer’s instructions. After 4 days of culture, 1 μCi/well of tritiated thymidine was added, incorporation of tritiated thymidine was measured in a beta-counter. A proliferation index >2 was considered positive.

MHC tetramer staining
Tetrameric-MHC complexes were kindly provided by dr. R Luiten and dr. H. Spits from the Netherlands Cancer Institute (Amsterdam, The Netherlands). Each tetramer was validated by staining against a CTL line specific for HLA-A2 in association with the peptide of interest. PBMC (1x10⁵ cells in 10 μl) were incubated with PE-labeled tetrameric-MHC complexes for 1 hour at RT. After washing the samples were analyzed by flow cytometry.

Cytotoxicity assay
Cytotoxic activity of DTH-infiltrated lymphocytes was measured using the chromium release assay as described previously. Briefly, target cells HLA-A2.1 positive BLM either transfected with control antigen G250, or with the antigens of study gp100 or tyrosinase were incubated with 100 Ci Na[51Cr]O⁴ (Amersham, Bucks, UK) for 45 min at 37°C. After extensive washing, chromium-labeled BLM (103) were mixed with unlabeled K562 cells (ratio 1:10) and added to lymphocytes (10⁵ cells) in triplicate wells of a round bottom microtiter plate (total volume 150 μl). After a 4-hour incubation, 100 μl of the supernatant was harvested and its radioactivity content was measured. The specific percentage of cytotoxicity was defined by: [(experimental release - spontaneous release) + (maximum release – spontaneous release)] x 100%.

IFN-γ ELISPOT assay
Production of IFN-γ in response to the peptides used for DC pulsing was determined by the ELISPOT assay as described previously. In short, CD8+ T cells (10⁶) and T2 cells (7.5 x 10⁴/well)
with the peptides (final concentration 50 μg/ml) were incubated for 20 h at 37°C on multiscreen HA plates (Millipore, Bedford, MA) which were coated with anti-human IFN-γ. Captured cytokine was detected by biotinylated mAb anti-human IFN-γ, avidin-peroxidase complex and peroxidase staining. Spot numbers were automatically determined with the use of computer-assisted video image analysis.

RESULTS

Patients Characteristics
In this study, a total of 19 stage IV melanoma patients were vaccinated, 9 with immature DCs and 10 with mature DCs. Patient characteristics are shown in table 1. Both groups were comparable considering age, sex and previous therapy. A higher number of patients vaccinated with mature DCs had a performance status 0, but in this group there were also more patients with a more advanced stage of disease (table 1).

Toxicity and side effects
No severe toxicity (common toxicity criteria grade III-IV) occurred. In patients vaccinated with immature DCs, mild fatigue, anorexia and nausea but no fever was observed. However, fever (38 °C) developed in 2 out of 10 patients vaccinated with mature DCs after the second vaccination, and in 7 out of 10 after the third vaccination. Furthermore, erythema was observed at the injection site of the second or third vaccination only after vaccination with mature DCs.

Proliferation and cytokine production of PBMC upon stimulation with KLH
To determine the ability of immature versus mature DCs to mount an immune response against KLH, PBMC collected and frozen after each DC vaccination were thawed and analyzed for the presence of KLH-reactive T cells in a proliferation assay. Patients receiving mature DCs (n=10) showed a strong proliferative response against KLH that could already be detected after a single DC vaccination (figure 2).

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Table 1. Patient characteristics. PS: performance status (WHO); RT: radiotherapy; HT: hyperthermia; MT: metastasectomy (in all patients of non-visceral metastases); IFN: interferon-α; DTIC: dacarbazine; RLND: regional lymph node dissection.

The observation that this response was not further augmented after the second and third DC vaccination or after administration of KLH protein i.d. suggests that a single DC vaccination was sufficient to obtain maximal proliferative responses against KLH.
This may also explain why we did not observe a clear correlation between the increase in proliferation and the number of injected DCs (table 2). By contrast, we observed no significant increase in KLH-specific proliferation of PBMC derived from patients vaccinated once with immature DCs (n=9) (figure 2). Proliferative responses measured after subsequent immature DC vaccinations, either i.v./s.c. or i.v./i.d. remained much less pronounced when compared to PBMC from patients vaccinated with equal numbers of mature DCs (table 2).

To determine the type of the induced immune response, PBMC obtained after 3 vaccinations with either immature or mature DCs were stimulated with KLH for 16 hours and cytokine production was measured. No cytokine production was observed by PBMC after 3 vaccinations with immature DCs (figure 2). However, PBMC from patients vaccinated with mature DCs produced high amounts of IL-2 (60 pg/ml), TNF-α (212 pg/ml), and IFN-γ (398 pg/ml) but no IL-4 in response to KLH (figure 2). From these results we conclude that mature DCs induce a T helper 1 (Th1) response.
Figure 2. KLH-specific proliferation of PBMC before and after DC vaccination. Patients were vaccinated either with immature DCs (a) or mature DCs (b). One representative patient for each DC subset is shown (closed bars PBMC without KLH; open bars PBMC with KLH). Results of the proliferation to KLH of all patients are shown in table 2. Cytokine production by PBMC of patients vaccinated 3 times either with mature (Mat-7; c, d) or immature DCs (Imm-6; e, f): unstimulated PBMC (c, e) and PBMC stimulated with KLH (d, f).
Chapter 2

Figure 3. Humoral responses against KLH. Representative humoral responses against KLH in the serum of patients vaccinated either with immature DCs (Imm-6) or mature DCs (Mat-3). Serum was obtained before (---), after each subsequent DC vaccination (----, number indicates number of DC vaccinations) and after vaccination with KLH protein (-----). Total IgG antibodies with specificity for KLH were detected by ELISA in KLH-coated microtiter plates. Isotype-specific secondary antibodies were used to detect IgG1, IgG2, and IgG4 (patient Mat-3). Results of total IgG against KLH of all patients are shown in table 2.

Humoral response against KLH in serum

Humoral responses against KLH (total IgG) were detected in serum of most patients (9 out of 10) vaccinated with mature DCs given i.v. and i.d. (table 2). After a single vaccination with KLH-pulsed DCs, IgG antibodies reactive with KLH were readily detectable by ELISA (figure 3). After subsequent vaccinations with mature DCs, the IgG antibody titers increased and remained elevated after vaccinations with the protein KLH at days 56, 84 and 112 (figure 3). In contrast, in serum of all the patients vaccinated with immature KLH-pulsed DCs, IgG antibodies reactive with KLH remained absent (figure 3, table 2) even after 3 DC vaccinations. To exclude the possibility that these patients were refractory to KLH, 2 patients received a subsequent single vaccination with KLH protein. High titers of IgG against KLH could be obtained demonstrating that their immune system was not affected (figure 3). Sera containing KLH antibodies after vaccination with mature DCs were further analyzed for subtypes of IgG (IgG1, IgG2 and IgG4).

We did not observe anti-KLH antibodies of the IgG4 subclass in any of the patients. In four patients we observed equal amounts of IgG1 and IgG2, whereas in 5 patients the levels of IgG2 were significantly higher compared to the levels of IgG1, indicating the presence of IFN-γ and hence a Th1 response (figure 3). Together, both antibody and proliferative responses against KLH demonstrate the superiority of mature DCs in inducing Th1 responses.
DTH reactions

No DTH skin reactions against immature DCs either unpulsed or pulsed with KLH and/or peptides were observed in patients vaccinated 3 times with immature DCs (table 2). In contrast, in all patients vaccinated (i.v./i.d.) with mature DCs, positive DTH reactions with indurations up to 12 mm were observed against mature DCs pulsed with KLH and/or peptides, and also in 8 out of 9 patients against unpulsed mature DCs (table 2). Moreover, in one patient vaccinated with mature DCs, a DTH with unpulsed immature DCs induced induration as well (data not shown). These data imply that vaccination with mature but not with immature DCs provoke a DTH response.

Detection of tumor peptide-specific T cells in peripheral blood and DTH

To determine the presence of antigen-specific T cells in peripheral blood, ELISPOT measuring IFN-γ-production by CD8+ T cells in response to the vaccinated tumor-derived peptides and tetramer staining were used.

In some of the analyzed samples (in 1 out of 4 tested patients vaccinated with immature DCs, 1 out of 5 patients vaccinated with mature DCs) a small increase in reactivity against the vaccinated peptides was observed after vaccination, either by ELISPOT or by tetramer staining (data not shown). Furthermore, this increase was not consistent after subsequent vaccinations. From these experiments we concluded that in peripheral blood no significant elevation of peptide-reactive T cells could be found.

Short-term T cell cultures (2 to 4 weeks) from biopsies of positive DTH reactions of seven patients vaccinated with mature DCs provided the opportunity to test T cell reactivity of the DTH-infiltrated T cells without restimulation in vitro.

No T cell outgrowth was observed from biopsies of DTH reactions against unpulsed DCs. From 4 out of 7 patients sufficient T cells were available after short time culture (i.e. 2–3 weeks) to perform cytotoxicity assays. T cells derived from a DTH biopsy of patient Mat-3 demonstrated specific lysis of gp100- and tyrosinase-BLM transfectants specifically (figure 4). DTH biopsy-derived T cells from the other 3 patients did not show specific lysis (data not shown). In 5/7 patients tetramer staining against the vaccinated peptides was performed on DTH biopsy-derived T cells. Since cultures from these biopsies consisted predominantly of T cells as determined by flow cytometry (data not shown), we performed single tetramer staining. DTH-infiltrated T cells from one of these 7 patients (Mat-10) stained positively with tetramer gp100:154 (2.5%) and gp100:280 (3%), and negatively with tyrosinase (figure 5).
Clinical outcome and correlation with immune responses

Of the immature DC group, 7 patients completed the DC vaccinations and 2 patients completed both parts of the protocol (Imm-1 and Imm-6). One of the 9 patients vaccinated with immature DCs was not evaluable for clinical response because of deteriorating performance status. All patients had either disease progression or stable disease with < 4 month duration as best response (table 2). The median overall survival was 7 months (range 1.5-19 months).

Of the mature DC group, all patients completed the DC vaccinations and 5 patients completed both parts of the protocol (Mat-1, Mat-3, Mat-4, Mat-9 and Mat-10). Three patients were eligible for maintenance therapy (Mat-3, Mat-4, Mat-10), two of them received two additional DC vaccination cycles with a 6 months interval (Mat-3, Mat-10).

Of the 10 patients vaccinated with mature DCs, one patient was not evaluable for clinical response because of deteriorating performance status, 4 patients had disease progression. Stable disease was observed in 3 patients with a duration of 4.5, 7.5 and 22 months, respectively (table 2). One patient (Mat-9) had a mixed response: 4 cutaneous metastases decreased in size (30%-50%), however one cutaneous metastasis increased with 35%. The median overall survival was 11 months (range 2->40 months).

In one patient (Mat-3) with stage M1c melanoma vaccinated with mature DCs, a partial response was observed in a distant lymph node metastasis with concomitant normalisation of serum LDH. This metastatic site was subsequently resected. Upon pathological examination the resected lesion showed areas of necrosis and an abundant infiltrate of CD8+ T cells in the tumor, which was still gp100 positive, but demonstrated a decreased expression of tyrosinase as compared to a cutaneous melanoma lesion which was resected prior to DC vaccination (data not shown). This patient is now in complete remission for more than three years.

Figure 5. Tetramer analysis by flow cytometry of T cells derived from a biopsy of a positive DTH reaction of patient Mat-10. Depicted is the forward scatter on the x-axis (double staining with CD8 FITC might interfere with the tetramer staining and predominantly T cells grow out of these biopsies) and on the y-axis tetramer PE staining. Tetramer staining 1 log above the negative population was considered positive.

In our study, the clinical outcome did not correlate with the pretreatment expression of gp100 or tyrosinase, neither with the percentage of positive cells, nor with the estimated staining intensity on the melanoma metastases (data not shown). Although the number of patients in this study is limited and does not allow statistical analysis it is intriguing that the best clinical outcome was observed in the two patients in whom tumor-specific T cell responses of DTH-infiltrating lymphocytes was demonstrated. Patient Mat-3 and Mat-10 had a progression free survival of more
than 40 and 22 months, respectively. For comparison, in the patients in whom no specific T cell reactivity could be observed, the median progression free survival was 2 months (range 1.5-7.5).

**DISCUSSION**

Our results in melanoma patients demonstrate a clear preference for the use of peptide-pulsed mature DCs as opposed to immature DCs in clinical vaccination studies. Monocyte-derived DCs matured with a cocktail of pro-inflammatory cytokines and PGE2 in vitro were superior in the induction of both humoral responses and proliferative responses against KLH in vivo. Analysis of KLH-specific T cells showed the production of the Th1 cytokine IFN-γ but not of the Th2 cytokine IL-4, and anti-KLH antibodies were of the IgG2 isotype which is also indicative for a Th1 response.

Despite the previously observed lack of IL-12 production and subsequent Th1 development of PGE2-matured DCs in vitro38, we conclude that vaccination with these fully matured DCs polarize T cells towards IFN-γ production in patients. The absence of T- and B cell responses to KLH after vaccination with KLH-pulsed immature DCs might be partly explained by our recent observation that immature DCs are not able to efficiently migrate to the T cell areas of lymph nodes15. Since lymph nodes are the primary site of interaction between B cells, T cells and DCs, DCs should migrate into the T cell areas of secondary lymph nodes. Migration and subsequent induction of an immune response of the mature DCs might be partly attributed to the use of PGE2 in our maturation cocktail.

Recently, PGE2 was described as a key factor in regulating the migratory capacity of DCs39,40 (and our own unpublished results). In addition to the lack of migration of immature DCs, the inability of these DCs to induce an immune response might also be due to the low production of DC-CK1 (CCL18), a chemokine which preferentially attracts both naïve T and B cells41,42.

We previously demonstrated that DC-CK1 production by immature DCs is significantly lower when compared to mature DCs25,41-43. Our observation that injection of antigen-pulsed immature DCs does not lead to significant immune responses confirms and extends findings of Jonuleit et al. who showed that immature DCs failed to induce peptide-specific cytotoxic T cells44. Furthermore, the inability of immature DCs to give rise to an immune response also indicates that monocyte-derived DCs do not mature after injection in vivo. Thus, for active immunization in cancer patients it appears to be critical that DCs are matured in vitro before injection. Recently it became clear that immature DCs are not simply ignored by the immune system but that antigen-pulsed immature DCs may blunt the capacity of the corresponding antigen-specific T cells to mount lytic activity in vitro45. In addition, it has been shown that repetitive stimulation with immature DCs may lead to tolerance by inducing IL-10-producing regulatory T cells46. In mice DCs can, under steady state conditions, induce T cell tolerance and thereby maintain peripheral tolerance to self-antigens47.
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<th>DTH a</th>
<th>Anti-KLH response</th>
<th>Clinical Response</th>
<th>PFS</th>
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<tr>
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<tr>
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<td>PD</td>
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<tr>
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<tr>
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<td>PD</td>
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<tr>
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<td>PD</td>
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<tr>
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<td>PD</td>
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<td></td>
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<tr>
<td>Imm-9</td>
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<td>1.5</td>
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<tr>
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<td>PD</td>
<td>1.5</td>
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<tr>
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<td>PD</td>
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<tr>
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<td>36/12 15/8 24/12 + +++ + SD</td>
<td>PD</td>
<td>22 e</td>
<td></td>
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Table 2. Immune and clinical responses after vaccination with immature and mature DCs. PFS: progression-free survival (months); PD: progressive disease; SD: stable disease; MxR: mixed response; n.e. not evaluated; n.d. not done. aDTH was considered positive as at least 2 out of the 4 DTH sites had a positive induration (+ induration $2<5$; ++ induration $5<10$; +++ induration $10$). b+ standard index (SI) $2<10$; ++ SI $10<20$; +++ SI $20$. cantibody titer, + designates $>1$ in 400. dcomplete response after excision of regressing tumor ereceived maintenance treatment with DC vaccinations.

Recent findings in humans also show circulating resident T cell populations with potent regulatory properties in the peripheral blood. Whether regulatory T cells were induced by our vaccination with immature DCs remains to be investigated. The superiority of mature DCs to induce an immune response was underscored by the observation that positive DTH reactions were only observed when the DCs were matured in vitro. In addition to DTH reactions to peptide/KLH-pulsed mature DCs, DTH reactions were also observed in
response to non-pulsed mature DCs. Similar findings have been reported by others and can most likely be attributed to the production of large amounts of chemokines and cytokines by the mature DCs themselves\textsuperscript{11,25,43}. Of note, cultures from biopsies of positive DTH reactions to unpulsed DCs did not give rise to T cells. Although significant differences were observed between vaccination with mature and immature DCs with respect to immunological response against KLH, the number of patients in our study is too small for a definite conclusion on the difference in clinical outcome between these vaccinations. Furthermore, 6/9 patients vaccinated with immature DCs received their vaccine i.v./s.c. whereas all patients vaccinated with mature DCs were treated i.v./i.d. Although the route of administration may play a role in the nature of the response, this seems less likely in our study since the observed immune responses in the three patients vaccinated i.v./i.d. with immature DCs did not differ from the patients vaccinated i.v./s.c.. Nevertheless, our results indicate improved clinical efficacy of peptide-pulsed mature DCs compared to immature DCs. Only in patients vaccinated with mature DCs an objective clinical response could be observed. A partial response was observed in a patient (Mat-3) with a distant lymph node metastasis with a concomitant normalization of serum LDH. After resection of the responding lesion, she currently is in ongoing remission for > 40 months. Another patient (Mat-10) achieved disease stabilization of 22 months in irresectable bulky retroperitoneal lymph node metastases upon vaccination with mature DCs. Prediction of clinical efficacy by immunological markers remains a challenge. Several clinical vaccination studies in cancer patients have reported T cell responses in the peripheral blood but usually in a minority of patients, or only after prolonged restimulation with antigen in culture\textsuperscript{49-53}. In our study we could not detect significant and consistent anti-tumor T cell reactivity in peripheral blood cells. This might be due to the fact that cells were tested without restimulation to prevent in vitro manipulation. Furthermore, the lack of antigen-specific T cells might be explained by the difference in the type of tumor antigens used, as the response to gp100 and tyrosinase in healthy individuals is much lower than to, for example, Melan-A/MART-1\textsuperscript{52,53}. We investigated the DTH reactivity as a tool to monitor the efficacy of our vaccine and its clinical outcome. Our results show that DTH-infiltrated T lymphocytes after short term culture without antigenic restimulation show peptide-specific reactivity as determined by MHC-tetramer positive T cells and cytotoxic reactivity to the vaccinated tumor antigens. Intriguingly, antigen-specific T cell responses were detected in the two patients with the best clinical outcome (Mat-3, Mat-10). In these patients gp100-specific T cells were detected by MHC-tetramer staining in cultured T cells from the biopsy of a DTH reaction (Mat-10) and MHC-tetramer positive cells by immunofluorescent staining on frozen biopsy sections (Mat-3, manuscript in preparation). Moreover, after a short time culture in vitro, these T cells were able to kill tumor cells expressing the antigens used for vaccination. The presence of antigen-specific T cells in the DTH suggests that a systemic response was induced. Furthermore, the fact that this was only observed in clinical responding patients suggests that the evaluation of T cell reactivity in positive DTH sites may be a powerful tool in the monitoring of
clinical T cell-directed vaccination studies in cancer patients.

In conclusion, we here demonstrate that maturation of DCs before injection is pivotal to induce antigen-specific immune responses in melanoma patients. Moreover, clinical responses were only observed in patients vaccinated with mature DCs. Remarkably, T cells isolated from positive DTH sites of the patients with the best clinical outcome were specific for the antigens used for vaccination. This suggests that T cells isolated from positive DTH sites might be very helpful in monitoring vaccination therapy but this method remains to be confirmed in larger clinical studies.

Acknowledgments

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Abbreviations: DC, dendritic cell; CTL, cytotoxic T lymphocyte; KLH, keyhole limpet hemocyanin; PBMC, peripheral blood mononuclear cells; IL, interleukin; GM-CSF, granulocyte monocyte-colony stimulating factor; HS, human serum; MCM, monocyte-conditioned medium; TNF, tumor necrosis factor; PGE2, prostaglandin E2; DTH, delayed type hypersensitivity; IFN, interferon; i.v., intravenous; i.d. intradermal; s.c., subcutaneous.

References


Magnetic resonance tracking of dendritic cells in melanoma patients for monitoring of cellular therapy


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Magnetic resonance tracking of dendritic cells in melanoma patients for monitoring of cellular therapy

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The success of cellular therapies will depend in part on accurate delivery of cells to target organs. In dendritic cell therapy, in particular, delivery and subsequent migration of cells to regional lymph nodes is essential for effective stimulation of the immune system. We show here that in vivo magnetic resonance tracking of magnetically labeled cells is feasible in humans for detecting very low numbers of dendritic cells in conjunction with detailed anatomical information.

Autologous dendritic cells were labeled with a clinical superparamagnetic iron oxide formulation or 111In-oxine and were co-injected intranodally in melanoma patients under ultrasound guidance. In contrast to scintigraphic imaging, magnetic resonance imaging (MRI) allowed assessment of the accuracy of dendritic cell delivery and of inter- and intra-nodal cell migration patterns. MRI cell tracking using iron oxides appears clinically safe and well suited to monitor cellular therapy in humans.

Cellular therapies using stem cells and immune cells are being increasingly applied in clinical trials. Accurate delivery of cells to target organs can make the difference between failure or success. Because of their pivotal role in initiating an immune response, dendritic cells are of widespread interest as a means of enhancing the endogenous immune response against tumor cells. Tumor antigen–loaded dendritic cell vaccines have been introduced in the clinic and have proven feasible and nontoxic, and both immunological and clinical responses have been observed1. However, effective immune induction is limited to a minority of patients. One possible explanation of this is insufficient delivery of dendritic cells to the target organs. For effective immunotherapy, dendritic cells must migrate throughout the vascular and lymphatic system to present their antigens to T cells located within lymph nodes. In independent studies, dendritic cells have been administered by different routes: intradermally, subcutaneously, intravenously or using combinations of these routes. Alternatively, dendritic cells can be injected directly into the lymph node2.

Thus far it has not been clear which route of administration is optimal. The design of
optimal dendritic cell therapy would therefore be facilitated by technologies for monitoring dendritic cell trafficking. Dendritic cells have previously been labeled with radionuclides for scintigraphic imaging, which is the only clinical cellular imaging modality approved by the US Food and Drug Administration (FDA). A major drawback of scintigraphy, however, is the lack of anatomical detail; it allows only gross anatomical determination of migration between lymph nodes without the ability to assess the intranodal distribution pattern of dendritic cells within each lymph node. Furthermore, accurate cell delivery, which may be essential for subsequent migration into nearby lymph nodes, cannot be properly evaluated owing to scintigraphy’s lack of spatial resolution. In contrast, MRI is well suited to obtain three-dimensional, wholebody, high-resolution images and is widely used in clinical practice. The most sensitive existing markers to label cells for magnetic resonance detection are (ultrasmall) superparamagnetic iron oxide ((U)SPIO) particles. Initially applied as a marker for cells of the reticuloendothelial system, including the liver and lymph nodes, these contrast agents are now either FDA-approved as a liver agent (SPIO; Feridex-USA; Endorem-Europe) or in late-phase clinical trials as a lymph node agent (USPIO; Combidex-USA; Sinerem-Europe). They are nontoxic and biodegradable. Recently, SPIO particles have been applied as a magnetic label to detect cells after local grafting or systemic injection, including dendritic cells labeled with a nonclinical-grade SPIO preparation through a two-step monoclonal antibody approach.

Figure 1. Study protocol. (a) Monocytes are obtained by cytopheresis from stage-III melanoma patients. (b) They are cultured and labeled with SPIO particles and 111In. (c,d) The cells are then injected intranodally into a (either cervical, inguinal or axillary) lymph node basin that is to be resected and their biodistribution is monitored in vivo by scintigraphy (c) and MRI at 3 Tesla (d). (e-g) The lymph node basin is resected and separate lymph nodes are visualized with high resolution MRI at 7 Tesla and histology (g).
These studies have all been performed in animal models. Translation of these techniques from animal models to humans is not straightforward because SPIO-labeling raises safety concerns associated with the use in patients of adjunct compounds, such as transfection agents.

In this study, we obviated these concerns by taking advantage of the fact that immature dendritic cells naturally endocytose clinically applied, FDA-approved SPIO-labels in substantial amounts. We found that cells could be labeled with high efficiency without affecting their function. We then investigated the biodistribution of SPIO-labeled dendritic cells applied as cancer vaccines in melanoma patients using MRI. In vitro-generated dendritic cells loaded with tumor-derived antigenic peptides were administered to stage-III melanoma patients as outlined in figure 1.20. Dendritic cells were labeled with In-oxine and SPIO (Endorem) separately and coinjected in a lymph node in the lymph node basin to be resected. This provided a unique opportunity not only to obtain magnetic resonance scans at 3 Tesla (T) before surgery, but also to generate high-resolution magnetic resonance images at 7 T of individual resected lymph nodes and to correlate the results with scintigraphy and (immuno)histopathology (figure 1). We show that magnetic resonance tracking of magnetically labeled cells is a clinically safe procedure that, because of its high resolution and excellent soft tissue contrast, appears ideally suited to monitor novel experimental cell therapies in patients.

**RESULTS**

**In vivo MRI and scintigraphy of dendritic cells**

We loaded dendritic cells with SPIO particles by co-culturing immature dendritic cells with 200 mg/ml SPIO, as immature but not mature dendritic cells are highly phagocytic. Several tests were conducted to determine whether the labeling procedure affected the cells’ viability or function. First, we confirmed that all cells had taken up a substantial amount of SPIO by the end of the culture period (figure 1b). Second, the phenotypes of SPIO-labeled and unlabeled cells appeared similar (figure 2a). Third, the random migration on fibronectin-coated wells showed that SPIO-labeled dendritic cells migrated as well as unlabeled- or In-labeled cells...
(figure 2b). Finally, SPIO-labeling of dendritic cells loaded with the melanoma-specific peptide gp100: 154-162 was found not to affect the peptide-specific production of interferon-γ by a gp100: 154-162-specific T-cell line22 (figure 2c), indicating that the antigen-presentation function of dendritic cells remained unaffected after labeling. Together these results demonstrate that SPIO labeling does not affect the cells’ phenotype or function.

Eight stage-III melanoma patients received an intranodal injection under ultrasound guidance of a mixture of 111In- and SPIO-labeled dendritic cells (ratio 1:1) 2 days before radical dissection of regional lymph nodes. Patients were imaged before and 2 days after the injection by both scintigraphic imaging and MRI to monitor delivery of the dendritic cells and their subsequent migration to nearby lymph nodes.

Scintigraphic imaging confirmed and extended previous findings that although a significant percentage of dendritic cells remained at the injection site, some did migrate to nearby lymph nodes. In four of eight patients, 1% to 40% of total 111In activity was found in regional lymph nodes draining the injected lymph node, indicating migration of 111In-labeled dendritic cells (figure 3d, table 1 and supplementary figure 1). The scintigraphic images were compared with in vivo magnetic resonance images obtained on a 3-T magnetic resonance system (figure 3). Comparison of gradient echo images before and after cell injection (figure 3a,c; patient 1) showed that the injected SPIO-labeled dendritic cells resulted in a significant decrease in signal intensity at the injection site. After injection, turbo spin echo (SE) images, which are relatively insensitive to SPIO-induced magnetic susceptibility effects, were also obtained (figure 3b).

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<th>Patient</th>
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<th>Number of LNs visualized on ex vivo scintigraphy</th>
<th>Number of LNs visualized on MRab</th>
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<td>1 (0%)</td>
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Table 1. Comparing scintigraphic and MR imaging for monitoring of cell migration. aIncluding the injected LN, between brackets the total percentage of 111In-activity at the site(s) distant from the injection depot is given (only for scintigraphy). bPart of the cells were injected in the LN, mostly in the perinodal tissue. cSPIO was detected only outside the LN.
Figure 3. In vivo scintigraphic and MRI. (a–c) Monitoring of the delivery of dendritic cells labeled with SPIO and $^{111}$In by MRI before and after intranodal injection in patient 1. (a) Gradient echo transversal magnetic resonance image before vaccination showing a right inguinal lymph node with a hyperintense signal area (1). (b) SE (technique much less sensitive for SPIO) transverse magnetic resonance image obtained from the same lymph node after vaccination. (c) Gradient echo transverse magnetic resonance images after vaccination in same position as b showing a decreased signal intensity of lymph node 1. (d–n) Monitoring of in vivo migration of SPIO and $^{111}$In-labeled dendritic cells with MRI and scintigraphy after injection in a right inguinal lymph node in patient 3. (d) In vivo scintigraphy 2 d after vaccination showing migration of the dendritic cells from the injection lymph node (1) to three following lymph nodes (2–4). (e–n) Five image pairs of a coronal gradient echo and SE image 2 days after vaccination showing migration of the dendritic cells from the injection lymph node 1 (e and f) to four following lymph nodes (g–n). Open arrows indicate lymph nodes that do not contain SPIO, on the SE images these nodes are dark-gray; on gradient echo images they are white. Closed arrows indicate lymph nodes that are positive for SPIO in the gradient echo magnetic resonance image. On gradient echo images SPIO-containing lymph nodes have a decreased signal intensity compared to SE images. The concentration of SPIO in lymph node 1 was very high, resulting even in a decreased signal intensity in the SE image. The lymph node that was identified by the scintigraphy as the injection lymph node (lymph node 1 in d) actually consisted of two distinct lymph nodes as evidenced by MRI (lymph nodes 1 and 5).
By comparing the iron-insensitive magnetic resonance spin echo sequence with iron-sensitive gradient echo sequences, one can reliably detect the presence of iron in a lymph node. In SE images, lymph nodes generally appear as dark-gray structures. In gradient echo images, they are white (compare open arrows in figure 3e,f; k,l and m,n; patient 3); a large decrease in signal intensity at the sites of the lymph nodes indicated the presence of SPIO-labeled cells (closed arrows in figure 3). In- and SPIO-labeled cells co-localized in the same areas, proving that the locations detected by MRI indeed represent injected and migrated dendritic cells (figure 3e–n). All lymph nodes that showed In activity were also positive in MRI. However, because MRI has a higher spatial resolution, the MRI images revealed more lymph nodes containing migrated dendritic cells as compared with the scintigraphic images (table 1). In addition, scintigraphic imaging saturates the image such that multiple adjacent lymph nodes may appear as one. An example is shown in figure 3. In patient 3, four positive lymph nodes were identified by scintigraphy. However, the magnetic resonance images revealed five lymph nodes, as the hot spot of lymph node 1 actually consisted of two separate lymph nodes, with the second one lying close to the injection node (figure 3d,g,h and supplementary video 1 online).

From the scintigraphy of the resected lymph node basin from patient 1, we could calculate that 2% of 7.5x10^6 dendritic cells migrated to draining lymph node number 4 (see supplementary figure 1). This particular node was also visible by MRI, indicating that as few as 1.5x10^5 migrated cells could readily be visualized. The node had a volume of 2.3x7.6x3.5 mm (90 voxels in one slice). Assuming that the distribution of SPIO-positive cells in the lymph node was homogeneous, as a rough estimate ~2x10^3 cells/voxel could be visualized. Because the distribution is inhomogeneous, the actual value may be larger.

Notably, the additional anatomical information provided by the magnetic resonance images could confirm that the injected dendritic cells were truly localized within the lymph node. In addition, MRI demonstrated that in three patients the dendritic cells were actually delivered not into the lymph node but in the perinodular fat (figure 4a,b). Notably, in all three patients no dendritic cell migration to draining lymph nodes was observed (table 1). Furthermore, in two patients, dendritic cells were injected only partly in the target lymph node and migration of dendritic cells was registered in only one of them.

The total number of SPIO-positive dendritic cells imaged by magnetic resonance

Figure 4. Monitoring of the accuracy of delivery of SPIO-labeled cells using MRI. (a) MRI before vaccination; the inguinal lymph node to be injected is indicated with a black arrow. (b) MRI after injection showing that the dendritic cells were not accurately delivered into the inguinal lymph node (black arrow) but in the vicinity, in the subcutaneous fat (white arrow).
was significantly correlated with the success of the intranodal injection (see supplementary figure 2, P<0.05). Thus, in contrast to MRI, which allows verification that hotspots are actually lymph nodes, scintigraphy cannot distinguish between correct and incorrect intranodal injections, leading to erroneous classification of injection sites as lymph nodes.

Analysis of the number of cases in which one technique was more accurate in imaging true dendritic cell–positive lymph nodes showed that MRI was significantly better than scintigraphy (P < 0.05). Thus, the detailed anatomical information (combination of high spatial resolution and excellent soft tissue contrast) of MRI is a clear advantage compared with scintigraphic imaging, allowing both verification of accurate delivery and monitoring of subsequent migration of SPIO-labeled cells.

**Ex vivo MRI and histology of resected lymph nodes**

As the melanoma patients were scheduled for regional lymph node dissection, a unique opportunity existed to investigate the biodistribution of the injected dendritic cells in more detail in individual resected lymph nodes by high-resolution MRI at 7T (figure 5a,c). All lymph nodes that contained 111In-positive dendritic cells as determined by a gamma probe were also positive for SPIO-labeled cells, validating the ability of MRI to detect and localize low numbers of injected and migrated SPIO-labeled cells. As expected when SPIO-labeled cells are present, the gradient echo images of these nodes showed much larger areas in the lymph nodes that are hypointense as compared with the SE images (see supplementary Videos 2 and 3 online).

Localization of SPIO-labeled dendritic cells in the lymph nodes was further confirmed by histology after Prussian blue staining of lymph node sections (figure 5b,d). There was an excellent correlation between the hypointense areas of images obtained by ex vivo MRI and the areas containing large numbers of SPIO-positive cells as visualized by histochemistry. Even low numbers of SPIO-labeled dendritic cells distributed over a larger area were readily detected by MRI (encircled areas in figure 5b,d). These ex vivo MRI and histology findings thus confirm that in vivo MRI is a sensitive and accurate technique for monitoring the biodistribution of SPIO-labeled cells.

Further histological evaluation of the lymph nodes beyond the injected node that were detected with autoradiography and MRI showed iron-containing dendritic cells in the paracortex. Large numbers of iron-containing cells were present in the sinuses of the lymph node. These findings indicate that injected dendritic cells entered the lymph nodes via their natural route through the afferent lymph vessels and sinuses (figure 5e). A significant proportion of the cells penetrated deep into the T-cell areas (figure 5f) throughout the lymph nodes, whereas no cells were found in the B-cell areas. SPIO-labeled dendritic cells in T-cell areas were often found to be surrounded by rosetting lymphocytes (figure 5g). The presence of rosettes of slightly enlarged T cells around SPIO-positive dendritic cells is indicative of T-cell activation, a requirement for effective dendritic cell vaccines.
Figure 5. Ex vivo magnetic resonance images and correlation with histology. (a–d) SE magnetic resonance image of the injected lymph node (a) and a nearby lymph node (c) obtained after resection from patient 1. SPIO-labeled dendritic cells were visualized by Prussian Blue staining in sections from the injected lymph node (b) and the nearby lymph node (d) at levels comparable to the magnetic resonance images. Areas with (dispersed) SPIO-labeled dendritic cells as detected at higher magnifications are encircled and correlated with the magnetic resonance images. The blue area in b that is visible even at low magnification represents the injection site. (e–h) Magnifications of specific regions of d. SPIO-labeled cells were detected in the sinus (e) and in T-cell areas (f) of the lymph node. (g) T cells form rosettes around vaccinated dendritic cells (a representative picture is shown). SPIO-labeled cells are negative for the macrophage marker CD68. (h) SPIO-labeled cells were positive for the dendritic cell marker S100. Blue represents SPIO-particles, brown indicates specific immunostaining.

The SPIO-labeled cells were negative for the macrophage marker CD68 (figure 5g), indicating that SPIO-positive cells were indeed injected dendritic cells and not macrophages that had phagocytosed SPIO particles released from dead cells. Moreover, SPIO-containing cells were also positive for the dendritic cell markers S100 (figure 5h) and CD83 (data not shown). Thus, SPIO-labeling does not prevent migration of injected dendritic cells to the T-cell area in the lymph node in vivo, and injected dendritic cells that migrate into the T-cell areas have productive interactions with resident T cells.

**DISCUSSION**

In this study, we followed the migration of autologous ex vivo-cultured mature dendritic cells after intranodal administration in eight stage-III melanoma patients scheduled for regional lymph node dissection. To the best of our knowledge, there is no previous report of effective tracking of ex vivo-labeled therapeutic cells in humans by noninvasive MRI. By co-injecting equal numbers of $^{111}$In- and SPIO-labeled cells, we demonstrated that MRI is at least as sensitive as scintigraphic imaging for detecting dendritic cell migration in vivo. Moreover, MRI is significantly better than scintigraphic imaging in that delivery of the dendritic cell vaccine to the intended
location can be verified and cells can be tracked more accurately.

We labeled dendritic cells in the immature state, following which they were allowed to mature, as immature but not mature dendritic cells are highly phagocytic. This obviated the use and clinical approval of transfection agents, which are now widely applied for efficient intracellular magnetic labeling of nonphagocytic cells in animal models.

The phenotypical and functional properties of the SPIO-labeled cells were unaltered as compared with unlabeled cells. Moreover, SPIO-labeled cells that had migrated into lymphoid tissue still expressed the maturation marker CD83, indicating that migrating dendritic cells remained mature and did not return to an immature state.

A major advantage of MRI over scintigraphic imaging is the high-resolution anatomical background contrast, which allows precise anatomical localization of SPIO-labeled cells at the actual injection site and after migration. A major advantage of scintigraphic imaging, however, is the possibility to quantify the amount of cells that have migrated from the injection site. Preferably, both techniques should be combined to obtain both quantitative and qualitative information on migration of therapeutically administered cells. Alternative in vivo approaches for noninvasive visualization of adoptively transferred cells, such as bioluminescent imaging or positron emission tomography, have no endogenous background contrast, lack the resolution to delineate fine anatomical details of tissues and organs or cannot be used clinically (bioluminescent imaging). The combination of both SPIO and radiouclide labeling allows both quantification (scintigraphy) and detailed anatomical localization (MRI) of migrated cells.

With these techniques we were able to detect as few as $1.5 \times 10^5$ cells in vivo, which is in concordance with a previous study in a preclinical porcine model using MRI to visualize injected iron fluorophore particle–labeled mesenchymal stem cells and which is far below the therapeutic range of $\sim 2 \times 10^6$ cells necessary for successful cardiac infarct engraftment. Furthermore, the combination of labeling techniques offers new possibilities to image differentially labeled subsets of cells simultaneously in vivo after their injection at one and the same site (for example, dendritic cell subsets or dendritic cells in different activation or maturation stages). This is particularly relevant for further optimization of cell-based anti-cancer therapies. Subsequent autoradiography, (immuno)histology and Prussian blue staining of tissue sections from resected lymph nodes to visualize individual $^{111}$In-labeled and SPIO-labeled cells would then offer a further means of validation to track mixed cell populations at the single-cell level.

Interestingly, we found that in only $\sim 50\%$ of the cases were dendritic cells correctly injected into the lymph node, despite ultrasonic guidance of the injection needle by a highly experienced radiologist. Subsequent migration could be observed only when dendritic cells were correctly injected into the lymph node, demonstrating the importance for cellular therapy of magnetic resonance verification of accurate delivery. Inadequate delivery may explain why only a limited proportion of patients is responding in ongoing clinical trials of dendritic cell vaccines. We found that MRI was significantly more accurate than scintigraphy for visualizing true dendritic cell–positive lymph nodes. These findings illustrate the power of additional anatomical information, which can also be of value for other fields of bio-
medical research. For example, the importance of magnetic resonance tracking of cell delivery has been recognized for bone marrow stem cell injections into infarcted myocardium of large animal models. MRI proved valuable for monitoring not only the accuracy of the injection but also the migratory capacity of antigen-loaded dendritic cells, as remote lymph nodes containing SPIO-labeled cells could be visualized individually. In contrast to scintigraphy, where a hot spot may represent one or more lymph nodes, MRI can detect all truly positive lymph nodes separately owing to its high spatial resolution and lack of saturation of images. In two patients, individual lymph nodes were missed by scintigraphy because they were located in the same vertical plane as the injection node. With MRI these lymph nodes could be detected separately, allowing correct evaluation of the migratory capacity of SPIO-labeled cells in vivo. That these lymph nodes were SPIO positive was confirmed by ex vivo MRI and histology. High-resolution MRI of these targeted lymph nodes ex vivo provided detailed information on the three-dimensional biodistribution of small numbers of cells and their migration into the paracortex of the lymph node, the location of the T-cell areas where the dendritic cell–T cell interaction takes place. These findings were confirmed by immunohistology after Prussian blue staining of lymph node sections.

Moreover, rosettes of slightly enlarged T cells around injected dendritic cells were present in several patients, demonstrating a functional interplay between SPIO-labeled dendritic cells and T cells. Thus, SPIO-labeling of dendritic cells did not affect the migratory behavior and functionality of the dendritic cells in vivo.

In conclusion, this clinical study demonstrates the potential of using MRI for tracking therapeutic cells in patients. Our approach could be easily extended to other clinical applications, including those based on monocyte, granulocyte and lymphocyte trafficking, monitoring of cellular transplants and tissue-restoration therapies based on stem cells and progenitors. The routine imaging protocols we used are readily available on common MRI systems. Therefore, cellular MRI may pave the way for many investigators and clinicians to obtain a more in-depth view of the underlying biodynamics of cellular treatment modalities.

**METHODS**

**Patients**

This study included melanoma patients enrolled in an ongoing protocol in which the in vivo immune responses of a dendritic cell vaccine are under study (KUN 99-150). Eligibility criteria included stage-III melanoma (according to the 2001 American Joint Committee on Cancer staging system), planned regional lymph node dissection for lymph node metastases, HLA-A2.1 phenotype, melanoma expressing the melanoma-associated antigens gp100 and tyrosinase, and World Health Organization performance status 0 or 1. Prior treatment was allowed, provided that a treatment-free period of at least 4 months was observed and all related toxicity had resolved. Patients with brain metastases, serious concomitant disease or a history of a second malignancy were excluded. The study was approved by our Institutional Review Board, and written informed consent was obtained from all patients. Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria. In total, 11 stage-III melanoma patients were included, of whom 2 developed clinical overt brain metastases between inclusion and start of treatment and could therefore not be included in the analysis. Of the other nine patients, one had severe claustrophobia precluding in vivo MRI, although scintigraphy and ex vivo MRI could be obtained. Toxicity was similar to previous dendritic cell vaccination studies and consisted of low-grade fever, mild flu-like symptoms and irritation at the site of injection after the vaccinations in some patients.
Preparation of SPIO- and \(^{111}\)In-labeled dendritic cells

Dendritic cells were generated from adherent peripheral blood mononuclear cells by culturing in the presence of interleukin-4 (500 U/ml) and granulocyte-monocyte colony stimulating factor (800 U/ml) (both Cellgenix). For SPIO-labeling, 200 mg Ferumoxide/ml (Endorem, Laboratoire Guerbet) was added 3 days after the onset of dendritic cell culturing. At day 5, dendritic cells were matured with autologous monocyte-conditioned medium supplemented with prostaglandin E\(_2\) (10 mg/ml, Pharmacia & Upjohn) and 10 ng/ml recombinant tumor necrosis factor-\(\alpha\) (Cellgenix) for 48h, as described previously\(^26,27\). Dendritic cells were pulsed with the melanoma peptides gp100:154-162, gp100:280-288, tyrosinase 369-376 as described previously\(^3\). Mature dendritic cells were labeled with \(^{111}\)In-oxine (Tyco Healthcare) in 0.1 M Tris-HCl (pH 7.0) for 15 min at 20\(^\circ\)C as described previously\(^3,26\), resulting in 5 \(\mu\)Ci activity per 7.5\(\times\)10\(^6\) cells. Cells were washed three times with PBS. Radiolabeling efficiency was determined by measuring activity in both the cell pellet and the washing buffer. Iron labeling efficiency was verified by Prussian blue staining. Virtually all cells endocytosed SPIO particles (figure 1). Total iron content of SPIO-labeled cells was assessed by a Ferrozin-based spectrophotometric assay following acid-digestion of labeled cell samples\(^11,28\). The iron content was 10–30 pg of iron per cell. Cell viability was determined by Trypan blue staining, showing comparable viability (more than 80%) for unlabeled dendritic cells and \(^{111}\)In and SPIO-labeled dendritic cells (data not shown).

Phenotypic and functional evaluation of dendritic cells

Fluorescence-activated cell sorting (FACS) analysis was performed using a Becton Dickinson FACSCalibur. The following fluorochrome-conjugated monoclonal antibodies were used: anti-HLA class I (W6/32), anti-HLA DR/DP (Q5/13), anti-CD80 (all Becton Dickinson), anti-CD83 (Beckman Coulter), anti-CD86 (Pharmingen), and anti-Chemokine Receptor 7 (kind gift of Martin Lipp). Random in vitro migration was tested as described previously\(^3\). Peptide-specific T-cell stimulatory capacity was tested by co-culturing dendritic cells with or without SPIO that were loaded with the gp100:154-162 peptide or an irrelevant peptide with a gp100:154-162 specific T cell line (dendritic cell: T cell ratio, 1:5)\(^22\). After 48 h the cytokines in the supernatant were analyzed with a cytometric bead array for human Th1/Th2 cytokines (BD Biosciences).

Treatment schedule

Within 2 weeks before vaccination a first (preinjection) baseline magnetic resonance scan was performed (see below). At day -7 peripheral blood mononuclear cells were obtained by leukapheresis for dendritic cell culturing. At day 0 patients received a single injection of \(^{111}\)In-labeled dendritic cells (7.5\(\times\)10\(^6\)) mixed with iron oxide–labeled dendritic cells (7.5\(\times\)10\(^6\), total volume 200 ml) directly into a lymph node of the lymph node region that was to be resected, using a 21 G sterile needle (0.8 50 mm, Microlance, Becton Dickinson). Intranodal injections were performed under ultrasound guidance.

One hour after injection the first scintigraphic image (see below) was acquired. At day 2, a second (post-vaccination) magnetic resonance scan was performed, followed by a second scintigraphic imaging session, followed by regional lymph node dissection. After surgery, the resected material was again imaged scintigraphically to verify whether all tissue containing radioactivity was indeed removed. Radioactive lymph nodes were dissected from the surgical specimen under guidance of a gamma probe (Europrobe, Eurorad) and then fixed in Unifix (Klinipath). Of these separate lymph nodes, high-resolution ex vivo magnetic resonance images were acquired at the end of day 2 and the beginning of day 3. The lymph nodes were sliced and embedded in paraffin at the end of day 3 and sections were cut at day 4 and processed for histology (iron-staining). As the lymph nodes were to be resected only 2 days after intranodal vaccination, the induction of an immune response might not have been optimal. For this reason patients simultaneously received melanoma peptide-loaded dendritic cells intranodally in a contralateral clinically tumor-free lymph node which was not to be resected. Patients received three more vaccinations at days 14, 28 and 42.

Scintigraphic imaging

In vivo and ex vivo planar scintigraphic images (256\(\times\)256 matrix, 174 and 247 keV \(^{111}\)In photopeaks with 15\% energy window) of the injection depot and corresponding lymph node basin were acquired with a gamma camera (Siemens ECAM) equipped with medium energy collimators, at day 0 and day 2. Migration was quantified by region-of-interest analysis of the individual nodes visualized on the images and expressed as the relative fraction of \(^{111}\)In-labeled dendritic cells
that had migrated from the injection depot to following lymph nodes after 2 days.

**MRI**
Patients were imaged using a 3 T whole body magnetic resonance system (Siemens Magnetom Trio) with a body array radiofrequency coil for signal reception. Magnetic resonance images were obtained with a gradient echo pulse sequence; the signals of three gradient echoes were combined into one T2*-weighted image with an average echo time of 15 ms (flip angle 36°, repetition time (TR) 1,060 ms, total acquisition time ~9 min, 30 slices, resolution 0.50x0.50x3.50 mm). In addition to T2*-weighted images, which are very sensitive to SPIO-induced magnetic susceptibility effects, SE images at corresponding slice locations were also acquired using a short echo time (TE) of 18 ms (resolution 0.83x0.50x3.50 mm, TR 2.5 s, total time ~6.5 min) as a reference control to ensure that the decreased signal intensity originated from the magnetic field inhomogeneities caused by SPIO. To keep radiofrequency power deposition within prescribed limits, hyperechoes were used in SE imaging. Ex vivo MRI of lymph nodes was performed using a 7T MR-spectrometer (Surrey Medical Imaging Systems) equipped with a 20-mm diameter radio frequency (RF) coil. The lymph nodes were placed in a plastic tube filled with Fomblin LC0810 (Ausimont), to reduce susceptibility artifacts at the tissue-air interface without a background proton signal. Multi-slice gradient-echo imaging was performed at two different echo times (TR 1/4 1,500 ms and TE 1/4 9 and 13 ms, voxel-size 1/4 0.12 0.12 0.5 millimeters, acquisition time 13 min). Subsequently multi-slice spin-echo imaging was performed at corresponding slice locations and voxel size (TR = 1,000 ms and TE = 15 and 28 ms, acquisition time 8.5 min).

**Statistical analysis**
Statistical analysis was performed using the Wilcoxon rank sum test for nonparametric distributions for paired and unpaired observations. To test the hypothesis that magnetic resonance is more accurate than scintigraphy, we coded our data for both methods: more lymph nodes visualized by magnetic resonance (MR = +1, scintigraphy = -1); more lymph nodes visualized by scintigraphy (MR = -1, scintigraphy = +1); or equal numbers (MR = scintigraphy = 0). When a hot spot in scintigraphy was qualified as situated outside lymphoid tissue by MRI, this was coded in favor of MRI (MR = +1, scintigraphy = -1). P < 0.05 was considered significant.

**Iron staining and immunohistochemistry of histopathological sections**
Sections (5 mm) of the radioactive resected lymph nodes were stained with Prussian blue to detect SPIO-labeled cells. Slides were stained with 2% potassium hexacyanoferrate (II)-trihydrate in 0.2 M HCl for 15 min and counterstained with 0.05% nuclear fast red in 5% aluminum sulphate. Immunohistochemistry was performed using antibodies against CD68 (KP1, DAKO), S100 (DAKO) and CD83 (Beckman Coulter). Bound antibody was visualized using powervision (Immunologic). Subsequently the slides were stained using Prussian blue and nuclear fast red.

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**SUPPLEMENTARY DATA**
Supplementary information is available at http://www.nature.com/naturebiotechnology/

**REFERENCES**


Supplementary figure 1. Monitoring of in vivo migration of SPIO and 111In-labeled DCs with MR imaging and scintigraphy after intranodal injection in patient 1. (a) GE transverse MR images before vaccination showing a right inguinal LN with a high signal intensity, which is presented in Figure 3a-c (arrow 1). (b) In vivo scintigraphy 2 days after vaccination showing two positive LNs. (c, f) GE transversal MR images before vaccination, (d, g) SE transverse MR images after vaccination show LN 2-4 with dark-gray signal intensity. (e, h) On GE transverse MR images after vaccination all these LNs show decreased signal intensity. (e) Ex vivo scintigraphy of the resected LN basin verified the MR findings. From this scintigraphy the percentage of migrated cells could be calculated (LN 1: 60%; LN2: 32%, LN3: 2%; LN4: 6% of injected DCs).
Supplementary figure 2. Number of LN positive for labeled DC imaged with MR and scintigraphy 48 hours after intranodal vaccination. Every symbol in the graph represents one patient. Based on the results of the MR imaging patients could be separated into three different groups. In the first group of patients the radiologist failed to inject the labeled cells correctly in the lymphoid tissue (missed), in the second group the labeled cells were only partly delivered into the target LN (partly) and in the third group injection was successful (correct). No such distinction could be made with scintigraphy where every hotspot is classified as being a LN. In the last column, the open symbols represent the patients that were categorized in group one (missed) by MR imaging. The total number of positive LNs imaged by MR in group three (correct) was significantly higher than the total number of LNs in group one and two (Wilcoxon rank sum test).
Vaccination of Advanced Melanoma Patients with Wild-type and Modified gp100 Peptides pulsed on Dendritic Cells


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Vaccination of advanced melanoma patients with wild-type and modified gp100 peptides pulsed on dendritic cells


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Dendritic cell (DC)-based immunotherapy is explored worldwide in cancer patients. Several strategies have been employed to load DCs with antigen, including peptide-loading. To increase immunogenicity of peptides, MHC class I binding affinity and stability of peptide-MHC complexes at the cell surface can be improved by modification of the amino acid sequence.

In this study, we compare the capacity of DCs loaded with wild-type or modified gp100 peptides with higher binding affinities to induce an immune and clinical response in advanced melanoma patients. Metastatic HLA-A2.1+ melanoma patients were vaccinated with mature DCs loaded with keyhole limpet hemocyanin (KLH) together with tyrosinase peptide and either wild-type (15 patients) or modified (12 patients) gp100 peptides.

All vaccinated patients showed a pronounced proliferative T-cell or humoral response against KLH. Gp100-specific T cell responses were monitored in peripheral blood by tetramer analysis and in post-treatment biopsies of delayed type hypersensitivity (DTH) skin tests by tetramer and functional analysis. Functional T cell responses against the vaccine antigens were found in 2 of 15 patients vaccinated with wild-type gp100-loaded DCs, versus 1 of 12 patients vaccinated with modified peptide-loaded DCs. These 3 patients also had the best clinical response, with long-term (>8 years) complete responses in two patients.

We conclude that vaccination with peptide-loaded DCs can result in long-term clinical responses in a minority of metastatic melanoma patients, and that the use of modified as compared to wild-type gp100 peptides for DCs loading does not result in relevant enhanced immune responses.

Dendritic cells (DCs) are well known for their unique capacity to induce activation of naïve tumor-specific T cells1. They play a critical role in determining the quantity and quality of the immune response to the antigen. For this reason, a growing number of clinical trials have been performed using tumor antigen-loaded DCs as a therapeutics vaccine in cancer patients (reviewed in ref. 2). We and other laboratories gained ample experience with monocyte-derived DCs in clinical immunization protocols3-9. Objective clinical responses and immune responses without significant toxicity have
been observed after vaccination with tumor-antigen loaded DCs\textsuperscript{3,4,9}. Although the clinical outcome of vaccination studies with antigen-loaded DCs are encouraging, DC vaccination is at an early stage, and several parameters need to be optimized\textsuperscript{10}. Several strategies have been employed to load DCs with antigen. The availability of class I-restricted peptides derived from tumor-associated antigens, such as gp100, tyrosinase, MAGE, and NY-ESO-1, led to the use of peptide-pulsed DCs in anti-tumor vaccination trials\textsuperscript{11}. However, natural epitopes are often poorly immunogenic. MHC class I binding affinity and stability of peptide-MHC complexes at the cell surface contributes to the immunogenicity of a cytotoxic T lymphocyte (CTL) epitope. The sequence at amino acid residues that are crucial for the interaction with HLA or with the specific T cell receptor (TCR) can be modified in order to increase the affinity for MHC class I and enhance the immunogenicity of peptides. In addition, modified peptides may increase the repertoire of CTLs reactive with the tumor antigens. For example, we have described the altered HLA-A2.1-binding gp100:154 epitopes, which have an improved immunogenicity and elicit wildtype epitope-reactive CTL\textsuperscript{12}. These modified peptides can subsequently be used for the preparation of more effective DC vaccines.

Here we compare the capacity of DCs loaded with wild-type gp100 peptides (gp100:154 KTWGGQYWQV; gp100:280 YLEPGPVT) or modified gp100 peptides (gp100:154 KTWGGQYWAV; gp100:280 YLEPGPVT), to induce an immune and clinical response in advanced melanoma patients. The modified gp100:154 peptide with alanine substitution at position 8 was selected from a panel of modified gp100:154 peptides based on enhanced MHC class I binding and superior immunogenicity compared to wild-type peptides in vitro and in vivo in HLA-A2.1 transgenic mice\textsuperscript{12}. The modified gp100:280 peptide with valine replacement at position 9 was selected based on high binding affinity to HLA-A2.1 and enhanced in vitro CTL induction in peripheral blood lymphocytes (PBLs) of HLA-A2.1\textsuperscript{+} melanoma patients\textsuperscript{13}. CTLs raised against both modified gp100:154 and modified gp100:280 recognized wildtype gp100\textsuperscript{12,13}. In this study, metastatic HLA-A2.1\textsuperscript{+} melanoma patients were vaccinated with mature DCs loaded with keyhole limpet hemocyanin (KLH) together with tyrosinase peptide and either wildtype or modified gp100 peptides and immune and clinical responses were monitored.

**MATERIALS AND METHODS**

**Patient criteria**

Inclusion criteria were: histologic evidence of metastatic melanoma, progressive disease, measurable disease parameters, focal or diffuse expression of gp100 (mandatory) and tyrosinase (optionally) in at least one metastasis as determined by immunohistochemistry, HLA-A2.1 phenotype, WHO performance status 0 or 1, and written informed consent. Patients were staged according to the 2001 AJCC staging system\textsuperscript{14}. Patients with clinical signs of brain metastases, serious concomitant disease or a history of second malignancy were excluded. Prior treatment was allowed, provided a treatment-free period of at least four months was observed and all related toxicity had resolved. Approval from the local regulatory committee was obtained.

**Clinical protocol and immunization schedule**

In eligible patients a leukapheresis was performed from which DCs were generated. Patients received treatment with either wildtype peptides-pulsed DCs (wildtype group) or modified peptides-pulsed DCs (modified group). The first ten patients were included in the wildtype group, the next group of patients in the modified group. The first vaccination cycle consisted of two parts.
In the first part, antigen-pulsed DCs were administered 3 times at bi-weekly intervals, intravenously (i.v.) and intradermally (i.d.). In the second part, patients without tumor progression received 3 monthly i.d. vaccinations with peptides alone (100 g) and KLH (2 g) as maintenance treatment for further T cell expansion. Prior to each vaccination 80 ml of blood was collected for immunological monitoring. A clinical response was defined as stable disease of at least 4 months or any partial or complete response. Stable disease and partial response were defined according to RECIST criteria15. Toxicity was assessed according to NCI common toxicity criteria. Progression free survival was calculated from the day of the first vaccination. Patients were evaluated for response after completing the first and second part of the protocol and every three months thereafter. Patients who remained free of disease progression were eligible for 2 maintenance cycles, each at six months interval and each consisting of 3 bi-weekly intranodal vaccinations in a clinically tumor-free, usually inguinal lymph node region under ultrasound guidance with mature DCs, pulsed with gp100- and tyrosinase peptides and KLH. Patients who received DCs pulsed with wild-type gp100 peptides in the first cycle received modified peptide-pulsed DCs in the first maintenance cycle and wild-type peptide-pulsed DCs in the second maintenance cycle. Patients who received DCs pulsed with modified gp100 peptides in the first cycle received wild-type peptide-pulsed DCs in the first maintenance cycle and modified peptide-pulsed DCs in the second maintenance cycle (figure 1).

**DCs: Preparation and characterization**

DCs were generated from peripheral blood mononuclear cells (PBMC) as described previously16,17. After leukapheresis, PBMC were used for the generation of monocyte-conditioned medium (MCM) and plastic-adherent monocytes were cultured in X-VIVO 15 medium (BioWhitaker, Walkersville, Maryland) supplemented with 2% pooled human serum (Bloodbank Rivierenland, Nijmegen, The Netherlands), IL-4 (500 U/ml) and granulocyte macrophage colony stimulating factor (GM-CSF, 800 U/ml) (both from Schering-Plough, International, Kenilworth, New Jersey). After the addition of KLH (10 g/ml; Calbiochem, San Diego, CA) on day 3, autologous MCM with prostaglandin E2 (PGE2, 10 g/ml, Pharmacia & Upjohn, Puurs, Belgium) and 10 ng/ml recombinant TNF- (kindly provided by Dr. Adolf Bender, Vienna, Austria) were added on day 7 (30%, v/v). This procedure resulted in mature DCs on day 9 as demonstrated by high expression levels of MHC class I and II, CD80, CD83 and CD86 and absence of CD1416.

**Cryopreservation of PBMCs and DCs**

PBMCs and DCs were frozen using a cryo 1°C freezing container (Nalgene, Rochester, NY, USA) which was put in -80°C for 24 hours, in freezing medium consisting of 50% XVIVO-15 (5% HS), 40% human serum albumin and 10% DMSO (final concentration, Sigma). Cells were frozen in 1 ml per vial containing a maximum of 40x10⁶ cells16. Cells were thawed in a 37°C water bath after which the cells were washed once in cold medium and once in medium of room temperature before further use. The recovery of viable mature DCs was 65±10%16.

**Peptide pulsing**

DCs were pulsed with HLA-A2.1-restricted wild-type peptides, gp100:154-167, gp100:280-288 or modified peptides (gp100:154-167 QA, gp100:280-288 AV) and wild-type tyrosinase 369-376. Pulsing was done directly after harvesting or after thawing18,20. On the day of vaccination, we added peptides (50 g/ml) for 90 min and kept DCs at 37°C/5% CO₂. Thereafter, fresh peptides (25 g/ml) were added and DCs were kept at room temperature for 60 min. After peptide loading, DCs were washed twice in 0.9% sodium chloride and resuspended in 0.2 ml and 1 ml for i.d and i.v. injections, respectively4.

**Antibodies and immunostaining**

To characterize and compare the phenotype of the DC populations, flow cytometry was performed using either FITC-conjugated or PE-conjugated mAbs. The following FITC-conjugated mAbs were used: anti-HLA class I (W6/32), and anti-HLA DR/DP (Q5/13); and PE-conjugated mAbs: anti-CD80 (Becton Dickinson, Mountain View, CA), anti-CD14, anti-CD83 (both Beckman Coulter, Mijdrecht, The Netherlands), and anti-CD86 (Pharmingen, San Diego, CA). For immunohistochemistry the following mAb were used: HMB-45 (Dako, Glostrup, Denmark) against gp100 and T311 (Novocastra, Newcastle, UK) against tyrosinase.

**Delayed type hypersensitivity**

One to two weeks after each of the three cycles of DC vaccinations a delayed type hypersensitivity (DTH) skin test was performed6. Briefly, unpulsed DCs, DCs pulsed with peptides (tyrosinase
and/or wildtype gp100:154 plus wildtype gp100:280), DCs pulsed with KLH, and DCs pulsed with peptides plus KLH (2x10^5 DCs each) were injected i.d. in the skin of the back of the patients at four different sites. The diameter (in millimeters) of induration was measured after 48 hours, each time by the same investigator (M.J.P.G.), and an induration of more than 2 mm was considered positive. From positive DTH sites, punch biopsies (6 mm) were obtained under local anesthesia. Biopsies were cut in half, one part for immunohistochemistry and the other part was cut in small pieces and cultured in RPMI/7%HS supplemented with IL-2 (100 U/ml). Every 7 days, half of the medium was replaced by fresh IL-2-containing RPMI/7%HS. After 2 to 4 weeks of culturing, T cells were tested for antigen recognition in a cytotoxicity assay or tested for tetramer binding.

Humoral response to KLH
Antibodies against KLH were measured in the serum of vaccinated patients by ELISA as previously described. Briefly, 96-well plates were coated overnight at 4°C with the protein KLH (25 μg/ml) in phosphate buffered saline (0.1 ml/well). After washing the plates, different concentrations of patient serum (range 1 in 100 to 1 in 500,000) were added for 1 hour at room temperature. After extensive washing, specific Ab (total IgG, IgG1, IgG2, and IgG4) labeled with horseradish peroxidase were allowed to bind for 1 hour at room temperature. Peroxidase activity was revealed using 3,3',5,5-tetramethyl-benzidine as substrate and measured in a microtiter plate reader at 450 nm. A positive signal at a 1 in 400 dilution of the patients’ serum was considered positive.

Proliferative response and cytokine production to KLH
Cellular responses against KLH were measured in a proliferation assay. Briefly, 1x10^5 PBMC, isolated from blood samples taken before each vaccination, were plated per well of a 96-well tissue culture micro plate either in the presence of KLH or without. After 16 hours of culture, supernatants (50 μl) were taken and IL-2, IL-4, IL-5, IL-10, TNFα, and interferon (IFN)γ were measured by a cytometric bead array (Th1/Th2 Cytokine CBA 1; BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. After 4 days of culture, 1 μCi/well of tritiated thymidine was added, incorporation of tritiated thymidine was measured in a beta-counter. A proliferation index >2 was considered positive.

MHC tetramer staining
Tetrameric-MHC complexes were kindly provided by dr. R Luiten and dr. H. Spits from the Netherlands Cancer Institute (Amsterdam, The Netherlands). Each tetramer was validated by staining against a CTL line specific for HLA-A2 in association with the peptide of interest. PBMC (1x10^5 cells in 10 μl) were incubated with PE-labeled tetrameric-MHC complexes for 1 hour at RT. After washing the samples were analyzed by flow cytometry.

Antigen recognition
Antigen recognition was determined by the production of cytokines and cytotoxic activity of DTH-derived cells in response to T2 cells pulsed with the indicated peptides or BLM (a melanoma cell line expressing HLA-A2.1 but without endogenous expression of gp100 and tyrosinase), transfected with control antigen G250, with gp100 or tyrosinase, or an allogeneic HLA-A2.1-positive, gp100-positive, and tyrosinase-positive tumor cell line (MEL624). Cytotoxic activity was measured using the chromium release assay. Cytokine production was measured in supernatants after 16 hours of coculture by the cytometric bead array (Th1/Th2 Cytokine CBA; BD Pharmingen).

Analysis of T cell frequencies in peripheral blood
The presence of tumor antigen-specific CD8+ T cells was analyzed using mixed lymphocyte-peptide cultures as described previously. Briefly, PBMC isolated before and after DC vaccination were thawed, divided over three groups, and incubated for 1 h at room temperature in Iscove’s medium (Life Technologies, Carlsbad, CA, USA) with 1% HS and 10 M of tyrosinase:369 peptide (YMDGTMSQV), wild-type gp100:154 peptide (KTIWGQYWQV), or wild-type gp100:280 peptide (YLEPGPVVTA). Next, these pulsed cells were washed, pooled, and distributed at 2x10^5 cells/0.2 ml in round-bottom microwells in Iscove’s with 10% HS and 10 M of tyrosinase:369 peptide. On day 7, 50% of the medium was replaced by fresh medium containing IL-2 and wild-type gp100:154 peptide. Wild-type gp100:280 peptide was added to the cultures at day 8 and tyrosinase:369 peptide at day 9. Tetramer labeling was performed on day 14 as described previously.
Statistical analysis
Data were analyzed statistically by means of analysis of variance and Student-Newman-Keuls test, or by means of Mann Whitney U nonparametric statistics. Statistical significance was defined as p<0.05.

RESULTS

Patient characteristics
Twenty-seven patients were included in the study. In the first DC vaccination cycle, 15 patients received wild-type peptide-pulsed DCs (supplementary figure 1, partly previously reported in ref. 4), and 12 received modified peptide-pulsed DCs. The clinical characteristics are presented in table 1. Median age in the wild-type group was 54 years (22-73), in the modified group 48 years (30-70). All patients had performance status 0, according to WHO criteria. Serum lactate dehydrogenase (LDH) was elevated in 4 patients in the wild-type group and in 4 patients in the modified group. According to AJCC criteria 9 patients had stage M1c, 4 M1b and 2 M1a in the wild-type group and 7 M1c and 4 M1b in the modified group, respectively. In the modified group 1 patient with stage IIIC disease was incorrectly included.

Characteristics and number of injected DCs
Patients vaccinated with wild-type peptide-pulsed DCs received an average of 27x10^6 DCs intravenously (i.v.) and 11x10^6 DCs intradermally (i.d.) per vaccination during the first cycle. Patients vaccinated with modified peptide-pulsed DCs received an average of 22x10^6 DCs i.v. and 12x10^6 DCs i.d. (table 2). After maturation, DCs of all patients showed a mature phenotype with high HLA-ABC, HLA-DR/DP, HLA-DQ, CD80, CD83, and CD86 expression (supplementary figure 1). There was no difference in the amount of injected DCs (p=0.3 for i.v. injected DCs, p=0.7 for i.d. injected DCs) or maturation status (p=0.2 to p=0.4), except for CD80 (p=0.02) and CD83 (p=0.01) that were expressed at lower levels in DCs pulsed with wild-type peptide.

Figure 1. Computer Tomography scanning images of the abdomen of patient mod-4.
The images show multiple liver metastases (upper image, arrows), which regressed after the first cycle of DC vaccinations and showed complete remission after the peptide vaccinations (lower image).

Clinical outcome and toxicity
Toxicity was limited to grade I-II fever, fatigue, flue-like symptoms and injection site reactions, which always resolved within 2 days after vaccination.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex</th>
<th>AJCC-stage</th>
<th>Localization &amp; Metastases</th>
<th>LDH</th>
<th>Response</th>
<th>PFS (m)</th>
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</tr>
<tr>
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<td>73/m</td>
<td>IV-M1b</td>
<td>Lung, skin</td>
<td>-</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>wt-2</td>
<td>56/m</td>
<td>IV-M1b</td>
<td>Lung</td>
<td>-</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>wt-3</td>
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<td>bulky LN</td>
<td>-</td>
<td>SD</td>
<td>13</td>
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<tr>
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<td>35/f</td>
<td>IV-M1c</td>
<td>Kidney, spleen, LN</td>
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<td>4.5</td>
</tr>
<tr>
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<td>IV-M1c</td>
<td>Sc, gut</td>
<td>-</td>
<td>PD</td>
<td>-</td>
</tr>
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<td>Sc, testicle</td>
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<td>SD</td>
<td>4</td>
</tr>
<tr>
<td>wt-8</td>
<td>59/m</td>
<td>IV-M1c</td>
<td>Bulky LN, sc</td>
<td>+</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>wt-9</td>
<td>33/f</td>
<td>IV-M1a</td>
<td>LN</td>
<td>-</td>
<td>SD</td>
<td>&gt;124</td>
</tr>
<tr>
<td>wt-10</td>
<td>63/m</td>
<td>IV-M1b</td>
<td>LN, lung</td>
<td>-</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>wt-11</td>
<td>72/m</td>
<td>IV-M1c</td>
<td>Liver</td>
<td>+</td>
<td>SD</td>
<td>8</td>
</tr>
<tr>
<td>wt-12</td>
<td>52/m</td>
<td>IV-M1a</td>
<td>Sc</td>
<td>-</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>wt-13</td>
<td>34/m</td>
<td>IV-M1b</td>
<td>Lung, LN</td>
<td>-</td>
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<td>IV-M1c</td>
<td>Liver</td>
<td>-</td>
<td>SD</td>
<td>6</td>
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<tr>
<td>wt-15</td>
<td>51/m</td>
<td>IV-M1c</td>
<td>Lung, sc</td>
<td>+</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>Modified group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mod-1</td>
<td>48/f</td>
<td>IIIc</td>
<td>LN, in-transits</td>
<td>-</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>mod-2</td>
<td>55/f</td>
<td>IV-M1c</td>
<td>Sc, LN, soft tissue</td>
<td>+</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>mod-3</td>
<td>43/m</td>
<td>IV-M1c</td>
<td>Sc, LN, soft tissue</td>
<td>+</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>mod-4</td>
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<td>IV-M1c</td>
<td>Liver</td>
<td>-</td>
<td>CR</td>
<td>&gt;100</td>
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<tr>
<td>mod-5</td>
<td>38/f</td>
<td>IV-M1c</td>
<td>Lung, liver, sc, LN</td>
<td>+</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>mod-6</td>
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<td>IV-M1b</td>
<td>Lung</td>
<td>-</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>mod-7</td>
<td>48/f</td>
<td>IV-M1c</td>
<td>Skin, liver, LN</td>
<td>+</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>mod-8</td>
<td>47/f</td>
<td>IV-M1b</td>
<td>Skin, lung</td>
<td>-</td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>mod-9</td>
<td>30/m</td>
<td>IV-M1c</td>
<td></td>
<td></td>
<td>PD</td>
<td>-</td>
</tr>
<tr>
<td>mod-10</td>
<td>70/m</td>
<td>IV-M1c</td>
<td>LN, skin, lung, adrenal</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>mod-11</td>
<td>52/f</td>
<td>IV-M1b</td>
<td>Lung, sc</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>mod-12</td>
<td>56/m</td>
<td>IV-M1b</td>
<td>Lung</td>
<td>-</td>
<td>SD</td>
<td>4</td>
</tr>
</tbody>
</table>

**Table 1. Patient characteristics.** 1Abbreviations: m, male; f, female; sc, subcutaneous; LN, lymph nodes; PD, progressive disease; SD, stable disease; CR, complete remission. 2Partial remission of a distant LN metastasis, CR after surgery for >10 years
In the wild-type group one patient (wt-9) had a 27% reduction in diameter of a distant lymph node metastasis, which was resected after the first maintenance vaccination cycle. This patient completed treatment according to protocol, and is free of disease at 10 years of follow up. Six patients in the wild-type group had stable disease for at least 4 months (4; 4.5; 4.5; 6; 8 and 13 months).

In the modified group one patient (mod-4) had a complete response of multiple histologically proven liver metastases after the first vaccination cycle (figure 1). This patient also completed protocol treatment and is currently free of disease at 8 years of follow up. One patient had disease stabilisation of 4 months duration.

<table>
<thead>
<tr>
<th>野型</th>
<th>改変型</th>
</tr>
</thead>
<tbody>
<tr>
<td>before</td>
<td>after 1 vacc.</td>
</tr>
<tr>
<td>1000</td>
<td>100</td>
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<tr>
<td>P=0.6</td>
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</table>

Figure 2. KLH-specific proliferation of PBMCs before and after DC vaccination.

Patients were vaccinated with DCs loaded KLH together with tyrosinase peptide and either wild-type (filled circles) or modified (open circles) gp100 peptides. KLH-specific proliferation was measured in PBMCs isolated before the first vaccination and after one, two, or three vaccinations. Proliferative response to KLH is given as proliferation index (proliferation +KLH/proliferation − KLH).

Proliferation and cytokine production of PBMCs upon stimulation with KLH

To investigate whether DCs loaded with modified or wild-type gp100 peptides have similar capacity to activate the patient’s immune system in general, humoral and cellular responses to the control protein KLH were measured in the peripheral blood of patients after each cycle of vaccination. Most patients mounted a potent proliferative and antibody response against KLH (table 2). KLH-specific IgG antibodies were induced after vaccination in 8 of 14 patients tested (57%) in the wild-type group and in 6 of 11 patients tested (55%) in the modified group. PBMCs collected after each DC vaccination were analyzed for the presence of KLH-reactive T cells in a proliferation assay. In the wild-type group 13 out of 14 patients tested showed a cellular response to KLH, whereas in the modified group a cellular response was induced in all patients (table 2). The magnitude of KLH-specific proliferation was similar in the wild type and modified group (figure 2). There were no significant differences between the groups at any time-point after vaccination (p=0.3 before vaccination; p=0.6 after vaccination 1; p=0.6 after vaccination 2; p=0.2 after vaccination 3). Interestingly, the patient that had no proliferative response (patient wt-8) did show an antibody response to KLH. Thus, all patients developed a response to KLH, either cellular or humoral, and there are no differences in KLH-specific immune responses after vaccination with DCs loaded with wild-type or modified gp100 peptides.
<table>
<thead>
<tr>
<th>patient no.</th>
<th>injected DCs (x10^6) (i.v./i.d.)</th>
<th>proliferative response against KLH</th>
<th>humoral response (IgG) against KLH</th>
<th>tetramer positive CD8+ T cells in DTH</th>
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<td></td>
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</tr>
<tr>
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<td>12/12</td>
<td>10/10</td>
<td>+++</td>
</tr>
<tr>
<td>wt-2</td>
<td>70/25</td>
<td>50/17</td>
<td>60/30</td>
<td>+++</td>
</tr>
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<td>30/14</td>
<td>18/8</td>
<td>20/10</td>
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<td>wt-7</td>
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<td>wt-9</td>
<td>30/7</td>
<td>30/8</td>
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</tr>
<tr>
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<td>13/7</td>
<td>+++</td>
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<td>30/15</td>
<td>20/10</td>
<td>20/10</td>
<td>+</td>
</tr>
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</tr>
<tr>
<td>mod-1</td>
<td>20/10</td>
<td>16/8</td>
<td>24/12</td>
<td>+++</td>
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<td>26/12</td>
<td>23/12</td>
<td>22/11</td>
<td>+++</td>
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<td>38/19</td>
<td>31/16</td>
<td>+</td>
</tr>
<tr>
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<td>-</td>
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</tr>
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<td>24/12</td>
<td>15/8</td>
<td>17/9</td>
<td>+++</td>
</tr>
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</table>
Table 2. Vaccine-induced antigen-specific immune responses (previous page). 1Proliferative response to KLH is given as proliferation index (= proliferation +KLH/–KLH). + 2>proliferation index (PI)<10; ++ 10>PI<20; +++ PI>20, at least one time point after vaccination. 2 Antibody titer, total IgG, + designates >1 in 400 times diluted serum. 3 Presence of functional tetramer-positive T cells in DTH is marked as +; 4a Ag-recognition of DTH derived T cells after stimulation with T2 cells loaded with the gp100 or tyrosinase peptides (peptide recognition), BLM transfected with the antigens (protein recognition) or a gp100 and tyrosinase-expressing tumor cell line (tumor recognition) as analyzed by IFNg-production; 4b Ag-recognition analyzed by cytotoxic activity (chromium release assay). Abbreviations: i.v. intravenously; i.d. intradermally; no, no outgrowth or outgrowth of less than 1x10^5 DTH infiltrating lymphocytes; n.t. not tested.

These results indicate that the immunogenicity of the DCs were comparable for both groups and therefore a possible difference in gp100 T cell reactivity upon vaccination between the two groups is unlikely to be the result of differences in the preparation and administration of the DCs.

Tumor antigen-specific T cell responses in post-treatment DTH

To investigate the immune response against tumor peptides generated in vaccinated patients, DTH challenges were performed with mature DCs loaded with tyrosinase and/or wild-type gp100 peptides. For patients receiving a second and third cycle of vaccination, the best immunological response over all received cycles of vaccinations was scored. In the group of patients vaccinated with wild-type peptide-loaded DCs, 11 of 13 patients tested (85%) showed indurations up to 22 mm against DCs pulsed with KLH and/or peptide. In the group vaccinated with modified peptide-loaded DCs, 9 of 10 patients tested (90%) showed indurations up to 23 mm against DCs pulsed with KLH and/or peptide. Biopsies were taken from DTH induration sites, which were cultured in low amounts of IL-2 without the addition of antigen. Outgrowth of DTH-infiltrating lymphocytes occurred in all the biopsies taken from patients treated with wild-type gp100, whereas only 63% of the biopsies in the modified group showed DTH-infiltrating lymphocytes outgrowth. The lack of outgrowth occurred in all different DTH, there was no preference for a DTH induced with a certain peptide. From 9 patients in the wild-type group sufficient T cells were available after short time culture (i.e. 2-4 weeks) to perform tetramer staining and functional assays (table 2). Biopsies of two patients (wt-4 and wt-9) contained gp100- and tyrosinase-tetramer positive CD8+ T cells that recognized gp100 or tyrosinase peptide and protein (previously shown in ref. 4). Tetramer staining and functional assays could be performed on DTH-infiltrating lymphocytes of four patients in the modified group. Only T cells derived from biopsies of patient mod-4 contained tetramer positive CD8+ T cells (figure 3a). These CD8+ T cells recognized gp100 or tyrosinase peptides, however, not the complete protein (figure 3b).
Figure 3. Tumor antigen-specific T cell responses in post-treatment DTH. (a) Tetramer analysis by flow cytometry of T cells derived from a biopsy of positive DTH reaction (DCs loaded with KLH and peptides gp100:154, gp100:280 and tyrosinase) of patient mod-4. Tetramer staining of 1 log above the negative population was considered positive. (b) IFN production by T cells of patient mod-4 derived from a biopsy of a positive DTH site (DCs loaded with peptides gp100:154, gp100:280 and tyrosinase) cocultured with T2 cell pulsed with gp100 peptides or tyrosinase peptides.

Thus, although the majority of patients in both the wild-type and modified group showed DTH-infiltrating lymphocytes at DTH sites, we could detect specific and functional CD8+ T cells in only one patient in the modified group and two patients in the wild-type group. Interestingly, these three patients also showed the best clinical outcome, with progression-free survival ranging from 13 (wt-4) to >124 (wt-9) months. In comparison, the progression-free survival of the patients without specific T cell reactivity in DTH sites ranged from 0 to 8 months.

Tumor antigen-specific T cell responses in peripheral blood

The presence of gp100- or tyrosinase-specific CD8+ T cells in blood was studied in 8 patients in the wild-type group by tetramer staining (data not shown). Although we detected gp100- and tyrosinase-specific CD8+ T cells in patient wt-4 by tetramer analysis, these T cells did not recognize gp100 or tyrosinase peptides or protein as determined by IFN production upon coculture with gp100/tyrosinase-loaded target cells. We could not detect tumor antigen-specific T cells in blood of the other patients tested.

The frequencies of antigen-specific T cells in blood are generally very low, and often we were unable to detect antigen-specific T cells in peripheral blood by direct tetramer staining, while functional tumor antigen-specific T cells were present in biopsies taken from DTH challenges. To further examine anti-gp100 and anti-tyrosinase CTL responses, we analyzed precursor frequencies of tumor antigen-specific CD8+ T cells in peripheral blood before and after one cycle of three bi-weekly DC vaccinations. These studies were performed in the three clinically responding patients with specific T cells in the DTH biopsies (wt-4, wt-9, mod-4) and in three non-responding patients (wt-5, mod-7, mod-12). Before vaccination, CTLp frequencies ranged from 1.7/10^7 to 1.6/10^5 of total blood CD8+ T cells (figure 4). CD8+ T cells directed against gp100:154 showed the highest frequencies in both clinical responders and non-responders before vaccination.
Fig. 4 Frequencies of tumor antigen-specific CD8^+ T cells in peripheral blood. CTLp frequencies were analyzed in peripheral blood of three DTH-responders (patients mod-4, wt-4, and wt-9) and three non-responders (patients mod-7, mod-12, and wt-5) before vaccination and after one cycle of three DC vaccinations by in vitro restimulation of PBMCs with antigenic peptides in limiting dilutions followed by tetramer analysis. CTLp frequencies could not be tested (NT) in patient wt-5 after vaccination due to lack of outgrowth after peptide stimulation.

After DC vaccination, frequencies of gp100:154- and tyrosinase-specific CTLs increased in all three responders, up to 31-fold, whereas frequencies of gp100: 280-specific CTLs increased only in patients mod-4 and wt-4. CTLp frequencies of non-responders increased only modestly after DC vaccination. Thus, the increase of peripheral blood T cell precursor frequencies correlate with the presence of tumor antigen-specific T cells in DTH biopsies upon vaccination.

**DISCUSSION**

In this study we compared mature DCs loaded with wild-type or modified gp100 peptides in their ability to induce immunological and clinical responses in metastatic melanoma patients. Although the number of patients in our study is limited, we observed no obvious advantage for the use of modified versus wild-type peptides in either endpoint.

Since both groups of patients were vaccinated with DCs that were matured and loaded with KLH in a similar way, no differences in KLH responses were expected. Indeed, patients vaccinated with wild-type or modified gp100 peptides showed similar cellular and humoral responses to the control protein KLH. In addition, inductions in DTH challenges were comparable in both groups. As reported before, induction was not predictive for clinical outcome. Outgrowth of DTH biopsies occurred more often in the wild-type group than in the modified group. In both the wild-type group and the modified group DTH challenges were performed with wild-type gp100 peptides. This may explain the lack of outgrowth in the modified group, although lack of outgrowth occurred not only in DTH challenges performed with gp100 peptides, but also in DTH challenges performed with tyrosinase peptide.

We observed tumor-specific T cells in DTH biopsies of only three patients, two vaccinated with wild-type peptides and one vaccinated with modified peptides. In one patient vaccinated with modified peptides during the first cycle, the best immunological response was found after the second cycle of DC vaccinations with wild-type peptide-loaded DCs. Interestingly, the best clinical outcome was observed in these three patients in whom tumor-specific T cell responses of DTH-
infiltrating lymphocytes were observed. This is in accordance with our previous findings that the presence of tumor-specific T cells in DTH biopsies correlates with clinical outcome.

For other modified peptides (gp100:209-217 and MART:26-35) it has been described that only a minority (~15-25%) of T cells raised against modified peptides cross-reacts with the wild-type epitope. However, for modified gp100:154 and gp100:280 we and others have demonstrated that in vitro and in HLA-A2.1 transgenic mice that CTLs raised against modified peptides recognized wild-type peptides. Our CTLp frequency analysis, which was performed with wild-type gp100 peptides, showed no differences in T cell expansion between the wild-type and modified group in the DTH-responders. All three patients showed increased precursor frequencies of gp100:154-specific CTLs. Two of these patients, one vaccinated with wild-type and one with modified peptides, showed a comparable increase in gp100:280-specific CTLs after one round of DC vaccinations. This suggests that vaccination with modified peptide raised CTLs cross-reacting with wild-type tumor antigens.

Recently, Speiser et al. demonstrated that, although vaccination with modified peptides resulted in higher T cell frequencies than vaccination with natural wild-type peptides, these T cells have lower tumor reactivity as demonstrated by lower avidity for the natural antigen and decreased T cell activation and effector function. This is in contrast with previous in vitro and mouse studies demonstrating that CTLs raised against modified peptides could recognize both wild-type and modified peptides as well as gp100-expressing melanoma cells. However, based on the results of Speiser et al, it may be beneficial to first vaccinate patients with mature DCs loaded wild-type peptides, which activates CTLs with strong tumor reactivity, followed by injection with mature DCs loaded with modified peptides in the second round of vaccination to improve expansion of these CTLs, thus inducing high number of functional tumor antigen-specific CTLs.

In conclusion, vaccination with peptide-loaded DCs results in clinically meaningful responses in a minority of metastatic melanoma patients, however, the use of modified as compared to wild-type gp100 peptides for DC loading does not result in relevant enhanced immune responses.

ACKNOWLEDGEMENTS

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REFERENCES

cells to lymph nodes in melanoma patients is determined by their maturation state. Cancer Research 63: 12-17
Supplementary figure 1. Study scheme and patient treatment.
Vaccination of colorectal cancer patients with CEA-loaded dendritic cells: antigen-specific T cell responses in skin tests


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Vaccination of colorectal cancer patients with CEA-loaded dendritic cells: antigen-specific T cell responses in DTH skin tests

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Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system. As such they are currently used in clinical vaccination protocols in cancer patients. We evaluated the ability of mature DCs pulsed with carcinoembryonic antigen (CEA)-peptide to induce CEA-specific T cell responses in patients with resectable liver metastases from colorectal cancer. CEA-specific T cell reactivity was monitored in peripheral blood, biopsies of vaccination sites and post-treatment DTH skin tests, and when available also in resected abdominal lymph nodes and tumor tissue.

Ten patients were vaccinated intradermally and intravenously with CEA-peptide pulsed mature DCs three times prior to resection of liver metastases. High numbers of CEA-specific T cells were detected in post-treatment DTH biopsies in seven out of 10 patients, which produced high amounts of interferon (IFN)-γ upon stimulation with CEA-loaded target cells. These responses were not found in biopsies of first vaccination sites, indicating a de novo T cell induction or at least a strong potentiation by the vaccine. In addition, CEA-specific T cells were detected in a resected lymph node in one patient, but not in peripheral blood or tumor tissue. Thus, vaccination with CEA-peptide loaded mature DCs induced potent CEA-specific T cell responses in advanced colorectal cancer patients. In this study, antigen-specific T cell responses were readily detected in DTH skin tests, much less in abdominal lymph nodes, and not in peripheral blood and tumor tissue.

Although progress has been made in the treatment of metastatic colorectal cancer using chemotherapy and targeted therapies, clinical outcome remains poor. Immunotherapy forms an alternative approach for the treatment of colorectal cancer. Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system. They are essential for the induction of antigen-specific T cell immunity and as such they have great potential for cancer immunotherapy. DC vaccination has shown to be feasible and safe, and both immunological and clinical responses have been reported.

Most clinical studies using DCs have been performed in melanoma patients. Only a few small studies investigated the immunogenicity of DC vaccines in colorectal cancer patients with variable results. The majority of clinical studies in cancer patients have been carried out using immu-
tured rather than mature DCs. We, as well as others, have demonstrated in comparative studies that only mature DCs are able to stimulate T cell responses14-16. These findings should be considered when interpreting recent negative clinical trials comparing immature DCs with other vaccination modalities17,18. Other pivotal questions, including the optimal DC culture method, dose, route of administration, etc., remain unanswered to date. To start to address these questions in small cohorts of patients, there is a need of validated assays that can monitor immunological outcome, since clinical responses occur in only a minority of patients. Most studies so far have focused on the monitoring of antigen-specific T cell responses in peripheral blood which, however, often requires in vitro restimulation due to low precursor frequencies. Tumor tissue and lymph nodes would be more interesting compartments to monitor these responses, but these tissues are often not available. We recently described a novel method of monitoring antigen-specific T cell responses: a short culture of biopsies of post-treatment delayed type hypersensitivity (DTH) reaction sites allowed a detailed analysis of tumor antigen-specific CD8+ T cell responses in stage IV melanoma patients19. We found that the presence of vaccine-induced melanoma-specific T cells in these biopsies significantly correlated with an improved clinical outcome.

Carcinoembryonic antigen (CEA) is expressed in almost all colorectal cancers, making it an attractive antigen for immunotherapy in patients with this tumor type 20. Here, we investigated the immunogenicity of CEA-peptide loaded mature DCs in colorectal cancer patients with resectable liver metastases. This model provided the unique opportunity to rigorously monitor and compare immune responses after vaccination in several relevant compartments of the body: peripheral blood, lymph nodes, tumor tissue and biopsies of DTH skin tests and vaccination sites.

MATERIALS AND METHODS

Study design
This was an open-label, single-institution, single-arm exploratory study in which monocyte-derived mature DCs loaded with CEA-peptide were administered to patients with resectable liver metastases of colorectal cancer (figure 1A). Approval from the local regulatory committee was obtained.

Objectives
The primary end point was to assess the immunogenicity of the vaccine. Secondary end points were the toxicity and the feasibility of CEA-specific vaccination in colorectal cancer patients and, specifically, the feasibility of using DTH-infiltrated lymphocytes in the monitoring of T cell responses in comparison with other relevant body compartments.

Patients
Inclusion criteria included: patients with liver metastases from CEA-expressing colorectal cancer scheduled for surgical resection, HLA-A0201 phenotype, Eastern Cooperative Oncology Group (ECOG) performance status 0—1, age ≥18 years, no clinical signs of extra-hepatic metastases, no prior chemotherapy, immunotherapy or radiotherapy within 2 months before planned surgery.

DC preparation
DCs were generated as described previously15, 21 with several modifications (figure 1a). Patients donated 0.5 l blood, from which a buffycoat was made (Sanquin Bloodbank Nijmegen, the Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated by PureCell (Medicult, Denmark) density gradient centrifugation (30 min, 4°C, 2100 rpm), adherent monocytes were cultured in Cellgro medium enriched with 500 U/ml interleukin (IL)-4 and 800 U/ml granulocyte macrophage colony stimulating factor (GM-CSF, all CellGenix, Freiburg, Germany). KLH (10 μg/ml; Calbiochem, USA) was added at day 3 of culture, and 2 days before harvesting we added the maturation cocktail [IL-4 (500 U/ml), GM-CSF (800 U/ml), prostaglandin E2 (PGE2; Pharmacia & Upjohn, Puurs, Belgium, 10 μg/ml), tumor necrosis factor alpha (TNF-α, 10 ng/ml), IL-1b (5 μg/ml) and IL-6 (15 ng/ml; all CellGenix)]. Cells
were harvested at day 7 and approximately 25% of the cells were put in a syringe for immediate vaccination; the remaining cells were frozen for the second and third vaccination and the DTH. From each batch of patient DCs, a sample was used for quality control. Release criteria were as previously described. DCs were pulsed with the wild type CEA-peptide CAP-1 (CEA571–579, YLSGANLNLI, Clinalfa, Switzerland) directly after harvesting or after thawing.

**Treatment schedule**

Vaccinations were administered three times at day 0, 7 and 14 (figure 1a). The patients received 5 x10^6 DCs intradermally (i.d.) in the upper leg, 5–10 cm from an inguinal lymph node. The remaining cells were given intravenously (i.v.) as a bolus injection at the same time point. If the patient consented, a biopsy was taken from the first vaccination site at day 7. On day 26 a post-treatment DTH test was performed (see below). At day 28, surgical resection of the liver metastases was performed. During the operation an accessible abdominal lymph node was excised and 6 mm punch biopsies were taken from the post-treatment DTH reaction sites. Before and after vaccination PBMCs were obtained. The same schedule of 3 weekly i.v./i.d. vaccinations followed by a post-treatment DTH was repeated twice at intervals of 6 months in the absence of recurrent disease.

**Immunologic monitoring**

CD4+ T cell responses against KLH were measured using a 3H-thymidine incorporation proliferation assay with PBMCs of the patients before and after vaccination. The index was calculated as the counts ratio between KLH-stimulated PBMC and non-stimulated PBMC. Post-treatment DTH reactions were performed as described previously. Briefly, CEA-peptide only (100 μg in 100 μl), DCs pulsed with CEApeptide, DCs pulsed with KLH and CEA-peptide, and DCs pulsed with KLH only (0.4–5x10^6 DCs each in approximately 100–200μl) were injected i.d., 5–10 cm from an inguinal lymph node at different sites, in the upper leg contralateral from the leg in which the DC vaccinations were performed. The maximum diameter of induration was measured after 48 h. T cell culture from DTH biopsies was performed in low dose IL-2 (100 U/ml; Proleukin, Chiron, the Netherlands) for approximately 2 weeks without ex vivo restimulation with antigen as described before.

Resected lymph nodes were disrupted and cell suspensions were made by gentle squeezing in a sterile open filter chamber (NPBI; Amsterdam, the Netherlands) in IMDM medium (Invitrogen; Paisly, UK) supplemented with 5% human serum (Sanquin; Nijmegen, the Netherlands). The cell suspension (2x10^6) was plated on a 24-well plate (Costar Badhoevedorp, the Netherlands; 5x10^5 cells/well), preincubated with sterile anti-CD3 ascites 16A9. When the cells were seeded, 1 μg sterile anti-CD28 (Immunotech, Marseille, France) in 1 ml RPMI/7% human serum was added. After 1 day medium was added, containing IL-2 (100U/μl), which was repeated every 3 days. T cells were tested after 1–2 weeks of culture. Tumor-infiltrating lymphocytes (TILs) were obtained and cultured according to the same protocol as the lymph node cell suspension (see above). DTH-derived cells (1x10^5 cells in 10 μl) or PBMC (1x10^6 cells in 10 μl) were incubated with PE-labeled CEA and cytomegalovirus (CMV) tetrameric-MHC complexes (Sanquin; Amsterdam, the Netherlands) for 60 min at room temperature. In the last 20 min of this incubation, fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies directed against CD8 (Becton Dickinson) were added. After washing, the samples were analyzed by flow cytometry. For peripheral blood at least 1x10^6 PBMCs were analyzed, for DTH-infiltrating T cells all available cells were analyzed. In all analyses CEA tetramer staining was compared with CMV tetrans as a negative control.

Production of cytokines by the total population of DTH-derived cells was measured in response to T2 cells pulsed with CEA-peptide or an irrelevant HLA-A2.1 binding peptide (tyrosinase or G250). Cytokine production was measured in supernatants after 16h by cytometric bead array (Th1/Th2 Cytokine CBA 1; BD Pharmingen). IFN-γ production was considered positive when IFN-γ levels of DTH-infiltrating T cells were more than 10-fold higher after co-incubation with CEA-loaded T2 cells compared with co-incubation with T2 cells loaded with irrelevant antigen.
Patient evaluation
Clinical follow-up consisted of history, physical examination, serum CEA-level, CT scanning of the liver and X-ray of the chest at 3-month intervals.

RESULTS

Patients
Ten patients were included (Table 1). Median age was 60 years (range 44–78); all patients had a performance status of WHO 0. Baseline serum CEA levels were abnormal in seven patients (median 17 μg/l, range 5.8–36.6). Six patients had synchronous liver metastases, four had metachronous liver metastases with intervals of 9, 10, 15 and 22 months, respectively, since surgery for the primary tumor.

Clinical outcome and toxicity
Toxicity consisted of mild flu-like symptoms. One patient (patient 5) had grade 2 diarrhea after the third vaccination during 3 days, which subsided following loperamide treatment. Of the 10 vaccinated patients, one showed evidence of lung metastases shortly before surgery (patient 5; table 2), upon which the procedure was cancelled, DTH biopsies were obtained, and the patient received chemotherapy. The remaining nine patients underwent surgery. Three appeared to have irresectable disease during surgery (patients 2, 3 and 10; table 2), surgery was aborted and patients received chemotherapy. Radical resection was performed in six patients. One of these (patient 7; table 2) had a relapse after 3 months. All other patients have no evidence of disease after a median follow-up of 14 months (range 3–23 months). To date, four patients have received additional vaccination cycles for maintaining immune responses (patients 1, 4, 6 and 8; table 2).

DC vaccine characteristics
In all patients sufficient amounts of buffy-coat monocytederived mature DCs could be obtained for three i.d. vaccinations (table 1). However, the yield was not always sufficient for simultaneous i.v. injections (see, for example, patient 1 or 5 in Table 1). Final DC vaccine products met the criteria of a mature phenotype5,15. All patients had a proliferative peripheral blood CD4+ T cell response against the control protein KLH, as expected for a mature DC vaccine15, indicating that the DCs were able to induce de novo T cell responses.
Table 1. Number of DCs injected per vaccination and induration (mm) of vaccination sites and post-treatment DTH skin tests

<table>
<thead>
<tr>
<th>Patient</th>
<th>Number of vaccinated DCs</th>
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<td>III</td>
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<td>9</td>
<td>4/0</td>
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The results of a representative patient who received three vaccination cycles are shown in figure 1b.

**CEA-responses in blood, tumor tissue and lymph nodes**

In none of the patients could an increase in CEA-specific T cells be detected in unstimulated peripheral blood by direct tetramer analysis (table 2). CEA-specific T cells were observed in one patient, however, these cells were also present before vaccination (supplementary figure 1d-e; patient 5). In five out of the nine patients who underwent resection of the metastases, low numbers of TILs were obtained. No specific anti-CEA or antitumor reactivity was observed by tetramer analysis and cytokine secretion (table 2). In five patients an abdominal lymph node was resected: a coeliac node (patient 1), a mesenteric node (patients 2 and 4) and a node of the hepatoduodenal ligament (patients 3 and 6). In one of the hepatoduodenal ligament nodes, which are considered the tumor-draining lymph nodes in case of liver metastases, a clear population of CEA-specific T cells was detected (patient 3, table 2, figure 2c). These T cells were able to produce IFN-γ and IL-2 when co-cultured with CEA-peptide loaded T2 target cells (figure 2c). In the other four patients, in whom a lymph node could be resected, no CEA-specific T cell reactivity could be observed (table 2).

**Post-treatment DTH: induration**

The induration sizes of the vaccination sites and the post-treatment DTH reaction skin tests that were performed with CEA-peptide alone or DCs loaded with CEA-peptide, KLH or both, are listed in table 1. Several trends are noteworthy. The first vaccination already induced induration in eight of 10 patients, suggesting that in these patients some form of immunity against CEA already existed before vaccination.
Figure 2. Tetramer analysis and CEA-specific cytokine production of all patients that had specific T cells in at least one of their DTH-sites. In all cases percentages shown in the tetramer analyses figures are percentages of tetramer positive cells of the CD8+ T cell populations. In the cytokine secretion figures, white bars are IFN-γ secretion and black bars are IL-2 secretion upon co-culture with T2 cells, loaded with CEA or an irrelevant peptide. For all tetramer analyses CMV tetramers were used as a control, which were all negative (not shown).
Secondly, in all patients induration size increased after subsequent vaccinations. Thirdly, the induration at the third vaccination site was usually larger compared with the induration at the post-treatment DTH sites, which were performed with lower numbers of the exact same CEA and KLH-loaded DCs (5x10^6 versus 0.05–0.5x10^6 DCs, respectively). Fourthly, post-treatment DTH skin tests that were performed with CEA-peptide alone induced induration in only two of 10 patients. These results suggest that the (amount of) DCs are of importance for induration to occur.

**Post-treatment DTH: CEA-specific T cell immunity**
In seven out of 10 patients, CEA-specific DTH-infiltrated T cells were detected after vaccination by tetramer analyses. Of all patients that had specific T cells in at least one of their DTH-sites an example is shown in figure 2a–h. In table 2 the presence or absence of CEA-specific T cells after vaccination is given per DTH-site for each patient. We found 0.3% (figure 2e) up to 98% (figure 2g) of CD8+ T cells to be CEA-specific in these DTH biopsies. When these T cells were co-cultured with CEA-peptide loaded T2 target cells they were able to produce large amounts of IFN-γ and IL-2 (figure 2a–h), but not IL-4 or IL-10 (not shown).

In none of the five patients in whom a biopsy from the first vaccination site was performed, CEA-specific T cell reactivity was found (table 2; patients 5–7, 9 and 10), despite the presence of induration in four of these patients (table 1; patients 5–7 and 9). This suggests that a possible pre-existing CEA-specific T cell reactivity did not have the potency to result in T cell outgrowth from the biopsy. However, in two of these patients CEA-specific T cells were readily detected in DTH skin tests after treatment (patients 5 and 6; table 2, supplementary figure 1).

In a patient with a history of CMV-infection, we found high numbers of CMV-specific T cells in peripheral blood, but not in a biopsy of a positive DTH reaction skin test (supplementary figure 2a-b). In this patient, no CEA-specific T cells could be detected in peripheral blood, but a large population was readily detected in the DTH biopsy (supplementary figure 2c-d), demonstrating the specificity of the DTH assay.

**DISCUSSION**
We vaccinated 10 patients with liver metastases of colorectal cancer with CEA peptide-pulsed mature monocyte-derived DCs prior to liver metastasectomy. Antigen-specific T cell responses were monitored in peripheral blood, T cell cultures from biopsies of vaccination sites and post-treatment DTH skin tests that were performed in skin sites remote from the injection sites, and when available also in abdominal lymph nodes and tumor tissue.

We here demonstrate that (1) mature CEA-peptide pulsed DCs induce potent CEA-specific T cell responses, (2) vaccination with buffy-coat derived, monocyte-derived DCs is feasible and (3) monitoring antigen-specific T cells in post-treatment DTH skin tests proved more sensitive when compared with blood, abdominal lymph nodes and tumor tissue. Functional CEA-specific T cells in short cultures of posttreatment DTH biopsies without in vitro restimulation could be demonstrated in 7 out of 10 patients.
Table 2. CEA-specific T cell responses after vaccination. The presence of CEA-specific T cells was scored positive (+) if both tetramer analysis and IFNγ release after co-incubation with CEA-loaded target cells were positive. Data from the first vaccination cycle are given. The criteria for determination of positive immune responses are described in patients and methods. Grey cells denote results of which the details are given in figure 2. a In this patient data from the DTH of the second cycle are given. The first post-treatment DTH-culture was not evaluable. b 3rd vaccination site. c In this patient data from the DTH of the second cycle are given. The first post-treatment DTH-culture did not show CEA-specificity. Abbreviations: LN, lymph node; nd, not done (no biopsy taken); ne, not evaluable (no tissue excised); NED, no evidence of disease.

<table>
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<th>patient</th>
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<th>CEA-pep</th>
<th>DC+KLH</th>
<th>DC+CEA</th>
<th>DC+CEA+KLH</th>
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<th>Tumor</th>
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No CEA-specific T cells were present in biopsies of first vaccination sites, indicating that specific T cell reactivity in the post-treatment DTH was induced by the vaccine. In previous reports spontaneous low-grade CEA-specific responses have been found infrequently in metastatic colorectal cancer patients25. The local induration that we observed after the first vaccination, which we have never observed in our melanoma studies using the same DC vaccine15, suggests that some form of pre-existing immunity against CEA was also present in our patients, although apparently this immunity was not strong enough to result in T cell outgrowth from this vaccination site. We cannot, therefore, be certain that de novo induction of anti-CEA immunity is caused by the vaccine; however, it is evident that at least a strong potentiation takes place.

We did not observe a correlation between the induration size of DTH reactions and CEA-specific T cell reactivity. This confirms our previous observations in melanoma patients19. Here, we found that antigen-specific T cell reactivity can even be detected in the absence of induration in a DTH reaction that was performed with CEA-peptide only. The absence of induration in this DTH reaction could be explained by a lack of CD4+ T cells in the absence of DC-produced chemokines and cytokines26. Together these data show that the DTH reaction is a complex assay. Yet, these findings also indicate that for the detection of antigen-specific T cells in cancer immunotherapy it may be beneficial to al-
ways obtain biopsies of DTH-reaction sites irrespective of induration size. We show here that adequate numbers of DCs can be cultured from buffy-coat derived monocytes. Compared to leukapheresis, which is so far the predominantly used method to obtain DCs for clinical vaccination studies, this is a more simple and less laborious procedure with little burden for the patient, although cell yields are lower. Nevertheless we observed CEA-specific IFN-γ producing T cells after three i.d. injections of 3x10^6 DCs only, indicating that low numbers of DCs may suffice. If the migration efficiency of these low numbers of DCs can be improved, this response may be further enhanced. Therefore, the use of buffy-coat derived DCs may facilitate clinical DC vaccination trials. In other studies with CEA-peptide pulsed DCs no CEA-specific immune responses were found or only in a minority of patients, which may be explained by differences in DC maturation state, the culture protocol, patient selection or immunomonitoring methods (peripheral blood only). Fong et al. investigated the immunogenicity of another type of DCs (Flt-3 Ligand expanded blood DCs) pulsed with an altered CEA peptide in patients with CEA-expressing colorectal and non-small-cell lung tumors. In this study, a correlation was found between clinical response and the post-vaccination expansion of CEA-specific T cells in peripheral blood. However, in contrast to our study, several restimulation steps in vitro were necessary for evaluation of the functionality of these cells. In our study, CEA-specific T cells were detected slightly above background in peripheral blood of only one out of 10 patients by direct tetramer staining. However, in this patient these cells were already present before vaccination. From these results it can be concluded that CEA-specificity could be detected in one in 10,000 CD8+ T cells by tetramer staining of peripheral blood mononuclear cells. Therefore, the fact that we could not find CEA-specific T cells in more patients may be due to lower frequencies of these cells in the circulation after vaccination. Perhaps a short in vitro culture period would have allowed the detection of lower CEA-specific T cell frequencies. Nevertheless, very low numbers of high-affinity T cells that are not detectable by means of direct tetramer-staining of peripheral blood may suffice for rejecting tumors in cancer patients, as shown by others. In another study these authors showed higher frequencies of vaccine- and tumor-specific T cells in metastases compared with peripheral blood after MAGE-3 vaccination in a melanoma patient. In our study we did not detect CEA-specific T cell responses in tumor samples. This may be explained by the limited amount of available tumor tissue, the short interval between vaccination and surgical resection or immuno-modulatory mechanisms in the tumor microenvironment that may preclude adequate T cell infiltration. Regional lymph nodes at the vaccination site may be another relevant compartment to monitor antigen-specific T cell responses, as has been shown in murine studies as well as in human melanoma studies. In our study an easily accessible abdominal lymph node was resected in five patients. In one patient functional CEA-specific T cells were found. In most patients no detectable CEA-response was found in lymph nodes, although they were present in biopsies of the DTH skin tests. This may be because the excised lymph nodes were not draining either the DC vaccination sites or the tumor. The small patient numbers and short follow-up of our series does not allow any
correlation between immunological results and clinical outcome. Given the positive correlation between specific T cell reactivity in DTH biopsies and clinical outcome in our previous study with melanoma patients vaccinated with peptide-pulsed DCs, our current results provide further support for the potential of DTH skin testing to monitor specific T cell reactivity upon antigen-specific cancer immunotherapy.

In conclusion, CEA-peptide pulsed mature DCs derived from buffy-coats can induce potent CEA-specific T cell reactivity in advanced colorectal cancer patients. In this study, DTH skin testing provided superior results in the monitoring of antigenspecific T cell responses compared with peripheral blood, abdominal lymph nodes and tumor tissue. These results warrant further studies with CEA-loaded DC vaccines in colorectal cancer.

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REFERENCES


Supplementary figure 1. Tetramer staining and cytokine production of T cells cultured from a DTH reaction site of patient 5 showed CEA-specific T cells after vaccination (b and c) which were not detectable before vaccination (in first vaccination site (a)); At the same time points tetramer analysis of peripheral blood mononuclear cells before (d) and after (e) treatment, revealed no increase in CEA-specific T cells after vaccination, although CEA specificity could be detected in as little as 0.01% of T cells. For all tetramer analyses CMV tetramers were used as a control, which were all negative (not shown).

Supplementary figure 2. Evaluation of CMV and CEA-specific T cell responses in blood and DTH reactions of patient 2 by tetramer analysis after vaccination, demonstrating the specificity of the DTH. A clear population of circulating CMV specific T cells (a) is not found in the DTH performed with CEA-peptide loaded DCs (b), whereas no specific T cells are detected in peripheral blood (c) by direct tetramer-staining, but 18.6% of CD8+ T cells in the DTH are CEA-specific (d).
Polyinosinic polycytidylic acid prevents efficient antigen expression after RNA electroporation of clinical grade dendritic cells


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Polyinosinic polycytidylic acid prevents efficient antigen expression after mRNA electroporation of clinical grade dendritic cells

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Tumor-derived peptides are used frequently as antigen (Ag) source in dendritic cell (DC) therapy in cancer patients. An alternative is to load DCs with tumor-associated Ag (TAA)-encoding RNA. RNA-loading obviates prior knowledge of CTL and Th epitopes in the Ag. Multiple epitopes for many HLA alleles (both MHC class I and class II) are encoded by the RNA and loading is independent of the patient’s HLA make-up. Here, we determined the optimal conditions for mRNA-electroporation of monocyte-derived DCs for clinical application in relation to different maturation cocktails.

The data demonstrate that TAA carcinoembryonic antigen, gp100 and tyrosinase are expressed already 30 min after electroporation with the encoding mRNA. Moreover, gp100-specific CTL are activated by gp100 mRNA-electroporated DCs. Importantly, we show here that the presence of polyinosinic–polycytidylic acid [poly(I:C)] in the maturation cocktail prevents effective protein expression of the electroporated mRNA as well as subsequent CTL recognition. This effect of poly(I:C) correlates with the induction of IFN-induced genes and innate anti-viral eVector molecules in DCs.

Together these data show that electroporation of mature DCs with TAA-encoding mRNA is attractive for use in DC vaccination protocols in cancer patients, but protein expression should be tested for each maturation cocktail.

Dendritic cell (DC) vaccination studies showed the feasibility and safety of DC vaccinations (reviewed in refs. 1 and 2). Clinical and immunological responses have been reported in some patients. One important aspect in DC-based vaccines is the maturation of DCs. The cocktails used commonly for DC maturation in clinical studies³ fail to induce IL-12p70 production in the DCs. For cancer vaccine development DCs producing IL-12p70 are desired because of the Th type 1 (Th1) and CTL inducing capacity⁴. It has been shown that ligation of particular Toll-like receptors (TLR) is a prerequisite to induce full maturation, enabling DCs to produce IL-12p70⁵. We and others have recently developed clinically applicable maturation cocktails, containing TLR ligands (TLR-L), to generate mature DCs with high migra-
tory and IL-12 producing capacity\(^6\). Another crucial aspect in DC-based vaccines concerns the efficacy of Ag delivery to DCs. To date, in most clinical studies DCs loaded with tumor lysates or defined tumor peptides have been used for the induction of anti-tumor immunity\(^8\)-\(^11\). Various methods have been designed to introduce whole tumor Ag into DCs, including tumor mRNA or synthetic mRNA\(^12\)-\(^14\). The advantage of endogenous expression of whole tumor Ag by the DCs is that T cell epitopes do not need to be specified, HLA type is not a limiting factor and multiple epitopes (both CTL and Th epitopes) can be presented. DCs transfected with mRNA encoding tumor-associated Ag (TAA) or with whole tumor mRNA can induce potent Ag- and tumor-specific T cell responses (reviewed in refs. 15 and 16). Electroporation of mRNA is an efficient way of transfection\(^15\),\(^16\), leading to clear expression of the introduced gene. This strategy can be optimized for the treatment of cancer patients using synthetic RNA encoding tumor Ag, by analyzing protein expression levels in the electroporated cells and by testing efficiency of protein expression after mRNA electroporation in combination with different, clinical grade, maturation cocktails.

We optimized the electroporation protocol for transfection of synthetic mRNA to obtain a clinical grade mature monocyte-derived DC vaccine. This protocol was used for the electroporation of mRNA encoding TAA. We found that transfection of TAA carcinoembryonic antigen (CEA), gp100 and tyrosinase leads to expression and presentation of these Ag by clinical grade mature monocyte-derived DCs. Moreover, we show that the presence of polyinosinic-polycytidylic acid [poly(I:C)], a TLR3 and melanoma differentiation-associated gene 5 (MDA-5) ligand, in the maturation cocktail interferes with effective transgene expression after mRNA electroporation of DCs and that this correlates with the upregulation of genes involved in an anti-viral response by poly(I:C). These results may improve the generation of efficient DC-based vaccines for use in cancer patients.

**RESULTS**

Expression and presentation of TAA after electroporation of mRNA differs for various tumor Ag

Electroporation is an efficient way to transflect DCs with mRNA\(^15\),\(^16\). Because studies comparing mRNA transfection of immature and mature DCs are contradictory\(^14\),\(^17\),\(^18\), we compared the efficiency of electroporation of immature and mature DCs with our settings. As shown in figure 1a the level of transgene expression was higher in mature DCs than in immature DCs. We decided to electroporate mature DCs with gp100, tyrosinase and CEA as TAA, because in our ongoing clinical trials peptides derived from these Ag are used to load DCs\(^19\)-\(^21\). First we studied the expression of TAA after electroporation of the encoding mRNA. As shown in figure 1b, 4 h after electroporation, gp100, tyrosinase and CEA proteins were clearly detectable in the electroporated DCs both by intracellular FACS analysis and by staining of cytopspins. All three TAA were already highly expressed 30 min after electroporation (figure 1c), but tyrosinase protein levels decreased to background levels within 24 h after electroporation (figure 1c), whereas both gp100 and CEA expression could still be measured 96 h after electroporation (data not shown).
Figure 1. Electroporation of DCs with mRNA encoding TAA results in expression of the respective proteins and presentation of gp100-derived epitope to specific CTL. (a) DCs were incubated with cytokine cocktail (mature DCs) or not (immature DCs). After 48 h, DCs were electroporated with mRNA encoding the tNGF-R. tNGF-R expression at the cell surface was determined by FACS analysis 24 h after electroporation. Histograms show staining with specific antibody [filled curve (mature DCs) and thick-lined curve (immature DCs)] or isotype control antibody (thin-lined curve) after electroporation by exponential decay pulse. (b) Cytokine cocktail-matured DCs (cDCs) were electroporated with mRNA encoding CEA, gp100 or tyrosinase by exponential decay pulse. Intracellular FACS analysis was performed 4 h after electroporation. Histograms showing isotype control antibody (thin-lined curve) and specific antibody (filled curves) are presented in the left panels. Cytospins were prepared 4 h after electroporation and stained with isotype control antibody (middle panels) or specific antibody (right panels). (c) cDCs were electroporated with mRNA encoding CEA, gp100 or tyrosinase by exponential decay pulse. At different time points after electroporation intracellular FACS analysis was performed. Median fluorescence intensity of the staining of positive cells is shown for one representative experiment of three performed. (d) cDCs were electroporated with mRNA encoding for CEA (control) or gp100 by exponential decay pulse. At different time points after electroporation 2.5x10^4 TIL1200 cells (gp100-specific CTL line) were incubated for 24 h with titrated amounts of electroporated DCs. IFNγ was measured in the supernatants by specific ELISA. Data shown are IFNγ production induced by gp100 mRNA-electroporated DCs, corrected for IFNγ production induced by CEA mRNA-electroporated DCs (control) and are mean ±SEM of triplicates from one representative experiment of four performed. IFNγ production induced by CEA mRNA-electroporated DCs (control) was 52, 174 and 57 pg/ml for 10,000 DCs and 59, 168 and 23 pg/ml for 1,000 DCs, when added 4, 24 and 48 h after electroporation. Stimulation indices were 16, 6 and 5 for 10,000 DCs and 6, 2 and 3 for 1,000 DCs, when added 4, 24 and 48 h after electroporation. Stimulation indices for the three experiments that were not shown were 9, 2 and 35 for 1,000 DCs added 24 h after electroporation.
The percentage of cells expressing gp100, tyrosinase and CEA was 81, 12 and 76%, respectively 24 h after electroporation and 59, 1 and 71%, respectively 48 h after electroporation.

To examine whether DCs electroporated with gp100-encoding mRNA are capable of presenting gp100-derived epitopes to specific T cells we used the T cell line TIL1200, specific for the gp100154 and gp100457 CTL epitopes presented in HLA-A2. DCs were electroporated with gp100-encoding mRNA and incubated with TIL-1200 cells at different time points after electroporation. As shown in figure 1d, high IFNγ-production was induced in specific CTL by gp100 mRNA-electroporated DCs as soon as 4 h after electroporation and this capacity to stimulate specific CTL was retained for at least 48 h.

Taken together the data presented here show that electroporation of clinical grade, monocyte-derived DCs in the mature state leads to expression of tumor Ag in the DCs and efficient presentation of TAA-derived epitopes to specific CTL for a durable time period.

**Protein expression after mRNA electroporation is dependent on DC maturation stimuli**

To date the most widely applied DC vaccines are matured with a defined cocktail of pro-inflammatory cytokines: IL-1β, IL-6, TNFα and prostaglandin E2 (PGE2) or with monocyte-conditioned medium (MCM) combined with TNFα and PGE2 (conventional DCs, cDCs). We used this cytokine mixture to optimize the electroporation protocol. Monocyte-derived cDCs produce very low to undetectable levels of IL-12p70. Recently, maturation cocktails containing TLR-L have been developed that induce the production of IL12p70 by DCs.

We tested the efficiency of transgene expression after electroporation of DCs with gp100-encoding mRNA after maturation with TLR3 and MDA-5 ligand poly(I:C) (pIC-DCs), TLR7/8L R848 (R848-DCs) and the combination (R + P-DCs). All maturation cocktails were supplemented with PGE2, because this is required to enhance migration for clinical application. All DCs (cDCs, pIC-DCs, R848-DCs and R+PDCs) were phenotypically mature as determined by FACS analysis (data not shown and ref. 6). However, poly(I:C) and/or R848 induced much higher levels of IL-12 than cytokine mixture (data not shown and ref. 6).

As shown in figure 2a, gp100 protein expression levels after gp100 mRNA electroporation were equal for R848-DCs and cDCs. In contrast, the presence of poly(I:C) in the maturation cocktail decreased the gp100 protein expression both at 4 and 24 h after electroporation. As shown in figure 2b, the capacity to activate gp100-specific T cells was also decreased for DCs matured with a cocktail containing poly(I:C). Poly(I:C) is a synthetic viral dsRNA analog and we have shown recently that treatment of DCs with poly(I:C) induces a strong upregulation of mRNA and protein for viral sensors, including the RNA helicases retinoic acid-inducible gene I (RIG-I) and MDA-5, and effector molecules like the double stranded RNA-activated serine/threonine protein kinase (PKR) and 2'-5'-oligoadenylate synthetase (2,5-OAS) (ref. 27 and unpublished data). PKR is known to inhibit protein synthesis and 2,5-OAS activates RNase L, that degrades single-stranded RNA.

Therefore, the mRNA expression levels of these genes induced by the different maturation cocktails were measured.
Figure 2 Polyinosinic–polycytidylic acid stimulation prevents effective protein expression after mRNA electroporation and increases expression of both viral sensors and effector molecules. DCs were matured with the conventional cytokine cocktail (TNFα, IL-1β and IL-6; cDCs), poly(I:C) (pIC-DCs), R848 (R848-DCs) or poly(I:C)/R848 (R + P-DCs), all with addition of PGE2. DCs were electroporated with gp100-encoding mRNA 48 h later. (a) Histograms of intracellular staining with specific antibody (filled curves) or with isotype control Ab (thick-lined curves) 4 and 24 h after electroporation. Numbers shown in the figures are median fluorescence intensity of gp100-staining (after subtracting median fluorescence intensity of isotype control staining). (b) 2.5 x 10⁴ TIL1200 cells were co-incubated with 400 DCs 24 h after electroporation and IFNγ was measured in supernatants 24 h later by specific ELISA. (c) mRNA levels of MDA-5, 2,5-OAS, PKR and RIG-I were determined using qPCR at several time points after electroporation (0 h is the time point of electroporation). (d) Protein expression of MDA-5, PKR and RIG-I was analyzed by western blot assay at different time points after electroporation. (e) Four and twenty-four hours after electroporation, DCs were stained with antibodies specific for MDA-5 and DC-SIGN, followed by Alexa 568- and Alexa 488-conjugated secondary Ab, respectively, and analyzed by confocal microscopy as described in “Materials and methods.” For DC-SIGN a cell surface staining was performed, whereas MDA-5 was stained intracellularly.
As shown in figure 2c, cocktails containing poly(I:C) strongly increased the expression of MDA-5, 2,5-OAS, PKR and RIG-I, whereas cytokine cocktail or R848 alone did not. Expression levels were upregulated 48 h after the induction of maturation (t = 0 h), the time point at which the cells were electroporated, and remained upregulated compared to cDCs and R848-DCs up to at least 24 h after electroporation.

Western blot analysis corroborated our findings by qPCR and demonstrated elevated protein levels of RIG-I, PKR and MDA-5 following exposure to poly(I:C) (figure 2d) at the time of electroporation and up to 24 h later. Furthermore, confocal microscopy analysis showed an increased expression of MDA-5 in the cytoplasm of DCs stimulated with a mixture containing poly(I:C) (figure 2e).

Collectively, these data show that the presence of poly(I:C) in the maturation cocktail prevents effective expression of TAA after electroporation of DCs with mRNA. The upregulation of genes involved in the induction of an antiviral state in the DCs and regulating protein expression levels is uniquely associated with the presence of poly(I:C) in the maturation cocktail and might be an explanation for this phenomenon.

**DISCUSSION**

In this study we show that CEA, gp100 and tyrosinase proteins are expressed already 30 min after mRNA electroporation. Furthermore, electroporated DCs present dominant epitopes derived from the encoded tumor Ag to specific CTL. Importantly, we show that the presence of poly(I:C) in the maturation cocktail prevents efficacious protein expression after electroporation of mRNA encoding TAA and subsequent CTL recognition. This correlates with upregulation by poly(I:C) of genes regulating protein expression levels by inducing degradation of mRNA and inhibition of protein synthesis. Dendritic cell vaccination trials have shown that DC vaccines can induce immune responses in cancer patients. However, as reviewed in ref. 28, many variables need to be optimized and standardized to further improve the efficacy of DC vaccination. One of these parameters is the way of loading the DCs with Ag. Electroporation of mRNA is an efficient way of antigen loading.\(^{15,16}\) We used synthetic mRNA encoding well-characterized TAA as a source to transfect DCs. This has the advantage that mRNA can be produced in batches for a large number of patients and it allows proper immune monitoring and comparison with peptide-loaded DCs.

It is now generally accepted that mature DCs should be applied for vaccination purposes.\(^{29,30}\) Currently, ex vivo generated monocyte-derived DCs, matured with a cocktail containing MCM, TNFα and PGE2 or IL-1β, IL-6, TNFα and PGE2, are used commonly in clinical studies. A disadvantage of this maturation cocktail is the poor IL-12p70 production by the DCs. We recently proposed a novel clinical grade maturation protocol in which TLR-L poly(I:C) and R848 are combined with PGE2 to generate DCs with both high migratory capacity and IL-12p70 production upon T cell encounter.\(^6\) For peptide loading of DCs this seems to be a good choice. However, we show here that protein expression after mRNA electroporation of DCs matured for 48 h by cocktails containing poly(I:C) is very ineffective. These results are in agreement with recently published data showing that protein expression after mRNA electroporation of
DCs matured for 24 h with a cocktail containing poly(I:C) is very inefficient. It is therefore necessary to test maturation cocktails and loading methods of DCs individually to find the optimal combination. We propose to mature the DCs with R848 and PGE2 if electroporation of mRNA is the Ag loading method. The observed interference with effective transgene expression by the presence of poly(I:C) in the maturation cocktail might be explained by the upregulation of genes involved in the innate anti-viral response. Poly(I:C) is a synthetic dsRNA and it has been shown that dsRNA inhibits protein synthesis in cell-free systems prepared from IFN-treated cells. Interferons trigger the development of a defensive response led by the enzymes 2,5-OAS and PKR. 2,5-OAS is activated by dsRNA and on its turn activates the latent ribonuclease RNAse L, which degrades single-stranded RNA. PKR inhibits initiation of protein synthesis. These two pathways regulate protein expression levels and hereby impair the replication of viruses. We here show that incubation of DCs with poly(I:C) induces expression of these two dsRNA-dependent enzymes: PKR and 2,5-OAS. These genes and the corresponding proteins are highly expressed at the time of electroporation and up to at least 24 h thereafter. Highly efficient induction of CTL responses by DCs co-electroporated with poly(I:C) and mRNA are not in disagreement with our data, but might be explained by translation of the mRNA into protein before the anti-viral response induced by poly(I:C) is full blown. The data provided here show that electroporation of DCs with mRNA encoding TAA is an efficient way of Ag loading, which is feasible and can be performed under cGMP conditions. We propose the use of TLR-L poly(I:C) and R848 in combination with PGE2 as maturation cocktail for peptide loading of DCs and omission of poly(I:C) from the cocktail for mRNA electroporation. Future clinical studies will demonstrate the efficacy of these mRNA-loaded DC vaccines in cancer patients.

MATERIALS AND METHODS
See supplementary material.

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Chapter 6
SUPPLEMENTARY MATERIALS AND METHODS

DC preparation from peripheral blood precursors and DC characterization

DCs were generated from PBMC prepared from leukapheresis products or from whole blood obtained from healthy donors and patients essentially as described previously\(^1\)\(^\text{-}^2\). The patient study was approved by the local medical ethical committee. No differences were observed in quality of the DCs and electroporation efficiency between buffy coat- and leukapheresis material-derived DCs and between healthy donor- and patient-derived DCs. Therefore we did not specify the source of PBMC used in each experiment. Plastic-adherent monocytes from leukapheresis or whole blood were cultured in X-VIVO 15™ medium (BioWhittaker, Walkersville, Maryland) supplemented with 2% pooled human serum (HS) (Sanquin, Nijmegen, The Netherlands), IL-4 (500 U/ml) and GM-CSF (800 U/ml) (both from CellGenix, Freiburg, Germany). On day 7, cells were either kept in the immature state or matured with 10 ng/ml recombinant TNFa, 5 ng/ml IL-1β and 15 ng/ml IL-6 (all CellGenix) (conventional DCs, cDCs); 20 μg/ml poly(I:C) (Sigma Chemicals Co., St. Louis, MO) (pDCs); 3 μg/ml R848 (PharmaTech, Shanghai, China) (R848-DCs); or 20 μg/ml poly(I:C) and 3 g/ml R848 (R+P-DCs). To all the before mentioned maturation cocktails 10 μg/ml PGE2 (Pharmacia & Upjohn, Puurs, Belgium) was added. All maturation cocktails gave rise to mature DCs on day 9, meeting the release criteria described previously\(^3\). After maturation more than 80% of the DCs expressed high levels of CD80 and CD86, analyzed by FACS analysis as described below. Immature DCs were also harvested on day 9 and expressed low levels of CD80 and CD86. Both immature and mature DC preparations contained more than 80% DCs.

Culture of gp100-specific CTL

TIL1200, a gp100-specific CTL line has been described\(^4\)\(^,\)^\(^5\)\(^,\)\(^6\) and was cultured in RPMI-1640 (Gibco BRL, Carlsbad, CA), containing 7% human serum and 100 U/ml IL-2 (Cetus Corp., Emeryville, CA). The TIL1200 line recognizes two gp100-derived epitopes: gp100154 (aa 154-162, KTWWGGYWWQV)\(^6\) and gp100457 (aa 457-466, LLDGTATTLRL)\(^4\).

Plasmids and in vitro mRNA transcription

Plasmids pGEM4Z-5'UT-CEA-3'UT-A64 and pGEM4Z-5'UT-tNGFR-3'UT-A64 were a kind gift from E. Gilboa (Duke University, Durham) [7] and pGEM4Z-5'UT-hgp100-3'UT-A64 was provided by Kris Thielemans (Free University Brussels, Belgium). pGEM4Z-5'UT-tyrosinase-3'UT-A64 was constructed by digestion of pGEM4Z-5'UT-tNGFR-3'UT-A64 with BglII and XbaI and digestion of pcDNA1.amp/tiyrosinase with BamHI and XbaI and insertion of the tyrosinase into the cut pGEM4Z. Plasmids were linearized with SpeI (pGEM4Z-5'UT-CEA-3'UT-A64) or NotI enzyme (pGEM4Z-5'UT-tNGFR-3'UT-A64, pGEM4Z-5'UT-hgp100-3'UT-A64 and pGEM4Z-5'UT-tyrosinase-3'UT-A64), purified with phenol/chloroform extraction and ethanol precipitation, and used as DNA templates. tNGFR mRNA was obtained by in vitro transcription with T7 RNA polymerase (mMESSAGE mMACHINE kit, Ambion, Austin, TX) according to the manufacturer's instructions. The transcribed RNA was recovered after DNaseI (Ambion) digestion with phenol/chloroform extraction and ethanol precipitation.

TAA-encoding mRNA (gp100, tyrosinase and CEA) was obtained from CureVac GmbH (Tübingen, Germany). RNA quality was verified by agarose gel electrophoresis, RNA concentration was measured spectrophotometrically, and RNA was stored at -40°C in small aliquots.

Electroporation of DCs

Immature or mature DCs were washed twice in PBS and once in OptiMEM® without phenol red (Invitrogen, Breda, The Netherlands). 20 μg RNA was transferred to a 4-mm cuvette (BioRad, Veenendaal, The Netherlands) and 10 x 106 cells were added in 200 μl OptiMEM® and incubated for 3 min before being pulsed in a GenePulser Xcell (Bio-Rad). Two methods of pulsing were applied and compared for electroporation of DCs: exponential decay pulse and square wave pulse. With exponential decay pulsation the voltage across the electrodes rises rapidly to the peak voltage and declines over time, whereas with square wave pulsation the pulse has approximately the same voltage at the end as at the beginning of the pulse. Electroporation settings were optimized for each pulse method by varying voltage and capacitance settings (exponential decay pulse) or voltage and pulse time settings (square wave pulse) using immature DCs and analyzing expres-
tion of the transgene and survival. We found that electroporation of 10 x 10^6 cells in 200 µl pulsed at 300 V, 150µF (exponential decay pulse) or 400 V, 0.6 ms (square wave pulse) resulted in the highest electroporation efficiency (data not shown). Immediately after electroporation, the cells were transferred to warm (37°C) X-VIVO 15™ without phenol red (Cambrex Bio Science, Verviers, Belgium) supplemented with 5% HS and left for at least 30 min at 37°C, before further manipulations were performed. Electroporation efficiency was analyzed by intracellular staining and FACS analysis for each separate TAA.

Flow cytometric analysis (FACS analysis)
To characterize and compare the phenotype of the DC populations, flow cytometry was performed. The following mAb or appropriate isotype controls were used: anti-HLA class I (W6/32), anti-HLA DR/DP (Q5/13) (both ascites), anti-CD80 (BD Biosciences, Mountain View, CA), anti-CD14, anti-CD83 (both Beckman Coulter, Mijdrecht, The Netherlands), anti-CD86 (BD Pharmingen, San Diego, CA), anti-CCR7 (kind gift of Martin Lipp, Max Planck Institute, Berlin, Germany) and anti-tNGF-R (mAb 20.4 (IgG1), used as concentrated hybridoma supernatant). All stainings with primary antibodies were followed by staining with FITC-labeled goat-anti-mouse (Zymed, San Francisco, CA).

For intracellular staining of the TAA the following mAb were used: NKI/beteb (IgG2b) (purified antibody) against gp100, T311 (IgG2a) (Cell Marque Corp., Rocklin, CA) against tyrosinase and Col-1 (IgG2a) (Lab Vision Corp., Fremont, CA) against CEA. For intracellular staining cells were fixed for 4 min. on ice in 4% (w/v) paraformaldehyde (Merck, Darmstadt, Germany) in PBS, permeabilized in PBS/2% bovine serum albumin (BSA)/0.02% azide/0.5% saponin (Sigma-Aldrich) (PBA/saponin), and stained with mAb diluted in PBA/saponin/2% HS, followed by staining with allophycocyanin-labeled goat-anti-mouse (BD Pharmingen). Flow cytometry was performed with FACSCalibur™ flow cytometer equipped with CellQuest software (BD Biosciences).

Cytospins and staining
Immunohistochemistry was performed on cytospins by incubation with the same antibodies as described above for intracellular staining, followed by incubation with biotinylated horse-anti-mouse (Vector, Burlingame, CA) and staining with Power-DAB (Immunologic, Duiven, The Netherlands). Cells were analyzed on a Leica DM LB microscope using Leica IM5000 software (Leica, Wetzlar, Germany).

Induction and determination of IFNg production by gp100-specific CTL
T cell stimulatory capacity was tested by coculturing 400 (figure 5C), 103 or 104 (figure 4B) DCs after electroporation with gp100-encoding or control mRNA with a gp100-specific T cell line (2,5 x 10^4 TIL1200 cells). After 24 h supernatants were tested for IFNg content using a standard sandwich ELISA. Coating Ab: mouse-IgG1-anti-hIFNg (clone 2G1; Pierce, Rockford, IL), detection Ab: biotinylated mouse-IgG1-anti-hIFNg (clone B133.5; Pierce). Streptavidin-HRP and TMB were used as enzyme and substrate, respectively.

RNA isolation
Total RNA was isolated from DC cultures using TRIZOL reagent (Invitrogen Life Technologies) according to the manufacturer's instructions, with minor modifications. RNA quantity and purity were determined using a NanoDrop® ND1000 Spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). The reverse transcription reaction was performed using Moloney Murine Leukemia virus reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. For each sample a “- RT” control was included in which the reverse transcriptase was replaced by ultrapure milli-Q (Invitrogen Life Technologies). The cDNA was stored at -20°C until further use.

Quantitative PCR
Quantitative analysis of gene expression in DCs was performed using SYBR Green-based qPCR. The qPCR reactions were performed in a 20 µl volume containing 10 µl SYBR Green mix (Applied Biosystems), 1.2 µl forward/reverse primer (300 nM endconcentration), 3.6 µl milli-Q and 4 µl cDNA dilution. Reactions were performed on an ABI 7000 Sequence Detection System (Applied Biosystems). Analysis was performed using 7000 system software (version 1.2X, Applied Biosystems). Primer sequences are available upon request and were designed using the freely accessible Primer Bank program6.

Western blot
Equal amounts of protein were separated by 12.5% sodium dodecyl sulfatepolyacrylamide gel electrophoresis and electroblotted onto nitrocellu-
lose membranes (Bio-Rad), followed by probing with the indicated antibodies. Anti-RIG-I and anti-protein kinase R (anti-PKR) antibodies were purchased from ProSci Incorporated and Becton Dickinson Transduction Laboratories, respectively. Rabbit polyclonal anti-MDA-5 was a kind gift of Paul Fisher (Departments of Pathology, Neurosurgery and Urology, Columbia University, College of Physicians and Surgeons, New York, USA) and production of this antibody was described previously. RIG-I, PKR and MDA-5 antibodies were used in 1:1,000, 1:500 and 1:10,000 dilutions, respectively. After washes, membranes were incubated with IRDye anti-mouse or anti-rabbit immunoglobulin G (IgG) (1:15,000) (Li-Cor Biosciences). Imaging was done using the Odyssey System.

Confocal Microscopy

DCs were harvested, washed and allowed to adhere to poly-L-lysinecoated coverslips in serum-free medium for 1 h at 37°C. Cells were fixed with 1% paraformaldehyde and blocked in PBS/2% HS/2% BSA (blocking buffer, BB) supplemented with 50 mM NH₄Cl. For cell surface staining, cells were incubated with mouse anti-human DC-SIGN (AZN-D1, 5 μg/ml, Beckman Coulter) in BB, washed and incubated with isotype-specific Alexa-labeled goat anti-mouse IgG1 in BB (Alexa 568, Molecular Probes). For intracellular staining, cells were fixed using 1% paraformaldehyde, permeabilized using 0.1% saponin in BB supplemented with 50 mM NH₄Cl, and incubated with polyclonal anti-MDA-5 followed by incubation with goat anti-rabbit IgG-Alexa 488 (Molecular Probes) in BB. After final washes, cells were sealed using Mowiol (Merck) and analyzed by confocal microscopy (Olympus Fluoview, FV1000).

REFERENCES SUPPLEMENTARY DATA


Immunogenicity of dendritic cells pulsed with CEA-peptide or transfected with CEA-mRNA for vaccination of colorectal cancer patients: a clinical study


Submitted for publication
Immunogenicity of dendritic cells pulsed with CEA-peptide or transfected with CEA-mRNA for vaccination of colorectal cancer patients: a clinical study

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Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system. We have demonstrated that vaccination of autologous ex vivo cultured DCs results in the induction of tumor-specific immune responses in cancer patients, which correlates with clinical response. Optimization of antigen-loading is one of the possibilities to further improve the efficacy of DC vaccination. Theoretically, transfection of DCs with RNA encoding a tumor-specific antigen may induce a broader immune response as compared to the most widely used technique of peptide pulsing.

In this clinical study, we compared RNA transfection with peptide pulsing as an antigen loading strategy for DC vaccination. Patients with resectable liver metastases of colorectal cancer were vaccinated intravenously and intradermally 3 times weekly with either carcinoembryogenic antigen (CEA) derived HLA-A2 binding-peptide loaded or CEA-mRNA electroporated DCs prior to surgical resection of the metastases. All DCs were loaded with keyhole limpet hemocyanin (KLH) as a control protein. Evaluation of vaccine-induced immune reactivity consisted of T proliferative responses and B cell antibody responses against KLH in peripheral blood. CEA reactivity was determined in T cell cultures of biopsies of post-treatment delayed type hypersensitivity skin tests.

Sixteen patients were included. All patients showed T cell responses against KLH upon vaccination. CEA peptide-specific T cells were detected in 8 out of 11 patients in the peptide group, but in none of the 5 patients in the RNA group.

In our study, DC CEA-mRNA transfection was not superior to DC CEA-peptide pulsing in the induction of a tumor-specific immune response in colorectal cancer patients.

Introduction

Dendritic cells (DCs) are the most potent antigen presenting cells, and are capable of inducing an effective anti-tumor immune response¹. Currently they are employed in clinical immunization protocols in cancer patients and objective clinical and immunological responses have been observed². However, a lot of crucial questions regarding the optimal preparation and administration of DC vaccines for clinical use remain unanswered to date³. One of these
questions concerns the optimal method of antigen-loading. In the vast majority of studies, tumor antigenic peptides have been used. This has the advantage that the monitoring of the immune response is focused on a limited and predefined number of epitopes. However, pulsing DCs with peptides limits the response to identified epitopes and therefore limits the treated patient group to certain specific HLA-types. In addition, peptide-pulsing does not account for post-translational modifications of the tumor antigens. These limitations may not exist for the expression of tumor antigens by DCs through RNA transfection, which has been shown effective in vitro and in vivo. Moreover, RNA transfection may lead to a more prolonged presentation of the antigen as compared to peptide-loading which appears to be short-lived. The feasibility, safety, and potential efficacy of non-virally RNA-transfected DCs has been shown in clinical studies with cancer patients. On the other hand, RNA transfection has potential drawbacks including a variable antigen expression and a low yield of viable cells after transfection, especially when using the technique of RNA electroporation. The true benefit of using RNA transfection over peptide loading should be derived from clinical trials. A clinical comparison of RNA-transfected DCs with peptide-pulsed DCs, however, has not been reported. Here, we compare the immunogenicity of an RNA-based DC vaccine versus a peptide-based DC vaccine in colorectal cancer patients. We included patients with resectable colorectal cancer liver metastases for the following reason. Vaccine therapy is likely to have greater efficacy in patients with low tumor volume compared to patients with widespread bulky metastases in whom immune suppression is often present. Patients with resected liver metastases have a high risk for relapse and may therefore potentially benefit from adjuvant treatment, such as vaccine therapy. Therefore we opted to test the immunogenicity of our method in this specific subgroup of patients.

PATIENTS AND METHODS

Study design
This was an open-label, single-institution, exploratory study in which monocyte-derived DCs loaded with CEA-peptide or electroporated with CEA-encoding mRNA were administered to patients with resectable liver metastases of colorectal cancer. It was planned that the first 10 patients would receive DCs pulsed with CEA peptides, and another 10 patients would receive DCs transfected with mRNA. Approval from the local regulatory committee was obtained. Trial registration: NCT00228189 (ClinicalTrials.gov).

Objectives
The primary endpoint was to compare the immunogenicity of CEA peptide-loaded DCs with DCs electroporated with CEA-mRNA. Secondary endpoints were the toxicity and the feasibility of CEA-specific vaccination in colorectal cancer patients.

Patients
Inclusion criteria included: patients with liver metastases from CEA-expressing colorectal cancer scheduled for surgical resection, HLA-A0201 phenotype, Eastern Cooperative Oncology Group (ECOG) performance status 0–1, age ≥18 years, no clinical signs of extra-hepatic metastases, no prior chemotherapy, immunotherapy or radiotherapy within 2 months before planned surgery.

DC preparation
DCs were generated either from buffy coats or from leukapheresis products. Monocytes were enriched from buffy coats as follows: patients donated 0.5 l blood, from which a buffy-coat was made (Sanquin Bloodbank Nijmegen, the Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated by PureCell® (Medicult, Denmark) density gradient centrifugation (20 min, 20°C, 2100 rpm) and monocytes were enriched by adherence to plastic. Monocytes were enriched from leukapheresis products by counterflow elutriation using Elutra-cell separator (Gam-
bro BCT, Inc.) and single-use, functionally sealed disposable Elutra sets, as described before\textsuperscript{14} and according to the manufacturer’s instructions. Monocytes were cultured in Cellgro® medium supplemented with interleukin (IL)-4 (500 units/ml) and granulocyte macrophage colony-stimulating factor (GM-CSF; 800 units/ml; all Cellgenix). KLH (10 μg/ml; Calbiochem, USA) was added at day 3 of culture, and 2 days before harvesting we added the maturation cocktail consisting of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}; Pharmacia & Upjohn, Puurs, Belgium, 10 ng/ml), tumor necrosis factor alpha (TNFα, 10 ng/ml) and IL-6 (15 ng/ml; all CellGenix). Release criteria were as previously described\textsuperscript{3}: more than 80\% of the DCs expressed high levels of CD80 and CD86. Mature DCs were electroporated with mRNA encoding CEA directly after harvesting as described below or pulsed with wild-type CEA-peptide CAP-1 (CEA\textsubscript{571-579}, YLSGANLNL, Clinalfa, Switzerland)\textsuperscript{13 ‘15} directly after harvesting or after thawing\textsuperscript{16 ‘17}.

**Electroporation of DC**

Mature DCs were washed twice in PBS and once in OptiMEM without phenol red (Invitrogen). Twenty micrograms of mRNA were transferred to a 4-mm cuvette (Bio-Rad) and 12x10\textsuperscript{6} cells were added in 200 μl OptiMEM and incubated for 3 min before being pulsed in a GenePulser Xcell (Biorad) by an exponential decay pulse of 300 V, 150 μF, as described before\textsuperscript{10}. Electroporation was done under good manufacturing practice conditions. For the first vaccination, 2 to 3 aliquots of 12x10\textsuperscript{6} DCs were electroporated with CEA-encoding mRNA. Electroporation efficiency was analyzed by flow cytometry. DCs for the first vaccination were injected 4 h after electroporation. DCs for subsequent vaccinations were frozen 2 h after electroporation, thawed at the day of vaccination, and incubated for an additional 2 h at 37°C before injection.

**Treatment schedule**

Vaccinations were administered three times at day 0, 7 and 14. The patients received 5 x 10\textsuperscript{6} DCs intradermally (i.d.) in the upper leg, 5–10 cm from an inguinal lymph node. The remaining cells were given intravenously (i.v.) as a bolus injection at the same time point. On day 26 a post-treatment DTH test was performed (see below). At day 28, 6 mm punch biopsies were taken from the post-treatment DTH reaction sites. Before and after vaccination PBMCs were obtained. The same schedule of 3 weekly i.v./i.d. vaccinations followed by a post-treatment DTH was repeated twice at intervals of 6 months in the absence of recurrent disease. Serum CEA levels were measured before and after completion of the vaccination protocol.

**Immunologic monitoring**

CD4\textsuperscript{+} T cell responses against KLH were measured using a \textsuperscript{3}H-thymidine incorporation proliferation assay with PBMCs of the patients before and after vaccination\textsuperscript{17}. The index was calculated as the counts ratio between KLH-stimulated PBMC and non-stimulated PBMC. Antibodies against KLH were measured in the serum of vaccinated patients by enzyme-linked immunosorbent assays (ELISA) as previously described\textsuperscript{15 ‘18}.

Post-treatment DTH reactions were performed as described previously\textsuperscript{19}. Briefly, CEA-peptide only (100 μg in 100 μl), DCs pulsed with CEA-peptide, DCs pulsed with KLH and CEA-peptide, and DCs pulsed with KLH only (0.4–5 x 10\textsuperscript{5} DCs each in approximately 100–200 μl) were injected i.d., 5–10 cm from an inguinal lymph node at different sites, in the upper leg controlateral from the leg in which the DC vaccinations were performed. The maximum diameter of induration was measured after 48 h. T cell culture from DTH biopsies was performed in low dose IL-2 (100 U/ml; Proleukin®, Chiron, the Netherlands) for approximately 2 weeks without ex vivo restimulation with antigen as described before\textsuperscript{19}. After 2 to 4 weeks of culturing, T cells were tested for antigen recognition or tested for tetramer binding. DTH-derived cells (1 x 10\textsuperscript{5} cells in 10 μl) were incubated with PE-labeled CEA and cytomegalovirus (CMV) tetrameric-MHC complexes (Sanquin; Amsterdam, the Netherlands) for 60 min at room temperature. In the last 20 min of this incubation, fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies directed against CD8 (Becton Dickinson) were added. After washing, the samples were analyzed by flow cytometry. In all analyses CEA tetramer staining was compared with CMV tetramers as a negative control.

Production of cytokines by the total population of DTH-derived cells was measured in response to T2 cells pulsed with CEA-peptide or an irrelevant HLA-A2.1 binding peptide (tyrosinase or G250) or in response to PBMCs electroporated with CEA mRNA. Cytokine production was measured in supernatants after 16 h by cytometric bead array (Th1/Th2 Cytokine CBA 1; BD...
Pharmingen). IFN-γ production was considered positive when IFN-γ levels of DTH-infiltrating T cells were more than 10-fold higher after co-incubation with CEA-loaded T2 cells compared with co-incubation with T2 cells loaded with irrelevant antigen.

Patient evaluation
Clinical follow-up consisted of history, physical examination, serum CEA-level, CT scanning of the liver and X-ray of the chest at 3-month intervals.

Statistical analysis
Data were analyzed statistically by means of analysis of variance and Student-Newman-Keuls test, or by means of Mann Whitney U nonparametric statistics. Statistical significance was defined as p<0.05.

RESULTS

Patients
Sixteen patients were included in the study. In the period 2004-2005 eleven patients received peptide-pulsed DCs (previously reported in ref. 13). One extra patient was included as planned in case a patient proved not to be evaluable. Due to a slow accrual of only 5 patients in the second phase of the study, as well as the results in these patients, the study was prematurely closed. Baseline characteristics between patients receiving peptide-pulsed DCs and RNA-loaded DCs were comparable (data not shown).

Clinical outcome and toxicity
Four out of 11 patients in the peptide-group and 1 out of 5 patients in the RNA-group had irresectable disease at surgery. In the peptide-group, one patient (pep-5) was diagnosed with lung metastases before surgery and one patient died of post-operative complications not related to the vaccination treatment (pep-9). No toxicity other than grade 1 flue-like symptoms, fever and injection site reactions were observed in both groups. Specifically, no diarrhea or other signs of autoimmune colitis were observed. Seven patients treated with peptide-pulsed DCs and two patients treated with mRNA-loaded DCs showed a transient and modest rise in serum CEA level upon vaccination, defined as an increase of 15% compared with baseline33.

Figure 1. DC characteristics. (a) Expression of HLA-ABC, HLA-DR/DP, HLA-DQ, CD80, CD83, CD86 and CCR7 was measured by flow cytometry on mature DCs for the first iv/id vaccination. Results are shown as mean SEM of the percentage of cells expressing the maturation markers of peptide-loaded DCs (grey bars) and mRNA-electroporated DCs (black bars). (b) CEA protein expression was measured by flow cytometry 2 h after electroporation with mRNA. Results are shown as the percentage of cells expressing CEA protein for each individual patient.
In the patients who underwent a radical resection of metastases the median progression-free survival after treatment with peptide-pulsed DCs and mRNA-loaded DCs was 18 months (range 1-77 months) and 26 months (range 13-41 months), respectively.

**DC vaccine characteristics**

In all patients sufficient amounts of monocyte-derived mature DCs could be obtained for three i.d. vaccinations (see Table 1). However, the yield was not always sufficient for simultaneous i.v. injections. Patients vaccinated with CEA peptide-pulsed DCs received an average of 4x10^6 DCs i.v. and 5x10^6 DCs i.d. per vaccination during the first cycle. Patients vaccinated with RNA-electroporated DCs received an average of 11x10^6 DCs i.v and 5x10^6 DCs i.d. (Table 1). Final DC vaccine products met the criteria of a mature phenotype with high HLA-ABC, HLA-DR/DP, HLA-DQ, CD80, CD83, CD86 and CCR7 expression (Figure 1a). There was no difference in the maturation status or amount of i.d. injected DCs. However, patients in the peptide group received less i.v. injected DCs (p<0.001). All patients had a proliferative peripheral blood CD4+ T cell response against the control protein KLH (Table 1), as expected for mature DC vaccine, indicating that the DCs were able to induce de novo T cell responses.

On average, 66% of the DCs electroporated with mRNA expressed CEA protein 2 h after electroporation (range 44-89%; Figure 1b).

**KLH-specific immune responses**

To investigate whether peptide-loaded DCs or mRNA-electroporated DCs have similar capacity to activate the patient's immune system in general, humoral and cellular responses to the control protein KLH were measured in the peripheral blood of patients. PBMCs collected after each DC vaccination were analyzed for the presence of KLH-reactive T cells in a proliferation assay. In both the peptide group and the RNA group all patients tested showed a cellular response to KLH. KLH-specific IgG antibodies were induced after vaccination in 8 of 10 patients tested (80%) in the peptide group and in 2 of 5 patients tested (40%) in the RNA group. Thus, all patients developed a response to KLH, either cellular or humoral. However, more patients vaccinated with peptide-loaded DCs developed a humoral response to KLH. These results indicate that there may have been differences in the immunogenicity of DCs in both groups. Nevertheless, all patients had a proliferative T cell response against KLH, indicating that the DCs were able to induce de novo T cell responses in all patients.

**Post-treatment DTH: CEA specific T cell immunity**

To investigate the immune response against tumor peptides generated in vaccinated patients, DTH challenges were performed with mature DCs loaded with CEA peptide or CEA mRNA. Biopsies were taken from DTH induration sites, which were cultured in low amounts of IL-2 without the addition of antigen. In eight out of 11 patients, CEA-specific DTH-infiltrated T cells were detected by tetramer analysis after vaccination. We found that 0.3% up to 98% of CD8+ T cells was CEA-specific in DTH biopsies of patients vaccinated with HLA-A2 binding peptide-loaded DCs (table 1; partially previously reported in ref. 13).
When these T cells were co-cultured with CEA-peptide loaded T2 target cells they were able to produce large amounts of IFNγ and IL-2, but not IL-4 or IL-10 (table 1 and ref. 13). An example of specific T cells in a DTH site is shown in figure 3a. In table 1 the presence or absence of CEA-specific T cells at DTH sites is presented for each patient. In none of the patients vaccinated with mRNA-electroporated DCs CEA-specific T cell reactivity was found in DTH-biopsies (figure 3b and table 1), despite the presence of skin induration in these patients (data not shown).

DISCUSSION

Colorectal cancer is an immunogenic type of cancer. Tumor-associated antigens have been identified, tumor-specific T cells have been isolated from patients and small proof-of-concept studies have shown that immunotherapeutic strategies can induce immunological and clinical responses. CEA is a tumor-associated antigen that is expressed by almost all colorectal cancers. Hence, it is an attractive antigen to use in clinical immunization protocols. However, because CEA is also expressed in normal tissues, and it is also shed from the surface of tumor cells, a high threshold of tolerance must be overcome. Previously, it has been demonstrated that DCs loaded with CEA-peptide can induce robust immune responses in colorectal cancer patients. Theoretically, using CEA-mRNA instead of peptides to load the DCs, may result in the induction of a much broader, more robust T cell repertoire, since more epitopes are expressed and posttranslational modifications can occur. For this reason, we compared CEA-peptide loaded DCs with CEA mRNA-electroporated DCs as vaccine in colorectal cancer patients. Previously, we have shown that with this technique clinical-grade DCs can be efficiently transfected with tumor antigens, resulting in prolonged antigen-presentation and efficient T cell activation. In all patients in the mRNA-group, CEA-expression was high upon electroporation of the DCs. The two DC vaccines were phenotypically identical. In addition, T cell reactivity against the control protein KLH was comparable in the two groups after vaccination, indicating that the T cell-stimulatory potential of
The two DC vaccines were comparable. However, in 8 out of 11 patients who were vaccinated with peptide-pulsed DCs we found CEA peptide-specific T cell reactivity in DTH skin tests upon vaccination, whereas we could not detect CEA peptide-specificity in the 5 patients who were vaccinated with mRNA-electroporated DCs.

Monitoring immune responses in patients who are vaccinated with mRNA-transfected DCs poses a difficulty because the precise epitopes are not known. Therefore, the absence of tetramer-positive T cells in the DTH tests should be interpreted with caution; it is possible that T cells with specificity for other epitopes are in fact induced.

Table 1. Vaccine-induced antigen-specific immune responses. 1 Proliferative response to KLH is considered positive when the proliferation index (PI; proliferation +KLH/proliferation -KLH) is >2, at least at one time point after vaccination. + 2>PI<10; ++ 10>PI<20; +++ PI>20, at least at one time point after vaccination. Antibody titer, total IgG, + designates >1 in 400 times diluted serum at least at one time point after vaccination. 2a Sera of first cycle not available, antibody titers measured after second cycle of DC vaccinations. 3Ag-recognition of DTH derived T cells after stimulation with T2 cells loaded with CEA peptides (peptide group and mRNA group) or PBMCs electroporated with CEA mRNA (RNA group) as analyzed by IFNγ-production. n.t. not tested

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<th>Patient</th>
<th>Injected DC (x10⁶) (i.v./i.d.)</th>
<th>Proliferative Response against KLH¹</th>
<th>Humoral Response (IgG) against KLH²</th>
<th>Tetramer positive CD8+ T cells in DTH</th>
<th>IFN-producing CD8+ T cells in DTH³</th>
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the two DC vaccines were comparable.
Figure 3. Tumor antigen-specific T cell responses in post-treatment DTH. Tetramer analysis by flow cytometry of T cells derived from a biopsy of a DTH reaction of patient pep-2 (a; CEA) and RNA-3 (b; DCs electroporated with CEA-encoding mRNA). Depicted is CD8 on the x-axis and CEA tetramer staining on the y-axis. Tetramer staining of 1 log above the negative population was considered positive. Tumor antigen-specific T cell responses of all patients are shown in table 1.

However, in a previous melanoma study, using the same DC culture and electroporation protocol but different antigens (gp100 and tyrosinase), we were able to demonstrate tetramer-specific T cells in 8 out of 11 vaccinated patients\textsuperscript{9}. In addition, we also used mRNA-electroporated PBMCs as stimulator cells in co-culture with DTH-infiltrated T cells upon vaccination for monitoring purposes. Nevertheless, we observed no T cell specificity after vaccination in the mRNA-group, which may depend on the antigen characteristics as well as the nominal epitope derived from the antigen.

In contrast to studies in melanoma\textsuperscript{9}, these results indicate that using CEA-mRNA electroporation as a DC antigen loading strategy is not superior to peptide loading. The reasons for this difference may be twofold. First, CEA is not processed through the MHC class II pathway whereas gp100 is, unless it is coupled to a lysosomal targeting signal\textsuperscript{32}. Second, a different T cell repertoire may be present in the vaccinated patients.

This study was not designed to allow meaningful conclusions on clinical efficacy. We did observe a transient increase in serum CEA upon vaccination in several patients, which may indicate a cytotoxic effect on CEA-expressing tumor cells\textsuperscript{33}.

CONCLUSION

In our study we could not find a benefit for CEA-mRNA electroporation over peptide-pulsing as a method for antigen loading of DCs for vaccination in colorectal cancer patients.

ACKNOWLEDGEMENTS

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Platinum-drugs break STAT6-mediated suppression of immune responses against cancer


Submitted for publication
Platinum-drugs break STAT6-mediated suppression of immune responses against cancer

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Tumor micro-environments feature immune inhibitory mechanisms that prevent T cells from generating effective immune responses. Therapeutic interventions aimed at disrupting these inhibitory mechanisms have been shown to result in enhanced anti-tumor immunity, but lack direct cytotoxic effects.

Here, we investigated the effect of cancer chemotherapeutics on immune inhibitory pathways. We observed that exposure to platinum-based chemotherapeutics markedly reduced expression of the T cell inhibitory molecule Programmed Death Receptor-Ligand 2 (PD-L2), for both DCs and tumor cells. This down-regulation of PD-L2 results in enhanced antigen-specific proliferation and Th1 cytokine secretion, plus enhanced recognition of tumor cells by T cells. We demonstrated that this down-regulation of PD-L2 is controlled by Signal Transducer and Activator of Transcription 6 (STAT6). Indeed, patients with STAT6-expressing head and neck cancer displayed significantly enhanced recurrence-free survival upon treatment with cisplatin-based chemoradiation compared to patients with STAT6-negative tumors, demonstrating the clinical relevance of platinum-induced STAT6 modulation.

We conclude that platinum-based anti-cancer drugs can enhance the immuno-stimulatory potential of DCs and decrease the immuno-suppressive capability of tumor cells. This novel double action of platinum compounds may extend their therapeutic application in cancer patients and provides a rationale for their use in combination with other immuno-stimulatory compounds.

Recently, it has become evident that chemotherapeutic drugs not only have a direct cytotoxic effect on tumor cells but may also potentiate the immune system via so-called off-target effects¹,². For example, direct immune-activating effects of cancer chemotherapy have been observed: treatment with low concentrations of several chemotherapeutics resulted in increased antigen cross-presentation, T lymphocyte expansion and T cell infiltration of tumors by to date unknown molecular mechanisms³,⁴. In addition, the cytotoxic drug oxaliplatin was shown to induce an immunogenic type of cell death in a Toll-like receptor 4/high-mobility group box 1-dependent manner⁵,⁶. However, the effect of treatment with cancer chemotherapy on
Figure 1. DCs exposed to platinum-based chemotherapy display enhanced T cell stimulatory capacity. (a) DCs were cultured in clinically relevant concentrations of chemotherapy during 48 hours of cytokine maturation and subsequently used in a mixed lymphocyte reaction with allogeneic peripheral blood mononuclear cells (PBMCs). T cell proliferation was measured by $^3$H-Thymidine incorporation after 5 days. Ctrl= control DCs (non-treated); MTX= methotrexate; Cyta= cytarabine; Vinc= vincristine; Irino= irinotecan; Eto= etoposide; Bleo= bleomycin; DTIC= dacarbazine; Gem= gemcitabine; 5-FU= 5-fluorouracil; Doxo= doxorubicin; Lena= lenalidomide; Carb= carboplatin (for all experiments: * p < 0.05, ** p < 0.01, *** p < 0.001; the mean and standard error of the mean are depicted). (b) Same experiment with different platinum-based chemotherapeutics. Ctrl= control DCs (non-treated); oxali=oxaliplatin; cis=cisplatin; carb=carboplatin. (c) Concentration-dependent effect of platinum exposure on T cell stimulatory potential of DCs. This effect was observed irrespective whether the platinum drugs were added during or after DC maturation (data not shown). Concentrations of oxaliplatin were 0.5, 2.0, 3.0 and 4.0 microgram/ml, respectively. (d and e) Secretion of interferon-$\gamma$ and IL-2 by T cells in MLR upon exposure to DCs cultured with or without platinum. (f) Enhanced T cell stimulatory potential of DCs matured with cytokines or TLR-ligands in the presence of platinum chemotherapy in an antigen-specific model. KLH-specific T cells were cocultured with KLH-loaded DCs, matured with cytokines or R848 and poly-I:C, in the presence or absence of platinum chemotherapy. The exposure to platinum compounds increased the T cell stimulatory capacity of cytokine-matured DCs to a level that was almost comparable to that of fully activated, TLR-matured DCs. cDC=cytokine-matured DCs; TLR-DC= TLR-ligands matured DCs. Ox= oxaliplatin. (g) Enhanced interferon-$\gamma$ secretion by T cells in MLR upon exposure to TLR-ligand matured DCs cultured with platinum as compared to platinum-untreated DCs.
immune inhibitory pathways that play a crucial role in tumor immune-escape is unknown.

RESULTS AND DISCUSSION

The T cell-stimulating potential of DCs is enhanced by platinum-based chemotherapy

We hypothesized that anti-cancer chemotherapeutics may influence the type of T cell-mediated immune responses induced by DCs, by acting on immune inhibitory pathways. Therefore, we determined the effect of a large number of clinically relevant concentrations of chemotherapeutic agents on the allogeneic T cell stimulatory potential of monocyte-derived DCs (supplementary table 1).

Surprisingly, DCs exposed to carboplatin during their maturation by cytokines induced a significantly higher T cell proliferation compared to all other chemotherapeutics tested (figure 1a). We also observed this similar enhanced immune-stimulating activity in a dose-dependent manner for the platinum compounds oxaliplatin and cisplatin, which are frequently used in the clinic (figure 1b and c, supplementary table 1). T cells that were activated by DCs exposed to platinum compounds produced significantly higher levels of interferon-γ (IFN-γ) and interleukin-2 (IL-2) compared to untreated DCs (figure 1d and e).

Notably, this increased T cell-stimulating activity of platinum-treated DCs was also observed in antigen-specific T cells using keyhole limpet hemocyanin (KLH) as a model antigen. In this antigen-specific context we found a profoundly enhanced T cell-stimulatory capacity when DCs were exposed to platinum compounds (figure 1f). Moreover, we observed that the T cell stimulatory capacity of DCs matured in vitro through Toll-like receptors (TLR) instead of cytokines, was also further enhanced by platinum-exposure (figure 1f).

This demonstrates that the observed effect of platinum compounds is independent from stimuli used to activate and/or mature DCs. The observed effect on TLR-DCs was not only limited to enhanced T cell proliferation but also supported by abundant IFN-γ production by these T cells (figure 1g).

The enhanced immunogenicity of platinum-treated DCs is dependent on PD-L2/STAT6

To investigate why platinum-treated DCs display enhanced immunogenicity, we determined their phenotype (figure 2a, shows effects of oxaliplatin; similar results were obtained with carboplatin and cisplatin, not shown).

No significant differences were observed in expression of MHC class I and II or the costimulatory molecules CD80 and CD86 on platinum-treated versus non-treated DCs. We also detected no relevant differences in pro- or anti-inflammatory cytokine production of DCs matured in the presence of platinum compounds in comparison with non-treated cytokine- or TLR-matured DCs (figure 2b and c, supplementary figure 1).

We therefore concluded that the enhanced immunogenicity is not caused by increased expression of MHC- or co-stimulatory-molecules or by increased pro-inflammatory cytokine secretion upon platinum treatment.
Figure 2. Platinum-based chemotherapeutics down-regulate PD-L2 expression on DCs, resulting in enhanced T cell activation in a STAT6-dependent manner. (a) Expression of MHC class I and II, co-stimulatory molecules CD80, CD83 and CD86 by DCs exposed to increasing clinically relevant concentrations of oxaliplatin during maturation with R848/poly-I:C (isotype grey; indicated antibody white). (b and c) Production of pro-inflammatory cytokines TNFα and IL-8 by DCs exposed to platinum chemotherapy during maturation. Supernatants were harvested 24 hours after stimulation. (d and e) Expression of T cell inhibitory molecules PD-L1 and PD-L2 (isotype grey; untreated DCs white; platinum-treated DCs dark-grey) and B7-H2/H3/H4 and IDO by DCs exposed to platinum chemotherapy during maturation. (f) MLR with DCs matured in the presence (black bars) or absence (grey bars) of oxaliplatin 5 μg/ml in the presence of blocking antibodies against PD-L1, PD-L2 or control immunoglobulin (# p < 0.05 compared with IgG control DCs). (g) MLR with DCs that were transfected with STAT6 siRNA or control-siRNA and subsequently matured with or without oxaliplatin 5 μg/ml.
Since DCs can also express several T cell inhibitory molecules, including PD-L1 and PD-L2, the enhanced T cell stimulatory potential upon platinum exposure could result from decreased PD-L expression. Indeed, treatment with oxaliplatin, cisplatin or carboplatin during DC maturation moderately reduced the expression of PD-L1, and profoundly reduced the expression of PD-L2 (figure 2d). Expression of other known inhibitory receptors remained unaltered upon platinum-treatment (figure 2e).

Short-circuiting of the PD-1 – PD-L1/2 axis by anti-PD-L1 and PD-L2 antibodies enhanced the capacity of mature DCs to stimulate T cells to a similar level when compared to platinum-treated DCs. However, the increased allo-stimulatory potential of DCs upon platinum treatment is abrogated in the presence of PD-L2 antibodies (figure 2f). This strongly supports the notion that down-regulation of PD-L1, and particularly PD-L2, is a major cause of the enhanced T cell stimulatory capacity of platinum-treated DCs.

Since STAT6 is a known regulator of PD-L2, we subsequently tested the T cell stimulatory activity of platinum-treated DCs exploiting small interfering RNA (siRNA) against STAT6 (figure 2g, supplementary figure 2). Despite the fact that inhibition was not complete, STAT6 knock-down DCs now lacked the previously observed enhanced T cell response upon platinum treatment, which was not observed when DCs were transfected with control siRNA.

Together, these data show that the enhanced immunogenicity of platinum-treated DCs depends on STAT6 inactivation resulting in PD-L2 down-regulation.

**Platinum dephosphorylates STAT6 in tumor cells resulting in decreased PD-L2 and subsequent increased T cell recognition**

Since many cancer cells are capable to induce an immunosuppressive microenvironment and to upregulate PD-Ls in order to evade anti-tumor immunity, we next tested if the same platinum compounds also affected PD-L expression on tumor cells. To mimic an immunosuppressive microenvironment in vitro, we exposed BLM melanoma cells to cytokine combinations known to induce PD-L1/2 expression on endothelial cells, i.e. IL-4 together with IFNγ, LPS or TNFα. Subsequent exposure to platinum compounds clearly reversed PD-L2 expression, but not PD-L1 expression (figure 3a, supplementary figure 3).

Because PD-L2 is regulated by STAT6 and nuclear factor κB (NFκB), we measured protein levels of STAT6 and phosphorylated (active) STAT6 in BLM tumor cells upon co-culture with IL-4 and several NFκB activating stimuli (figure 3b, supplementary figure 4). We found that cisplatin caused a strong dephosphorylation of STAT6. Although several STAT molecules are constitutively expressed in multiple types of cancer and play an important role in the proliferative capacity and anti-apoptotic and immune-evasive potential of cancer cells, a direct STAT-inactivating mechanism induced by cytotoxic chemotherapy has not been reported before.

To further explore whether chemotherapy-induced reversal of an immunosuppressive tumor microenvironment not only reduces PD-L2, but also effectively enhances susceptibility of tumor cells to cytotoxic T cells, we compared the recognition of platinum-treated and non-treated gp100
Figure 3. Platinum-based chemotherapeutics downregulate PD-L2 on tumor cells via STAT6 dephosphorylation, resulting in enhanced tumor cell recognition by tumor antigen-specific T cells. (a) PD-L1 and PD-L2 expression by BLM melanoma cells cultured with or without platinum chemotherapy for 24 hours (cis= cisplatin 10μg/ml). (b) Western blot of BLM melanoma cells treated with or without IL-4, IFNγ, LPS, TNFα and cisplatin 20μg/ml for 8 hours, as indicated. (c) IFNγ production of gp100-specific T cells upon co-culture with gp100-expressing BLM melanoma cells, pre-incubated with or without cisplatin 10μg/ml for 24 hours. (d) STAT6 expression by squamous cell carcinoma of the head and neck. (e) STAT6-negative tumor with immune infiltrates as internal positive control. (f) Kaplan-Meier estimates of recurrence-free survival of patients with (n=35) or without (n=21) tumor STAT6 expression (p=0.038). The time to recurrence was analyzed in a cohort of head and neck cancer patients that had been treated with cisplatin and radiotherapy in our institute in the period of 2003-2007.

antigen expressing melanoma cells by gp100-specific T cells. As expected, we observed that platinum-induced reduction of PD-L2 coincides with enhanced T cell recognition as measured by antigen-specific IFNγ production (figure 3c). Together, these findings indicate that platinum compounds not only directly affect immune cells, but also modulate an immunosuppressive microenvironment by dephosphorylating STAT6 in tumors, resulting in down-regulation of PD-L2 and subsequent enhanced tumor cell killing by cytotoxic T lymphocytes.

Tumor STAT6 dictates response to platinum-based cancer treatment

Finally, to verify the clinical relevance of platinum-induced STAT6 modulation in tumor cells in cancer patients, we analyzed
tumor STAT6 expression in a cohort of patients with squamous cell carcinoma of the head and neck that had been treated in our institute with cisplatinum in combination with radiotherapy (figure 3d and e, supplementary table 2). After a median follow-up of 68 months we observed a clinically highly relevant and statistically significant difference between patients with STAT6-negative tumors and STAT6-positive tumors with a 3-year recurrence-free survival of 48% and 80%, respectively (figure 3f). These data demonstrate the significance of platinum-induced STAT6 modulation in the tumor microenvironment in cancer patients.

The PD-1/PD-L pathway is of pivotal importance in regulating the immune balance between T cell activation and inhibition\(^\text{10}\). High PD-L expression by antigen-presenting DCs can result in the induction of tolerant or anergic T cells\(^\text{10,15,16}\). A wide variety of human cancers express PD-Ls, which are known to correlate with poor prognosis\(^\text{17-20}\). Antibody blockade of the PD-1/PD-L pathway results in enhanced tumor-specific T cell expansion and activation\(^\text{21,22}\). Our findings show that treatment with platinum compounds down-regulate these PD-L inhibitory molecules, which not only results in enhanced T cell stimulation by dendritic cells, but at the same time enhances the sensitivity of the tumor for lysis by cytotoxic T cells. The importance of our findings is underscored by recent evidence that not only pro-inflammatory cytokines such as TNFα\(^\text{23}\), but also IL-4 and IL-13 are abundantly present in the tumor microenvironment and induce phosphorylation of STAT6 in tumor cells, resulting in an incompetent Th2 type of immune milieu\(^\text{24}\). We propose that platinum-based chemotherapy may revert this incompetent Th2 into effective Th1 anti-tumor immunity. These findings may contribute to the design of innovative treatment schedules for cancer patients by the combination of platinum compounds with known immunotherapeutic approaches.

**METHODS**

**DC culture**

Peripheral blood mononuclear cells were obtained from leukapheresis material from melanoma and colorectal cancer patients participating in clinical DC vaccination studies (Clinical Trials.gov identification numbers NCT-00243529 and NCT-00228189)\(^\text{25,26}\). Informed consent for experimental use of the cells was obtained from all patients. DCs were generated from adherent peripheral blood mononuclear cells by culturing in X-VIVO 15 medium (Lonza, Verviers, Belgium) supplemented with 2% human serum (Sanquin, Amsterdam, the Netherlands) in the presence of interleukin-4 (IL-4, 500 U/ml) and granulocyte-monocyte colony stimulating factor (800 U/ml, both Strathmann, Hamburg, Germany), as previously described\(^\text{27}\).

For experiments concerning Keyhole Limpet Hemocyanine (KLH)-specific immune responses, the DCs were loaded on day 3 of the culture with KLH (10 µg/ml; Calbiochem, USA).

At day 5, DCs were matured with a cytokine cocktail (prostaglandin E2 [Pfizer, New York, NY, USA, 10 µg/ml], tumor necrosis factor alpha [TNFα, 10 ng/ml], IL-1β [5 µg/ml] and IL-6 [15 ng/ml, all CellGenix, Freiburg, Germany]) or with Toll-like receptor 3 and 7 ligands (poly-I:C, Sigma-Aldrich, St. Louis, USA 20 µg/ml and R848, Axxora, Lausan, Switzerland 3 µg/ml) for 48 hours. Chemotherapy was added either after the maturation or during the maturation, as indicated. Supernatants were harvested after 24 hours for cytokine analysis.

**Mixed lymphocyte reaction**

DCs were plated in sterile 96 well U-bottom plates (Corning, New York, USA), 2x10^4/well in RPMI medium (Invitrogen, Carlsbad, USA), supplemented with 5% human serum. Peripheral blood mononuclear cells of a healthy voluntary donor were freshly isolated by density gradient centrifugation and added to the wells, 1x10^5/well. Supernatants were harvested after 48 hours for cytokine analysis, as indicated. After 5 days 1 µCi/
well of 3H-thymidine was added to the culture for 8 hours, after which proliferation was stopped by storing the culture plate at -20°C. Incorporation of 3H-thymidine was measured in a β-counter. In some experiments blocking antibodies against PD-L1 and PD-L2 (both from e-Bioscience, San Diego, USA) were added to the culture in a final concentration of 10 μg/ml, as indicated. Normal mouse serum was used as isotype control. Antibodies against PD-L1 and PD-L2 were preincubated with the DCs for 30 minutes before adding the PBMCs.

**Cytokine measurement and flow cytometry**

Cytokine production was measured by cytometric bead array in the supernatants of the MLR (Th1/Th2 Cytokine CBA 1; BD Pharmingen or Flowcytomix BenderMed, Vienna, Austria) or DC culture (Inflammation kit CBA; BD Pharmingen or Flowcytomix, BenderMed).

**Flowcytometry**

The following antibodies were used for analysis of DC and tumor cell phenotype: HLA-ABC (hybridoma W6/32), HLA-DR/DP (hybridoma Q5/13), CD80, CD86, (all Becton-Dickinson, Franklin Lakes, USA), CD83 (Beckman Coulter, Fullerton, USA), PD-L1-PE (eBioscience, San Diego, USA), PD-L2-PE, IgG1-PE (both Becton-Dickinson).

**Tumor cell culture**

The human melanoma cell line BLM was cultured in DMEM medium (Invitrogen, Breda, the Netherlands) supplemented with penicillin G, streptomycin sulfate, and amphotericin B (Invitrogen) and 7% human serum, in a concentration of 0.5x10^6 cells in T25 flasks or 1x10^6 cells in T75 flasks (Corning). Twice weekly the cells were harvested and plated again 1:10. For PD-L expression upon platinum-treatment, cells were cultured in the presence of oxaliplatin, carboplatin and cisplatin in concentrations as indicated. After 24 hours the cells were washed twice with phosphate-buffered saline (PBS), upon which trypsin (Invitrogen) was added for 30 seconds which was blocked with the human serum-containing medium. Then the cells were harvested. For western blot analysis cells were washed twice in PBS, centrifuged 1500 rpm for 5 minutes, and lysis buffer was added, on ice (see below).

**In vitro chemotherapy treatment**

The chemotherapeutic agents that were used in the experiments are indicated in supplementary table 1. All drugs were used in clinically meaningful concentrations.

**Transfection of siRNA against STAT6**

Small interfering RNA (ON-TARGETplus) for the STAT6 gene were obtained from Dharmacon (Lafayette, CO). In the case of DC, ~1x10^6 immature monocyte-derived DCs were replated on day 3 in a well of a 24-well plate, and transfected at day 5 with 2 μM siRNA and 6 μl DharmaFECT4 transfection reagent, essentially following the manufacturer’s directions. Knockdown efficiency was assessed at the protein level by means of Western blot analysis. Cells were stimulated on day 6 with poly-I:C and R848 for 48 hr after stimulation these cells were used in mixed lymphocyte reactions, as described above.

**Preparation of protein lysates and Western blotting**

Regardless of celltype, 1x10^6 cells were lysed in 100 μl phosphatase-inhibiting lysisbuffer containing 10 mM Tris/HCl pH 7.8, 5 mM EDTA, 50 mM NaCl, 1 mM NaVO_4, 10 mM pyrophosphate, 50 mM NaF, 1% Triton X-100, 1mM PMSF, 10 μg/ml aprotonin, 10 μg/ml leupeptin and 1X Roche protease inhibitor cocktail (Roche Diagnostics Nederland BV, Almere, the Netherlands). Before polyacrylamide gel electrophoresis, reducing sample buffer (62.5 mM Tris/HCl pH 6.8, 25% v/v glycerol, 2% w/v sodium dodecyl sulfate, 0.01% w/v bromophenol blue, 5% v/v β-mercaptoethanol) was added 1:1 to a lysate equivalent of ~200,000 cells. Samples were subjected to polyacrylamide gel electrophoresis using the MiniProtean system (BioRad, Hercules, CA) and further processed for Western blot analysis. After blocking, membranes were incubated with one of the following antibodies: mouse monoclonal anti-β-actin (1:10,000; Sigma-Aldrich, St Louis, MO), mouse monoclonal anti-pSTAT6...
(pY641; 1:250; BD Biosciences Pharmigen) or rabbit polyclonal anti-STAT6 antibody (S-20; 1:500; Santa Cruz Biotechnology). After washing, the membranes were incubated with polyclonal goat anti-rabbit Alexa Fluor-680 (Molecular Probes, Eugene, OR) and goat anti-mouse IRDye800CW (LI-COR Biosciences, Lincoln, NE) as a secondary antibody, and analyzed with the LI-COR Odyssey Imaging system (LI-COR Biosciences, Lincoln, NE). Integrated intensities were analyzed using Excel (Microsoft Corp., Redmond, WA). Whenever necessary, membranes were stripped in 0.2 M glycine/1% w/v sodium dodecyl sulfate, pH 2.5, blocked, and reprobed and processed as described above.

Generation of CD8+ gp100-specific T cells
The vectors pGEM4Z-TCRa296 and pGEM4Z-TCRP296, a kind gift from Dr. N. Schaft (University Hospital Erlangen, Germany), encode the TCR α and β chains originating from a gp100:280-288/HLA-A2-specific CTL clone29. Gp100-specific T cells were generated by transferring the TCR α and β chain to T cells by electroporation of RNA, resulting in transient expression of the TCR chains as described previously30. Briefly, the DNA vectors were linearized with SpeI enzyme and purified by phenol/chloroform extraction and ethanol precipitation, and used as DNA templates for in vitro transcription. In vitro RNA synthesis was done with T7 RNA polymerase (mMESSAGE mMACHINE T7 kit; Ambion, Austin TX, USA) according to manufacturer's instructions. After DNase treatment, RNA was purified by phenol/chloroform extraction and isopropanol precipitation. RNA concentration was measured spectrophotometrically and RNA was stored at -20°C. RNA quality was verified by agarose gel electrophoresis.

CD8+ T cells were isolated from PBMC of an HLA-A2.1 positive donor. Monocytes were removed via adherence and CD8+ T cells were isolated from the non-adherent cell population by positive isolation using FITC-conjugated anti-human CD8 (BD Biosciences, San Jose, CA, USA) and anti-FITC microbeads (Anti-FITC Multisort kit, Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions.

For RNA electroporation, the CD8+ T cells were washed once with PBS and once with OptiMEM without phenol red (Invitrogen Gmbh, Karlsruhe, Germany). 10-12x10^6 cells were incubated for 3 min with 15-20 μg of RNA in 200 μl OptiMEM in a 4-mm cuvette (Biorad). Subsequently, cells were pulsed in a Gene pulser Xcell (Biorad). Pulse conditions were square-wave pulse, 500 V, 5 ms. Immediately after electroporation, the cells were transferred to X-VIVO-15 medium without phenol red (Cambrex, Belgium) supplemented with 2% human serum. After 4 hrs incubation at 37°C, cells were washed and frozen in FCS and 10% DMSO in liquid nitrogen. Expression of the gp100 TCR in the T cells was verified by flow cytometry using PE-conjugated anti-TCRβ14 mAb (Coulter Immunotech, Marseille, France), which recognizes the gp100 TCR. Functionality of the gp100-specific T cells was shown by upregulation of the early activation marker CD69 after overnight stimulation with gp100:280-288 peptide loaded HLA-A2+ immature DCs.

Gp100-specific activation of CD8+ T cells
For gp100-specific activation BLM cells and BLM-gp10014 were activated for 20 hours with IL-4 (300U/ml, Strathmann) and/or IFN (400U/ml, Endogen, Woburn, USA) with or without cisplatin 5 μg/ml (added 15 minutes before IL-4/IFNγ). After 20 hours of activation BLM-gp100 or BLM (7x10^3 per well) were washed and co-incubated with CD8+ gp100:280-288-specific T cells (5x10^4 per well) in round-bottom 96-well plates. After overnight incubation IFNγ production was measured using a standard sandwich ELISA. Gp100-specific T cells produced no IFNγ when co-cultured with BLM melanoma cells that were not transfected with gp100 (not shown). Coating Ab: mouse-IgG1-anti-hIFNγ (clone 2G1; Pierce, Rockford, IL), detection Ab: biotinylated mouse-IgG1-anti-hIFNγ (clone B133.5; Pierce). Streptavidin-HRP and TMB were used as enzyme and substrate, respectively.

STAT6 staining of tumor sections
Paraffin-embedded slides of 58 patients with locally advanced squamous cell carcinoma of the head and neck that had been treated with cisplatin (weekly 40 mg/m^2) in combination with accelerated radiotherapy were stained for STAT6 expression. Inflammatory cells that stain for STAT6 were used as internal control. Percentage of tumor cells with cytoplasmic staining, regardless of intensity, was estimated. Based on the distribution, 50% positive cells was chosen as cut-off level: tumors were considered STAT6-positive when more than 50% of the cells had cytoplasmatic staining.

The staining procedure was as follows. Slides were deparaffinized by 5-minute incubation in xylol, and washed in 100% ethanol, and hydrated in
PBS. Slides were then incubated in 3% hydrogen peroxide for 10 minutes and hydrated in PBS. The slides were then placed in preheated to boiling temperature Sodium citrate, 0.05%, pH 6, and incubated for 10 minutes and rinsed in 1× PBS. They were then incubated overnight with the primary antibody STAT6 diluted in Normal antibody Diluent (Immunologic, Duiven, the Netherlands) overnight at room temperature (dilution 1:80) at 4 °C. The secondary antibody was PowerVision Poly-HRP—Anti Ms/Rb/Rt IgG (Immunologic, Duiven, the Netherlands) which was used for 30 minutes by RT, followed by DAB (DAB plus, Power DAB) substrate for 5 min, and counterstained with hematoxylin and mounted with Xylol.

Clinical study

We retrospectively constructed a patient database using data obtained in the period of 2003-2007 in our institute. Eligible patients had histologically proven locally advanced squamous cell carcinoma of the head and neck without distant metastases, which was treated with weekly cisplatin 40mg/m² in combination with a 6-week course of radiotherapy. Histological tumor material for STAT6-staining obtained before treatment had to be available. The primary endpoint was recurrence-free survival, which was defined as the period between start of treatment and the occurrence of a relapse, not including a second primary or death due to other causes. The following characteristics were registered in the database: tumor location, TNM-stage, age and sex (supplementary table 2). We collected data of 58 patients. Tumor and patient characteristics were evenly distributed between STAT6-positive and STAT6-negative cases. Two patients were excluded: one patient because of inadequate tumor material for STAT6-staining, one patient because only material was available that was obtained after treatment.

Statistical analysis

Data were analyzed statistically by means of analysis of variance (ANOVA) and Student-Newman-Keuls-test. Statistical significance was defined as $p < 0.05$. The recurrence-free survival curves were estimated by the Kaplan–Meier method and compared by means of the log-rank test.

Supplementary Information is linked to the online version of the paper.

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REFERENCES


Supplementary data

Supplementary figure 1. Cytokine production by monocyted-derived DCs. Cells were matured by R848/Poly-I:C in the presence of clinically relevant concentrations of platinum compounds (numbers on X-axis denote µg/ml). Levels are means with SD of 3 independent experiments.

Supplementary figure 2. Knock-down efficiency of DCs transfected with control or STAT6-siRNA as measured by western blot analysis. The ratio of the integral intensity of STAT6 protein expression, normalized to the integral intensity of actin is shown.
Supplementary figure 3. PD-L2 expression by BLM melanoma cell lines. Cells were treated with IL-4 combined with TNFα/IFNγ/LPS, with or without cisplatin 10 µg/ml (light-grey-isotype control, dark-grey PD-L2).

Supplementary figure 4. Western blot analysis of BLM melanoma cells. Expression of total STAT6, phosphorylated STAT6 and actin was measured 30 minutes, 1 hour, 2 hours, 8 hours and 24 hours after stimulation with IL-4/IFNγ and cisplatin 20 µg/ml.
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**Supplementary table 1. Characteristics of the chemotherapeutic agents used in the in vitro experiments.**
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</table>

Supplementary table 2. Patient characteristics

SUPPLEMENTARY REFERENCES


A pilot study on the immunogenicity of dendritic cell vaccination during adjuvant oxaliplatin/capecitabine chemotherapy in colon cancer patients


British Journal of Cancer 2010, In Press
A pilot study on the immunogenicity of dendritic cell vaccination during adjuvant oxaliplatin/capecitabine chemotherapy in colon cancer patients

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Dendritic cell (DC) vaccination has been shown to induce anti-tumor immune responses in cancer patients, but so far its clinical efficacy is limited. Recent evidence supports an immunogenic effect of cytotoxic chemotherapy. Preclinical data indicate that the combination of chemotherapy and immunotherapy may result in an enhanced anti-cancer activity. Most studies have focused on the immunogenic aspect of chemotherapy-induced cell death, but only few studies have investigated the effect of chemotherapeutic agents on the effector lymphocytes of the immune system.

Here we investigated the effect of treatment with oxaliplatin and capecitabine on non-specific and specific DC vaccine-induced adaptive immune responses. Stage III colon cancer patients receiving standard adjuvant oxaliplatin/capecitabine chemotherapy were vaccinated at the same time with keyhole limpet hemocyanin (KLH) and carcinoembryonic antigen (CEA)-peptide pulsed DCs.

In 4 out of 7 patients functional CEA-specific T cell responses were found at delayed type hypersensitivity (DTH) skin testing. In addition, we observed an enhanced non-specific T cell reactivity upon oxaliplatin administration. KLH-specific T cell responses remained unaffected by the chemotherapy, whereas B cell responses were diminished.

The results strongly support further testing of the combined use of specific antitumor vaccination with oxaliplatin-based chemotherapy.

Until recently, the commonly held opinion was that chemotherapy and immunotherapy should not be combined because of the myelosuppressive effect of most cytotoxic agents. However, it has now become evident that chemotherapeutics can exhibit several beneficial effects on the immune system¹. For example, treatment with gemcitabine results in increased antigen cross-presentation, T lymphocyte expansion and T cell infiltration of tumors², and 5-fluourouracil has been described to upregulate tumor antigen expression on colorectal and breast cancer cells³. Furthermore, suppressive regulatory T cells are depleted by several chemotherapeutics also resulting in enhanced T cell reactivity⁴,⁵. In addition, treatment with non-myeloablative lymphodepleting chemotherapy results not only in reduced regula-
tory T cell frequencies, but also provides ‘space’ in the bone marrow for naive T cells to proliferate upon adoptive T cell transfusion. Using this strategy, significant clinical results have been obtained in metastatic melanoma patients. Pioneering work by Zitvogel and colleagues has shown that the cytotoxic agents oxaliplatin and doxorubicin induce immunogenic cell death, since upon treatment with these agents tumor cells transport calreticulin to their cell surface. The exposure of calreticulin provides a signal that is recognized by dendritic cells (DCs) and ultimately results in phagocytosis of the tumor cells. Exposure of tumor cells to oxaliplatin results in the release of High Mobility Group Box 1 (HMGB1) protein, that activates DCs in a Toll-like Receptor-4 (TLR4) dependent manner. The clinical impact of this immune pathway in colorectal cancer patients is emphasized by the recent observation that patients carrying the TLR4 loss-of function allele Asp299Gly exhibit reduced progression-free and overall survival in response to oxaliplatin as compared to patients carrying the normal TLR4 allele. Previous studies in animal models provide a rationale for the combination of chemotherapy and immunotherapy in colon cancer. In a murine model, T cell responses against the colon cancer-associated antigen thymidylate synthase that were induced by a peptide vaccine were not hampered by the administration of 5-Fluorouracil (5-FU). The combination of peptide vaccination and 5-FU resulted in a significant delay in tumor growth as compared to treatment with peptide or 5-FU alone. Similarly, it was shown in a murine colon cancer model that the addition of 5-FU/leucovorin or irinotecan to bone marrow-derived DC vaccination did not diminish the immunogenicity of the vaccine. Small proof-of-concept clinical trials in cancer patients indicate that the efficacy of anticancer vaccines may indeed be enhanced by chemotherapy, however additional studies on scheduling and appropriate combinations are warranted.

Platinum-based chemotherapy represents a cornerstone in the systemic treatment of many types of cancer. Beside their direct cytotoxic effects, platinum anti-cancer drugs may also exert their clinical effect through indirect activation of the immune system via induction of immunogenic tumor cell death. Most studies however, mainly focused on the effect of chemotherapy on tumor cells and antigen-presenting cells. Little attention has been paid to the effect of chemotherapy on the effector lymphocytes of the immune system. Although neutropenia is a common side effect of chemotherapy in the treatment of solid tumors, lymphopenia is rarely observed. A stimulatory effect of chemotherapy on tumor immunogenicity without impairing immune effector cell function would provide a strong rationale to develop novel chemo-immunotherapeutic strategies. For this reason, we investigated whether an oxaliplatin-based chemotherapy regimen combined with antigen-specific vaccination in cancer patients can result in tumor antigen-specific immune reactivity.

PATIENTS AND METHODS

Study design
This was an open-label, single-institution, single-arm exploratory study in which patients with stage III colon cancer received adjuvant treatment with monocyte-derived mature DCs loaded with CEA-peptide in combination with standard oxaliplatin/capecitabine chemotherapy. Approval from the local regulatory committee was obtained.
**Objectives**

The primary endpoint was to assess the immunogenicity of the vaccine during oxaliplatin/capecitabine chemotherapy. Secondary endpoints were the toxicity and the feasibility of CEA-specific vaccination in colon cancer patients, during chemotherapy.

**Patients**

Inclusion criteria included: patients with stage III colon cancer, HLA-A0201 phenotype, ECOG performance status 0-1, age above 18 years, initiation of adjuvant chemotherapy less than 8 weeks since surgery for the primary tumor, no prior chemotherapy, adequate bone marrow, kidney and hepatic function, and written informed consent.

Exclusion criteria included: the use of immunosuppressive drugs, a history of second malignancy and other serious concomitant diseases preventing the safe administration of study drugs or likely to interfere with the study endpoints.

**DC preparation**

DCs were generated as described previously. Peripheral blood mononuclear cells (PBMCs) were isolated by PureCell® (Medicult, Denmark) density gradient centrifugation (30 min, 4°C, 2100 rpm), adherent monocytes were cultured in Cellgro® medium enriched with 500 U/ml Interleukin (IL)-4 and 800 U/ml granulocyte macrophage colony stimulating factor (GM-CSF, all CellGenix, Freiburg, Germany). KLH (10 μg/ml, Calbiochem, USA) was added at day 3 of culture and 2 days before harvesting we added the maturation cocktail (prostaglandin E₂ [PGE₂, Pharmacia & Upjohn, Puurs, Belgium, 10 μg/ml], tumor necrosis factor alpha [TNFα, 10 ng/ml], IL-1β [5 μg/ml] and IL-6 [15 ng/ml, all CellGenix]). Cells were harvested at day 7 and part of the cells were loaded with peptide and put in a syringe for immediate vaccination; the remaining cells were frozen for the second and third vaccination and the DTH. Of each batch of patient DCs a sample was used for quality control. Release criteria were as previously described.

DCs were pulsed with the wild type CEA-peptide CAP-12 (CEA571-579, YLSGANLNL, Clinalfa, Switzerland) directly after harvesting or after thawing.

**Treatment schedule**

Patients received 8 cycles of oral capecitabine 2000 mg/m² days 1-14 and oxaliplatin 130 mg/m² intravenously at day 1. Cycles were repeated every 3 weeks. Prior to the first cycle patients underwent a leukapheresis for collection of peripheral blood mononuclear cells for the DC culture. At day 4, 10 and 17 of the first cycle of chemotherapy patients received 3 vaccinations intradermally (i.d.) and intravenously (i.v.) (5x10^6 i.d./10x10^6 i.v.) with CEA-peptide loaded dendritic cells (figure 1). In preclinical models it has been shown that i.v. administered DCs provide a better antitumor response against visceral metastases, whereas non-visceral metastases respond better to i.d. administered DCs. Because both lymphogenic and hematogenous spreading occur in colorectal cancer, we chose to combine both routes of administration. After completion of the vaccinations a DTH skin test was performed on day 19, followed by biopsies of DTH injection sites on day 22.

**Immunologic monitoring**

- **Analysis of peripheral blood immune cell subsets**
  Within the CD45+ leukocyte population, PBMCs were stained with antibodies against CD14, CD19, CD3, CD8, CD4 and CD56 (for distinction of monocytes, B cells, T cells, CTLs, Thelper lymphocytes and NK cells, respectively). After washing, the samples were analyzed by flowcytometry.

- **Monitoring of non-specific T cell stimulatory capacity**
  PBMCs were obtained by density-gradient centrifugation at day 1, 4, 10, 17 and 22. Cells were plated in 96-wells U-bottom plates, 2x10^5/well, in RPMI/human serum 5% and Phytohaemagglutinin (PHA) was added (1 μg/ml). After 24 hours supernatant was harvested for cytokine production analysis. After 3 days 1 μCi/well of 3H-thymidine was added to the culture for 8 hours, after which proliferation was stopped by storing the culture plate at -20°C. Incorporation of 3H-thymidine was measured in a β-counter. The in-
dex was calculated as the counts ratio between PHA-stimulated PBMC and non-stimulated PBMCs.

- Monitoring of KLH-specific CD4+ T cell responses

CD4+ T cell responses against KLH were measured using a ^3H-Thymidine incorporation proliferation assay with PBMCs of the patients at day 1, 4, 10, 17 and 22. The index was calculated as the counts ratio between KLH-stimulated PBMCs and non-stimulated PBMCs.

- Delayed type hypersensitivity reactions

Post-treatment DTH reactions were performed as described previously. Briefly, CEA-peptide only (100 µg in 100 µl), DCs pulsed with CEA-peptide, DCs pulsed with KLH and CEA-peptide, and DCs pulsed with KLH only (0.4-5x10^5 DCs each in approximately 100-200 µl) were injected i.d., 5-10 cm from an inguinal lymph node at different sites, in the back of the patients. The maximum diameter of induration was measured after 48 hours. T cell culture from DTH biopsies was performed in low dose IL-2 (Proleukin®, Chiron, Netherlands 100U/ml) for approximately 2 weeks without ex vivo restimulation with antigen as described before.

- MHC Tetramer staining

DTH-derived cells (1x10^5 cells in 10 µl) or PBMCs (1x10^6 cells in 10 µl) were incubated with PE-labeled CEA and cytomegalovirus (CMV) tetrameric-MHC complexes (Sanquin, Amsterdam, Netherlands) for 60 minutes, room temperature. In the last 20 minutes of this incubation, FITC-conjugated monoclonal antibodies directed against CD8 (Becton Dickinson) were added. After washing, the samples were analyzed by flow-cytometry. For peripheral blood at least 1x10^6 PBMCs were analyzed, for DTH-infiltrating T cells all available cells were analyzed. In all analyses CEA tetramer staining was compared with CMV tetramers as a negative control.

- Cytokine secretion

Production of cytokines by the total population of DTH-derived cells was measured in response to T2 cells pulsed with CEA-peptide or an irrelevant HLA-A2.1 binding peptide (tyrosinase or G250). Cytokine production was measured in supernatants after 16 hours by cytometric bead array (Th1/Th2 Cytokine CBA 1; BD Pharmingen). IFNγ production was considered positive when IFNγ-levels of DTH-infiltrating T cells were more than tenfold higher after co-incubation with CEA-loaded T2 cells compared to co-incubation with T2 cells loaded with irrelevant antigen.

RESULTS

Patients and toxicity

Seven patients were included in this study with a median age of 55 years (range 47-75). All patients had stage III colon cancer. Most patients experienced grade I fever and flu-like symptoms after vaccination. One patient had a grade 2 allergic reaction of unknown cause after two vaccinations, consisting of generalized exanthema with fever and pruritus for which he was treated with antihistamines.

Figure 2. Frequencies of PBMC subsets during treatment with oxaliplatin and capecitabine and DC vaccination. Means with standard deviation are depicted, (a) total T cells (CD3+), (b) T helper cells (CD4+), (c) cytotoxic T cells (CD8+), (d) natural killer cells (CD3-/CD56+), (e) monocytes (CD14+) (f) B cells (CD19+).
Figure 3. Proliferative non-specific T cell response upon PHA-stimulation during treatment with oxaliplatin/capecitabine and DC vaccination. (a) In all patients an increase in proliferation was observed during treatment. (b) Also the IFNγ-production upon PHA-stimulation increased during treatment (data of 4 tested patients are given; the means with standard deviation are depicted).

The third vaccination was without symptoms. Other toxicities (grade 2 nausea/vomiting, one patient; grade 1 diarrhea, one patient; grade 3 neurotoxicity, one patient) were considered to be related to the chemotherapy. With a follow-up of 10-35 months (median 18 months), all patients are recurrence-free.

Peripheral blood subsets during treatment
Peripheral blood frequencies of mononuclear cells did not significantly decrease during treatment with oxaliplatin/capecitabine (figure 2, supplementary figure 1). The total number of T cells (CD3+), T helper cells (CD4+), cytotoxic T cells (CD8+), B cells (CD19+), monocytes (CD14+) and Natural Killer cells (CD3-/CD56+) remained stable during chemotherapy.

Non-specific immune reactivity during oxaliplatin/capecitabine
To test whether treatment with oxaliplatin and capecitabine affects overall T cell reactivity that could influence the efficacy of DC vaccination, we tested the PHA-induced T cell proliferative response at different time points during treatment (figure 3). Surprisingly, we found a striking increase in the proliferative capacity of peripheral blood T cells, in most cases directly after infusion of the oxaliplatin (figure 3a). In addition, the IFNγ-release upon PHA-stimulation increased (figure 3b). Since this has not been reported before after DC vaccination, and since we also observed this in a control group of patients that were treated with platinum compounds only (supplementary patients and methods and supplementary figure 2), we conclude that this is a platinum-effect and not due to the DC vaccine. When peripheral blood lymphocytes of 3 healthy donors were stimulated with PHA after 24 hours of in vitro culture with oxaliplatin, we did not observe an enhanced effect of the platinum treatment (supplementary figure 3).

KLH-specific immune responses during oxaliplatin/capecitabine
To test whether de novo antigen-specific T and B cell immune responses can be induced during treatment with oxaliplatin and capecitabine, we loaded the DCs with
the control antigen KLH and measured the proliferative T cell response and antibody response (figure 4, table 1). We observed a robust CD4+ proliferative response against KLH in all patients, comparable to previous studies in colorectal cancer and melanoma patients who were vaccinated with the same vaccine without chemotherapy (figure 4a)\(^1\),\(^7\),\(^11\),\(^21\). However, in all but two patients antibody responses against KLH were absent (figure 3b). In the patients with an antibody response, this reaction was only weak. This is in contrast to our previous studies in which we could detect a response in almost all vaccinated patients\(^2\).

**CEA-specific immune responses during oxaliplatin /capecitabine**

We next determined the vaccine-induced tumor antigen-specific immune response in DTH biopsies as previously published\(^2\),\(^3\),\(^4\). In 4 out of 7 patients we found CEA-specific T cells by means of tetramer analysis (figure 5a, table 1). In a previous cohort of colorectal cancer patients that were treated with the same vaccine without chemotherapy we found CEA-specific DTH-infiltrating T cells in 5 out of 11 patients after one vaccination cycle\(^1\). Thus, the efficacy of the vaccine does not seem to be negatively influenced by chemotherapy in terms of the induction of tumor antigen-specific immunity. Furthermore, the functionality of the T cells remained unaffected since they produced high amounts of IFN\(\gamma\) and IL-2 upon coculture with CEA-loaded target cells (figure 5b).

**DISCUSSION**

Despite ample evidence for chemotherapy-induced immunogenic cell death, much less is known about the effect of chemo-
therapy on the effector lymphocytes of the immune system \textit{in vivo} in cancer patients. Therefore we conducted a clinical pilot trial to test the immunogenicity of a DC vaccine in stage III colon cancer patients treated with standard adjuvant oxaliplatin and capecitabine chemotherapy. We found that robust \textit{de novo} KLH-specific T cell responses could very well be induced during the chemotherapy regime. However, B cell responses were hampered when compared to previous studies using the same vaccine without chemotherapy\textsuperscript{21,23}. No additional toxicity was observed, apart from the side effects that frequently occur for both treatment strategies separately.

In 4 out of 7 patients we observed a functional CEA-specific T cell response upon vaccination with CEA-loaded DCs. Although the number of patients is limited, this is comparable to a previous cohort of colorectal cancer patients that were treated with the same vaccine without chemotherapy\textsuperscript{17}. To date, none of the treated patients have had a relapse. However, given the adjuvant nature of the treatment, the short follow-up and the limited numbers of patients, no conclusion can be drawn on the clinical efficacy of adding DC vaccination to standard adjuvant chemotherapy. Other studies investigating the combination of immunotherapy and chemotherapy have been performed in colorectal cancer patients. Kaufman and colleagues vaccinated metastatic colorectal cancer patients with a CEA-ALVAC vaccine in combination with irinotecan, 5-fluorouracil and leucovorin and found that the CEA-specific T cell response was unaffected by the chemotherapy\textsuperscript{14}. Similar observations were made, using the same chemotherapeutic agents but different vaccines\textsuperscript{25,26}.

Surprisingly, the non-specific T cell proliferative capacity increased upon chemotherapy treatment in most patients shortly after infusion of the oxaliplatin. Previously, others have found a similar effect for gemcitabine in a murine model\textsuperscript{2}, but for platinum-based compounds this has not been reported before. Although oxaliplatin has been described to induce an immunogenic type of cancer cell death, which could result in DC activation and subsequent non-specific T cell activation\textsuperscript{9,27}, we consider this mechanism less likely in our study since the patients were treated in an adjuvant setting, that is in the absence of macroscopic tumor.

A possible explanation for the increased T cell activation may lie in our recent finding that platinum compounds dephosphorylate Signal Transducer and Activator of Transcription 6 (STAT6)\textsuperscript{28}.

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\textbf{Table 1. Antigen-specific immune responses.} Summary of all tested KLH and CEA-specific immune responses upon vaccination and oxaliplatin/capecitabine treatment. nt: not tested
This protein is of crucial importance for the induction of T helper 2 immunity and hence for the development of B cell responses. Inactivation of STAT6 may result in enhanced T helper 1 responsiveness and at the same time in decreased B cell activation, as observed in this study. The fact that we did not find this effect when lymphocytes of healthy donors were stimulated with PHA after 24 hours of culture in the presence of oxaliplatin, suggests that this mechanism mainly works through antigen-presenting cells and not lymphocytes. However, it is questionable whether the in vitro conditions properly mimic the in vivo situation. More research is needed to decipher the exact effects of platinum compounds on immune cells.

Our study is the first to investigate the combined use of specific vaccination with oxaliplatin-based chemotherapy. Oxaliplatin appears to be a promising drug to include in chemo-immunotherapeutic regimens, since it induces an immunogenic type of tumor cell death resulting in enhanced DC activation. Our findings that the immune effector cells remain unaffected and that T cell proliferative capacity following PHA stimulation even increases upon oxaliplatin treatment further support this strategy.

Although DC vaccinations frequently induce immune responses, objective clinical remissions are rare. One possible explanation is that DC vaccination may be more effective in residual microscopic disease, since it lacks a direct cytotoxic effect that is likely required in macroscopic disease. On the other hand, also cytotoxic chemotherapy does not result in complete tumor eradication in the majority of patients with metastatic solid tumors. Therefore the combination of DC vaccination with cytotoxic chemotherapy is an attractive strategy. Our data demonstrate the feasibility of the combination of these two strategies, and provide the rationale for future studies that investigate the clinical efficacy of this strategy.

ACKNOWLEDGEMENTS
We kindly thank Christel van Riel, Michel olde-Nordkamp, Jeanette Pots and Michelle van Rossum for their assistance. This work was supported by grants from the Netherlands Organization for Scientific Research (92003250) to W.J.L. and the Sascha Swarttouw-Hijmans Foundation. None of the authors has competing financial interests.

REFERENCES


Supplementary Patients and Methods
Because we found enhanced T cell proliferation after oxaliplatin infusion, during DC vaccination, we questioned whether this could be caused by the DC vaccine or by the oxaliplatin. Furthermore, we questioned whether this could be an oxaliplatin-specific or general platinum effect. For this reason we obtained PBMCs from 2 patients with squamous cell head and neck carcinoma and 1 patient with cervical cancer that were treated with weekly cisplatin (40 mg/m²) in combination with local radiotherapy and tested non-specific T cell proliferative capacity by PHA stimulation. Patients gave written informed consent. Blood was obtained before and 3-4 days after infusion of cisplatin.

PBMCs were obtained by density-gradient centrifugation. Cells were plated in 96-wells U-bottom plates, 2x10⁵/well, in RPMI/human serum 5% and Phytohaemagglutinin (PHA) was added (1 µg/ml). After 24 hours supernatant was harvested for cytokine production analysis. After 3 days 1 µCi/well of 3H-thymidine was added to the culture for 8 hours, after which proliferation was stopped by storing the culture plate at -20°C. Incorporation of 3H-thymidine was measured in a β-counter. Production of cytokines was measured in supernatants after 16 hours by cytometric bead array (Th1/Th2 Cytokine CBA 1; BD Pharmingen). Data were analyzed statistically by means of analysis of variance (ANOVA) and Student-Newman-Keuls-test. Statistical significance was defined as p < 0.05.

In a similar experiment PBMCs were obtained from three healthy voluntary donors by density-gradient centrifugation. The monocytic fraction was removed by plastic adherence during 1 hour at 37°C. Cells were plated in 96-wells U-bottom plates, 2x10⁵/well, in RPMI/human serum 5% either in the presence or absence of oxaliplatin (Eloxatin) at a concentration of 4 µg/ml. After 24 hours the cells were washed and PHA was added (1 µg/ml). Proliferation was measured according to the above-described protocol.

Supplemental Figure 1. Flow cytometrical determination of lymphocyte subpopulations. Gating of lymphocytes on CD45 (red) is verified by back gating from the respective marker against SS. Lymphocyte subpopulations are determined in the respective quadrants. Monocytes were determined from the CD45/SS plot (green). FS, Forward scatter; SS, side scatter.
Supplementary figure 2. PHA responses in cancer patients treated with platinum compounds. (a) Proliferative response of PBMCs to phytohaemagglutinin (PHA) of cancer patients before and after treatment with cisplatin or oxaliplatin (n=5). Production of (b) IL-1 and (c) TNFα of PHA-stimulated PBMCs of cancer patients before and after treatment with cisplatin (n=3; * p < 0.05, ** p < 0.01).

Supplementary figure 3. Proliferative responses of peripheral blood lymphocytes of three healthy donors to PHA. The cells were pre-incubated for 24 hours in the presence or absence of a clinically relevant concentration of oxaliplatin (the means of three experiments with standard deviations are given; ** p < 0.01).
Dendritic cell vaccines in melanoma: From promise to proof?

Lesterhuis WJ, Aarntzen EH, De Vries JJ, Schuurhuis DH, Figdor CG, Adema GJ, Punt CJ

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Dendritic cell vaccines in melanoma: from promise to proof?

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Dendritic cells (DCs) are the directors of the immune system, capable of inducing tumour antigen-specific T- and B cell responses. As such, they are currently applied in clinical studies in cancer patients. Early small clinical trials showed promising results, with frequent induction of anti-cancer immune reactivity and clinical responses. In recent years, additional trials have been carried out in melanoma patients, and although immunological responses are often reported, objective clinical responses remain anecdotal with objective response rates not exceeding 5-10%.

Thus, DC vaccination research has now entered a stage in between ‘proof of principle’ and ‘proof of efficacy’ trials. Crucial questions to answer at this moment are why the clinical responses remain scarce and what can be done to improve the efficacy of vaccination. The answers to these questions probably lie in the preparation and administration of the DC vaccines. Predominantly, cytokine-matured DCs are used in clinical studies, while from preclinical studies it is evident that DCs that are activated by pathogen-associated molecules are much more potent T cell activators. For sake of easy accessibility monocyte-derived DCs are often used, but are these cells also the most potent type of DCs? Other yet unsettled issues include the optimal antigen-loading strategy and route of administration. In addition, trials are needed to investigate the value of manipulating tolerizing mechanisms, such as depletion of regulatory T cells or blockade of the inhibitory T cell molecule CTLA-4. These issues need to be addressed in well-designed comparative clinical studies with biological endpoints in order to determine the optimal vaccine characteristics. DC vaccination can then be put to the ultimate test of randomized clinical trials.

Here, we review the immunobiology of DCs with emphasis on the different aspects that are most relevant for the induction of anti-tumour responses in vivo. The different variables in preparing and administering DC vaccines are discussed in this context and the immunological and clinical results of studies with DC vaccines in melanoma patients are summarized.

1 Introduction

Melanoma is one of the more immunogenic cancer types and many strategies to enhance specific or non-specific immunity in melanoma patients have been explored in clinical studies¹.
DCs are activated and subsequently the immune system is alerted\(^2\). It then depends on the size of the tumour and its immunomodulatory characteristics, whether the immune system is able to eradicate the cancer. Often malignant growth is a slow and silent process that fails to provide a ‘danger signal’ necessary for the activation of the immune system. The goal of DC vaccination is to mend this inattention of the immune system by providing it with ex vivo ‘educated’ DCs; appropriately activated and loaded with tumour antigen. The underlying principle is that DCs are the most potent antigen presenting cells of the immune system that play a central role in the induction-phase of antigen-specific immunity. DCs acquire and process antigen and migrate to the lymphoid organs where they present the antigen to the specific arm of the immune system, resulting in the induction of primary T- and B cell responses. Because of these unique qualities they represent an interesting tool in cancer immunotherapy. The possibility to generate DCs in large amounts for clinical use has accelerated research in this field, and immunological and clinical responses have been reported in melanoma patients after vaccination with DCs (table 1-2)\(^3\)-\(^5\). Several years ago it was already estimated that more than thousand cancer patients had received some form of DC-based vaccination\(^6\). In contrast to other systemic therapies in cancer treatment, it is not possible to pool these patients in a meta-analysis. This is due to the enormous diversity in terms of vaccine preparation and administration and immunomonitoring. Although much progress has been made in several of these areas over the past years, there seems still room for significant improvement before an optimal DC vaccine is to put to the ultimate test of large scale clinical studies.

**2.1 DC subsets**

Two distinct categories of DCs exist: plasmacytoid DCs and conventional or ‘myeloid’ DCs\(^7\). Plasmacytoid DCs are circulating cells with a plasmacytoid morphology that are capable of producing large amounts of type I interferons upon activation by microbial stimuli\(^8\). In addition, they can differentiate into DCs that are capable of activating naïve T cells against allo-antigens\(^9\) and exogenous antigens\(^10\). In the context of cancer, plasmacytoid DCs have been implicated in the induction of both immunity and tolerance, and their potential role in vaccination strategies in cancer patients still has to be determined\(^11\).

Conventional DCs or ‘myeloid’ DCs\(^12\) can be further divided into migratory DCs, which actively sample the peripheral tissues and migrate to draining lymph nodes to present antigens to T cells, and lymphoid-tissue-resident DCs which capture local (foreign and self-) antigens and present it to local T cells\(^13\). Examples of lymphoid-tissue-resident DCs are splenic and thymic DCs. Migratory DCs derive from both CD34\(^+\) precursor cells and monocytes. Monocytes can differentiate into DCs upon transendothelial migration\(^14\). The presence of GM-CSF, in addition to other pro-inflammatory cytokines, can differentiate both CD34\(^+\) precursor cells and monocytes into DCs. Recently, it was proposed to recognize a third distinct group of conventional DCs, ‘inflammatory’ DCs: cells that are not present in the steady state, but that appear under the influence of inflammation or infection\(^15\).

**2.2 DC subsets in clinical trials**

Monocyte-derived DCs have been used in most vaccination studies because of the
relative ease with which large quantities of cells can be obtained. Usually, one leukopheresis is enough to obtain approximately 100-150x10^6 cells. Yields are much lower with CD34+ selection and repeated leukophereses are often necessary to obtain enough cells. The same accounts for circulating blood DCs, which can be obtained from peripheral blood after in vivo Flt-3L expansion and negative selection ex vivo.

Banchereau and colleagues have reported impressive immunological and clinical results using Flt3-Ligand expanded CD34+ DCs. In other studies with CD34+ DCs, using different maturation and vaccination regimes, clinical responses were observed less frequently. The same variable outcomes are seen in studies with monocyte-derived DCs (table 1 and 2, and section on clinical results). These differences may well be related to the differences in culture and maturation protocols, than to the differences in DC subsets. Due to the fragmented nature of the available data and the absence of direct comparative studies, it is not possible at this moment to draw any firm conclusion on the most optimal DC subset to be used in clinical trials. In recent years, evidence is accumulating that considerable cross-talk takes place between the different DC subsets, and that perhaps it is beneficial even to combine different subsets of DCs, as has been done in the above mentioned studies.

3.1 Maturation

The term ‘maturation’ refers to the phenotypic and functional reaction of DCs upon encountering danger signals. Maturation can be induced by pro-inflammatory cytokines such as IL-1 or IL-6, by interaction with T cells and by interaction with pathogens. DCs can detect pathogens through pattern recognition receptors such as Toll-like receptors (TLR). The TLR family consists of several receptors that recognize molecular patterns of pathogens, for example bacterial lipopolysaccharide (through TLR4) and single stranded viral RNA (through TLR7).

In the past 10 years, the term ‘mature’ DCs has generally been used to describe T (helper 1) cell stimulatory DCs. Immature DCs were considered to be primarily involved in the recognition and uptake of antigen. Upon receiving maturation signals these immature DCs would then change their chemokine receptor repertoire and upregulate their co-stimulatory molecules, thus acquiring the phenotype of mature DCs that are capable of migration to the lymph nodes and activation of T cells. In the absence of maturation signals, DCs would not upregulate their co-stimulatory molecules and would thus remain anergy- or tolerance-inducing antigen-presenting cells.

Although this concept may not be entirely wrong, recent findings show that this is probably an oversimplification (for a comprehensive review on this topic see ref. 21). For instance, in the steady state, that is in the absence of ‘danger’, immature DCs have been shown to be capable of circulating through the tissues and lymphoid organs, encountering and capturing both self-antigens and innocuous environmental antigens. It is suggested that through this mechanism immature DCs play a critical role in the continuous induction of peripheral tolerance, thereby preventing both auto-immunity and hyperreactivity. In addition, although the expression of co-stimulatory molecules is one of the phenotypic markers of mature DCs, the induction of a tolerogenic immune response depends on the presence of these molecules as well. In a chimeric murine model it was shown that although cytokine-
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matured DCs demonstrated upregulation of co-stimulatory molecules and induction of T cell proliferation, the activated T cells did not fully develop into IFN-producing effector cells. Only when the DCs were activated through Toll-like receptors (TLRs), also referred to as 'licensed' DCs, the induced antigen-specific T cells were able to fully develop into effector cells. The 'licensing' of DCs is not only restricted to pathogen-derived signals, since activation by bystander T helper cells may also suffice.

Another level of complexity is added by the timing and duration of the maturation signal. For example, murine bone marrow-derived DCs that are exposed to lipopolysaccharide for 48 hours have the same expression of costimulatory molecules CD80, CD86 and CD40 as compared to DCs that are exposed to lipopolysaccharide for 8 hours and also have a comparable migratory capacity and allostimulatory potential. However, the former exhibit a decreased IL-12 production potential correlating with low antigen-specific T cell responses after vaccination in mice. In addition, both the timing of the activation signal and the exposure to antigen are of crucial importance for optimal antigen presentation: only the simultaneous presence of apoptotic cells and TLR ligands to DCs resulted in efficient antigen-presentation and subsequent T cell activation.

With respect to the type of TLR ligands, it has been shown that combinations of different TLR ligands can have a synergistic effect on the immunogenic potential of DCs in vitro and in vivo. For example, a combination of triggering via TLR3 and TLR 7 leads to a ten- to hundred-fold higher IL-12 production, when compared to DCs that have been activated by one of the respective TLRs alone. In concordance with these data, Querec et al. recently showed that broad immunity that was induced by a Yellow fever vaccine was dependent on triggering multiple TLRs simultaneously (notably TLR 2,7,8,9). In terms of IL-12 production, it appears that monocyte-derived DC activation via TLR3 and 7 leads to the most potent TH1 T cell responses. However, migratory capacity is somewhat hampered in these cells as compared to cytokine-matured DCs, which can be restored by co-culturing the DCs in PGE.

If DCs with similar expression of costimulatory molecules can exert entirely different functions, what then determines the nature of the induced T cell response? The ability of DCs to produce IL-12, TNFα and IL-6 is thought to be important for the induction of robust T cell responses and to bypass suppressor T cell-induced tolerance. In addition, several factors such as IL-10, vitamin-D3 and corticosteroids can skew the DCs into a more suppressive T cell type of inducer. However, it still remains poorly understood which mechanisms truly determine the nature of the T cell response as instructed by the DCs.

Taken together, these findings show that the process of maturation is highly complex and that different maturation stimuli do not lead to one common 'mature DCs', but that there are in fact a lot of different types of mature DCs that exert different functions ranging from the induction of strong Th1-type responses to the induction of tolerance. These data also show that the maturation stage of DCs cannot be fully characterized by the expression of costimulatory molecules and surface-MHC. When reporting clinical DC vaccination trials it is therefore of crucial importance that the phenotypic and functional characteristics of the DCs are carefully described.
Thus far, T cells have been the primary target of DC vaccination protocols. In the last few years more evidence is accumulating that also DC-induced activation of natural killer (NK) cells and natural killer T (NKT) cells can eradicate cancer. NKT cells and DCs activate each other communally. DCs activate NKT cells by presentation of exogenous (microbial) and endogenous glycolipid antigens, thereby inducing IFNγ release and CD40-ligand upregulation by the NKT cells. Reciprocally, NKT cells activate DCs via CD40-CD40L interaction. This in turn causes the DCs to produce IL-12, which further activates the NKT cells (reviewed in ref. 42). Because glycolipid antigens induce both NKT and DC activation in this manner, they offer an interesting target in cancer immunotherapy. The synthetically produced, marin sponge-derived glycolipid -galactosylceramide (GalCer) has been studied in murine and human studies. A single injection of GalCer combined with a model antigen induced strong CD4+ and CD8+ T cell responses, through activation of DCs in a TLR-independent but NKT cell-dependent manner. The results were confirmed using tumour vaccines instead of a model antigen.

Thus far, one phase I dose-escalation study with clinical-grade GalCer (KRN 7000) has been carried out in 24 patients with solid tumours, in which no clinical responses were observed.

### 3.2 Maturation of DCs in clinical trials

In early clinical trials only immature DCs were used. Some of these DCs might have been ‘semi-mature’ due to the addition of fetal calf serum in the culture medium. Although objective clinical remissions were observed in these studies, there is now strong evidence that immature DCs should no longer be used in clinical practice. In a comparative study in metastatic melanoma patients cytokine-matured, peptide-pulsed DCs were superior to immature DCs, with no immune induction against the control protein keyhole limpet hemocyanine (KLH) in the latter arm of the study, while all patients that were vaccinated with cytokine-matured DCs showed a strong T cell and B cell response against KLH. In addition, only in patients vaccinated with mature DC, delayed type hypersensitivity (DTH) responses against the vaccine were observed. Using immature DC in vaccination protocols in cancer patients might in fact be hazardous due to the induction of tolerance instead of immunity.

Several maturation methods have been applied with maturation being defined by a high expression of mature DC-specific surface markers (which is a rather limited description as discussed in the previous paragraph). Most widely used is a cytokine cocktail that includes TNFα, with any of the following cytokines in any combination: IL-1β, IL-6, prostaglandin-E2, or the supernatant of activated autologous monocytes (Monocytes Conditioned Medium). There is some evidence that culturing DCs with IL-15 may lead to a type of mature DCs that induces stronger Th1 effector type of immune responses, however no comparative studies have been reported yet. Lastly, CD40-ligation has been used as a method of activation of DCs in a clinical setting.

None of these different maturation methods has shown to be clearly superior, which is mainly due to the fact that there are no direct comparative studies, neither in animal models nor in cancer patients. It is important to realize that to date no trial has been published in which TLR-matured, truly Th1-polarized DCs were...
used in cancer patients. In vitro data are promising\textsuperscript{63}, but clinical results with TLR-matured DCs are eagerly awaited.

4.1 Antigen-processing and presentation

Intracellular endogenous antigens are usually presented in MHC class I, whereas exogenous antigens are usually presented in class II by antigen-presenting cells (figure 1a)\textsuperscript{64}. Of crucial importance for DC-based vaccines in cancer immunotherapy is the finding that internalized antigens from exogenous sources, such as apoptotic or necrotic tumour cells\textsuperscript{65,66} are also presented in MHC class I to cytotoxic T cells, a process referred to as cross presentation (figure 1a)\textsuperscript{67,68}. Thus, tumour antigens derived from necrotic or apoptotic tumour cells can be presented by the DCs to both CD4+ (in MHC class II) and CD8+ T cells (in MHC class I), which implies that a broad effector and memory immune response can be induced against tumour antigens.

Apoptosis is a physiological ‘silent’ immunologic event, pertaining millions of cells per second. However, with regard to the immunogenicity of apoptotic cancer cells the data are less clear\textsuperscript{69-73}. Recently it was shown in a murine model that chemotherapy-induced apoptosis results in cross-presentation and T cell activation, thereby preventing tumour outgrowth in a prophylactic setting\textsuperscript{74}. The same authors showed in another study that apoptotic tumour cells induce immune responses in a TLR-4 dependent manner via secretion of HMBG1\textsuperscript{75}. The importance of these findings is that apoptosis of cancer cells may be an immunologic event without the need of an extra ‘danger signal’, and that this can be accomplished by the administration of cytotoxic drugs or radiotherapy.

4.2 Antigen-loading in clinical trials

In most clinical DC vaccination studies synthetic MHC class I-binding peptides have been used\textsuperscript{15,17,18,49,52,56,76-86}, with class II-binding peptides being added in some (figure 1b)\textsuperscript{57,87}. There is convincing preclinical evidence that targeting both cytotoxic T cells and T helper cells is of crucial importance for the induction of a strong and sustained anti-tumour T cell response\textsuperscript{88,89}. However, no clinical trial has yet been performed in which MHC class I antigen-loaded DCs are compared to the combination of both MHC class I and II antigen-loaded DCs.

Instead of HLA-binding peptides, whole antigenic proteins can be used. The DCs processes the protein into peptides, which has the advantage that multiple epitopes are presented in both MHC class I and II and that there is no HLA-restriction. Unfortunately, little recombinant proteins are clinically grade available\textsuperscript{90}. Autologous\textsuperscript{49,91-96} or allogeneic\textsuperscript{61,97-99} tumour cell lysates have also been applied as a source of antigens. This has several advantages: the antigen expression by the tumour does not need to be defined and a wide array of both MHC class I and II epitopes are presented including tumour-unique antigens. Possible drawbacks include the presentation of auto-antigens, the requirement of a sufficient volume of tumour tissue and difficulties in monitoring tumour-specific T cell responses since the antigens relevant to T cell responses are not known. Palucka et al. partly circumvented the latter problem by using large peptide libraries to pinpoint the dominant T cell responses in advanced melanoma patients after vaccination with allogeneic tumour lysate-loaded DCs\textsuperscript{61}. 152
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Figure 1. Antigen presentation and antigen loading. (a) Antigens can be processed by the DC through three major pathways. Exogenous antigens will be internalized by the DC and enter the endocytic pathway in which they are targeted to lysosome-related MHC class II-rich compartments. In these compartments the antigens are degraded and loaded onto MHC class II molecules. During maturation of the DC the MHC-peptide complexes are released to the surface, thus making the cell ready for antigen presentation to CD4+ T helper cells (route 1 in the figure). Intracellular endogenous antigens, such as unstable self-proteins or viral proteins, are cleaved into peptides in the proteasome and subsequently translocated into the lumen of the endoplasmatic reticulum (ER) by transporters associated with antigen processing (TAP), where stable MHC class I-peptide complexes are assembled. Upon binding of the peptide, the complex is released from the endoplasmatic reticulum and transferred to the cell surface (route 2 in the figure), where it is presented to CD8+ cytotoxic T cells. Lastly, DCs have the unique capacity to present exogenous antigens, such as necrotic or apoptotic tumour cells, in MHC class I to cytotoxic T cells, a process referred to as cross presentation (route 3 in the figure). (b) Either by transfection with DNA or RNA encoding melanoma antigens, or pulsing with tumour lysate, antigenic proteins or peptides melanoma antigens can be loaded onto the DCs for antigen presentation.

Transfection of DCs with RNA concerns a novel antigen-loading technique, with either tumour-derived RNA or synthetic RNA encoding specific melanoma-associated antigens being used. A benefit of this technique lies in the presenta-
tion of several MHC class I epitopes and sometimes also MHC class II epitopes, depending on the presence of an endosomal targeting sequence\textsuperscript{104}. Also, it may lead to a more prolonged presentation of the antigen as compared to peptide-loading which appears to be short-lived\textsuperscript{105}. Potential drawbacks of RNA transfection include a variable expression and a low yield of viable cells after transfection, especially with respect to the most widely used technique of RNA electroporation. However, several studies have shown that this technique is feasible and results in highly efficient DC-transfection\textsuperscript{103,106-109}. Furthermore, anti-tumour T cell responses and clinical responses have been reported in patients vaccinated with DCs electroporated with tumour-derived RNA\textsuperscript{110,111}. In a study with 33 metastatic melanoma patients vaccination with autologous tumour lysate-loaded DCs resulted in a slightly higher response rate compared to peptide-pulsed DCs, (3 versus no partial remissions, respectively)\textsuperscript{93}. Equal immunogenicity was demonstrated for peptide- and RNA-pulsed DCs in colorectal cancer patients\textsuperscript{112}. However, immature DCs were used in both studies. Mature DCs have not been used in clinical studies comparing different antigen-loading techniques. Therefore at this moment the optimal method for antigen loading is unknown.

Antigens that target DCs in vivo would obviate the need for laborious ex vivo culturing protocols\textsuperscript{113}. Although this appears to be feasible through DC-specific molecules such as the C-type lectins DC-SIGN\textsuperscript{114} and DEC-205\textsuperscript{115} using model antigens, it will probably take some years before this can be applied in clinical experiments in cancer patients. In addition, a DC maturation stimulus should be applied in vivo. Another approach concerns the targeting of intratumoural DCs by delivering danger signals in situ, which can be combined with chemokine treatment in order to increase the number of intratumoural DCs\textsuperscript{116}. In addition, local tumour-destructing therapies can induce antigen-release in situ, for example by using radiotherapy\textsuperscript{117}, chemotherapy\textsuperscript{118} or radiofrequency ablation\textsuperscript{119}. The immunogenicity of these methods may then be further enhanced by the local delivery of DC-activating signals\textsuperscript{120,121}.

5.1 Migration
DCs are the sentinels of the immune system and therefore need efficient migratory capacity. In peripheral tissues they continuously sample the environment for antigens. After antigen uptake DCs must migrate to the secondary lymphoid organs, in particular the lymph nodes for presentation of the antigens to the adaptive arm of the immune system. The lymph node homing chemokine receptor CCR7 is essential for DC migration to the lymph nodes\textsuperscript{122,123}. CCR7 guides the DCs towards and through the lymphatic vessels to the lymph nodes in response to chemotactic gradients of its ligands CCL19 and CCL21 that are expressed by lymphatic vessels and lymph node-residing cells (figure 2a)\textsuperscript{124}. Expression of CCR7 is upregulated upon DC maturation, resulting in an enhanced migratory capacity of matured DCs as compared to immature DCs\textsuperscript{73}. In addition, inflammatory signals such as prostaglandin-\textsubscript{E\textsubscript{2}} are needed to further sensitize CCR7 to its ligands\textsuperscript{125,126}. These findings are of importance for DC-based vaccination, since they show that it may be beneficial to culture DCs in the presence of prostaglandin-E\textsubscript{2} in order to get a good migratory capacity after vaccination, even though prostaglandin-E\textsubscript{2} has been de-
scribed to skew DCs under some circum-
stances towards a Th2 type of immune re-
sponse40. Interestingly, in a murine model DC migra-
tion could be increased up to ten-fold after
pretreatment of the injection site with
TNFa or unloaded DCs, due to the
upregulation of CCR7-ligand CCL21 in
lymphatic endothelial cells, resulting in a
superior magnitude and quality of the T
cell response123.

5.2 Route of DC administration in
clinical trials
For the effective induction of immunity it
is obligatory for the DCs to interact with T
cells, which takes place in the peripheral
lymphoid organs: mainly in the lymph
nodes but to some extent also in the
spleen. Recent evidence suggests that also
the bone marrow may be a site for primary
immune responses127. As different routes
lead to different sites of accumulation of
the vaccinated DCs, these issues are of
importance when considering the route of
delivery of the DCs (figure 2b). Murine
models have shown that after intravenous
injection the majority of DCs accumulate
in the spleen and to a lesser extent in the
lungs, kidneys and liver, while hardly any
DCs end up in peripheral lymph nodes128.
In vivo studies in cancer patients have
shown that after intradermal injection ap-
proximately 2-4% of the DCs migrate to
draining lymph nodes129. There is now
convincing evidence from several inde-
pendent human studies that migration af-
after subcutaneous injection is much lower
compared with intradermal injection84,129-
131.

Intranodal injection results in a much
higher amount of DCs accumulating in
lymph nodes, not only in the injected
node, but also in subsequent draining
nodes129. Migration to these subsequent
nodes via the physiological pathway
through lymph vessels can be as much as
80%. However, the magnitude of DC mi-
gration that is observed after intranodal
injection is more variable than after intrad-
ermal vaccination129. Intranodal injections are usually adminis-
tered under ultrasound guidance. Suppos-
edly, this would result in an assured ac-
curate delivery, which was the reason for
some studies to prefer this route of ad-
ministration54. However, using paramag-
netic beads-labeled DCs that can be
tracked in patients after injection by MR
imaging, we recently found that intranodal
injection is complicated by inaccurate de-
lymph nodes. These results explain the
variability in migratory outcome after in-
tranodal administration and underscore
the importance of accurate delivery133. Re-
cently, Lesimple and colleagues showed
that intralymphatic delivery into a lymph
vessel in the dorsum of the foot is also
feasible134.

Migration of administered cells however,
is only a surrogate endpoint. The true
value of the different routes of delivery
can only be determined with immune re-
sponse or even clinical response as an
endpoint. One study in melanoma patients
found a small benefit in peptide-specific T
cell responses after intranodal injection as
compared to intradermal or intravenous
injection78. Another study in advanced
melanoma patients showed no benefit for
intranodal vaccination as compared to in-
tradermal vaccination: of 22 evaluable pa-
tients a positive DTH reaction against the
vaccine was detected in 7/10 intradermal
vaccinated patients and in 3/12 intranodal
vaccinated patients135. Fong et al compared
vaccination with DCs enriched from peripheral blood mononuclear cells injected via three different routes in advanced prostate cancer patients: intradermal, intravenous and intralymphatic injection via a canule in a lymphatic channel in the dorsum of the foot. Antigen-specific T cell responses were observed regardless of the route of delivery, although IFNγ production after antigen stimulation in vitro was only demonstrated in the intradermally and intralymphaticly vaccinated patients. However, the intravenous route gave rise to a more pronounced antibody response. In an important study performed by the same group intravenous injection of DCs was shown to be essential for immune responses against visceral melanoma metastases, whereas for subcutaneous vaccination this was true for non-visceral metastases.

**Figure 2. DC migration and administration.** (a) DCs that reside in the periphery migrate to draining lymph nodes through efferent lymphatic vessels following chemotactic gradients of CCL19 and 21. These chemokines are expressed by lymphatic endothelial cells and lymph node-residing cells. Blood DCs enter the lymph nodes through high endothelial venules (HEV). After interaction with DCs, the T cells leave the lymph node through efferent lymphatic vessels and disseminate throughout the body via the circulation. (b) Several routes of administration are used in clinical DC vaccination trials: intradermal, subcutaneous, intratumoral, intralymphatic, intranodal and intravenous.
These findings indicate that the combination of different routes of administration may be beneficial to target different tumour locations in the entire body. In conclusion, to date no specific route of administration has unequivocally been shown to be superior in terms of induction of immune or clinical responses.

6.1 Tolerance

The immune system has several pathways to tune down immune responses in order to prevent autoimmunity or excessively long or vigorous inflammatory reactions. These many pathways include antigen-presentation by tolerizing DCs (as discussed above), the suppressive activity of so-called regulatory T cells (Tregs) and tolerance induction via inhibitory molecules on T cells. According to current insights there are two major distinct populations of Treg: naturally occurring CD4+/CD25+ Treg which by the time they leave the thymus already have a suppressive potential, and induced Treg that are ‘conventional’ CD4+ or CD8+ T cells but transformed into cells with an immunosuppressive function under the influence of tolerizing conditions. Tregs can affect immune responses at the level of antigen-presentation and during the effector phase of T cells at the site of inflammation or tumour growth. Treg function is controlled by cytokines and cross-talk with antigen-presenting cells, but also by direct interaction with pathogens via TLRs. The mechanisms by which Tregs exert their suppressive function are not completely elucidated, but it may happen in a cell-cell contact dependent manner via camp as second messenger as well as through cytokines such as IL-10 or TGF-β. The clinical importance of Treg in cancer has been demonstrated by Curiel et al. A high number of Treg in tumours of ovarian cancer patients correlated with poor survival. In other tumor types, these data have been confirmed. Furthermore, depletion of naturally occurring Treg by anti-CD25 or anti-GITR monoclonal antibodies in murine models resulted in enhanced therapeutic efficacy of a cancer vaccine. Since T effector cells also upregulate CD25 upon activation (the β-chain of the IL-2 receptor), a potential drawback of targeting Tregs via CD25 may be the depletion of newly activated T effector cells along with the Tregs. It may therefore be a meticulous task to find the optimal dosing and timing of anti-CD25 treatment to deplete Tregs via anti-CD25 antibodies without affecting the activated T cell population.

Another important pathway through which tolerance or anergy may be induced involves inhibitory molecules on the T cells, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) or programmed death-1 receptor (PD-1). The ligands of CTLA-4 are the DC costimulatory molecules CD80 and CD86. Binding of these costimulatory molecules to the inhibitory molecule CTLA-4 instead of the activating T cell molecule CD28, results in suppression of T cell activation and proliferation. Activated T cells express CTLA-4. Since the affinity of CTLA-4 for CD80 and CD86 is higher than that of CD28, eventually the tolerogenic pathway prevails, thereby terminating immune reactions. PD-1 is another inhibitory T cell surface molecule that upon binding to its ligands
PD-L1 and PD-L2 induces anergy or tolerance\textsuperscript{149}. Interestingly, PD-L1 and 2 are expressed by several types of cancer, which may allow the tumour to escape from immune surveillance\textsuperscript{151,152}. This phenomenon can be reversed in mice by treatment with an anti-PD-L1 antibody\textsuperscript{152,153}. Although this has been confirmed in vitro for the human situation, studies using PD-L1 blockade in cancer patients have not been reported yet\textsuperscript{154}.

### 6.2 Breaking tolerance in melanoma patients

Several new drugs have entered the clinic that target immunosuppressive mechanisms and may therefore be relevant in combination with anti-cancer vaccines. Treg can be efficiently depleted in prostate cancer patients with denileukin diftitox, a compound that consists of a diphteria toxin coupled to IL-2, resulting in enhanced antigen-specific T cell responses after DC vaccination\textsuperscript{155}. Denileukin diftitox as a single agent has shown promising immunological and clinical results in phase I and II studies in ovarian cancer [Barnet ASCO 2006]. In melanoma however, thus far the data are conflicting, with one study failing to show any efficient Treg\textsuperscript{156}, while another preliminary study showed tumour regression in two patients [Chesney ASCO 2006]. Mahnke et al. found enhanced antigenic immune responses after peptide vaccination and a significant reduction in Treg frequencies in melanoma patients after denileukin diftitox\textsuperscript{157}. The combination of DC vaccination with denileukin diftitox has not yet been reported in melanoma patients. This also holds true for the combination with anti-CD25 antibodies, despite the fact that these are commonly used in transplant patients.

The recent unexpected results with the anti-CD28 monoclonal antibody TGN 1412-trial again demonstrated the enormous potency of drugs that manipulate the co-stimulatory pathways\textsuperscript{158,159}. Blockade of CTLA-4 has been shown to induce durable objective responses in metastatic melanoma patients, at the cost of autoimmune side effects\textsuperscript{160-163}. Anti-CTLA-4 treatment in melanoma patients did not result in depletion or decreased suppressive activity of Treg (which highly express CTLA-4), suggesting that the anti-tumour efficacy of the treatment is caused by an increased T cell activation and not by inhibition or depletion of Treg\textsuperscript{164}. Since treatment with anti-CTLA-4 is antigen non-specific, the combination with a vaccine could potentially direct the T cell response in a more specific manner, thereby diminishing autoimmune side effects. There is anecdotal information that anti-CTLA4 treatment after DC vaccination may indeed enhance DC vaccine-induced T cell responses\textsuperscript{163}, however clinical trials that are specifically designed to answer this question are lacking.

### 7.1 Clinical results of DC vaccination studies in melanoma

Proof-of-principle studies were performed in the late nineties, showing the feasibility and the potential efficacy of DC vaccination in cancer patients\textsuperscript{49,50,56}. Since then numerous small studies have been performed, especially in melanoma patients (table 1 and 2). Given the enormous variations in culturing protocols and frequency, dose and route of administration, it is not possible to pool all these data in a meta-analysis. Nevertheless, some general observations can be made. Objective response rates in these selected patients do not exceed 5-10\%, with disease stabilization and mixed responses being observed more often. As discussed above, there is convincing evidence from preclinical and
clinical studies that immature DCs are not proper T cell activators, and could even induce tolerance. Strikingly however, this does not translate into a dramatic difference in clinical outcome when studies with immature DCs are compared with studies in which matured DCs have been used (table 1 and 2). Perhaps this hints at the relatively low immunogenicity of the mature DC vaccines that are currently applied: using cytokines to mature the DCs without activation by Toll-like receptor triggers.

In a recent phase III multi-institutional, randomized controlled trial the standard chemotherapy regimen for patients with metastatic melanoma, DTIC, was compared with cytokine-matured DC vaccination and no difference in survival was observed after inclusion of all patients, upon which the study was discontinued. How can this disappointing result be explained? As reported by the authors themselves, the DCs displayed a variable maturation status and the subcutaneous route of administration that was used is inferior. In addition, not all patients received the anticipated numbers of DCs. Others have pointed out the enormous complexity of performing a multi-center study with patient-specific vaccines and the difficulties in establishing a standardized vaccine product as a possible explanation of the negative outcome of this study. Together these factors may explain why no clinical benefit was found for the DC vaccine. In a subgroup-analysis a correlation was found between response to DC vaccination and HLA-type, with a favorable outcome for the HLA-2+/HLA-B44 haplotype. Although this trial could be interpreted as a negative trial for DC vaccination, in our view equality with the standard therapy for the last 30 years is perhaps not a bad starting point, given the fact that there are many parameters regarding DC vaccination that can still be optimized, as we have discussed above.

7.2 Immunological results of DC vaccination studies in melanoma

Successful development of DC vaccines in cancer patients much depends on obtaining biological information that correlates with clinical efficacy. A clear correlation between immunological response and clinical outcome has been observed in some studies, but not in all. Monitoring immune responses in DC vaccination trials is difficult and laborious, since very low frequencies of high-affinity melanoma antigen-specific T cells in peripheral blood may be sufficient for tumour rejection. These frequencies can be as low as 1 in 40,000 T cells. These low responses are often not detected by the most frequently used techniques such as Elispot-analysis and direct tetramer-staining of peripheral blood lymphocytes. In order to detect these low frequencies Coulie et al. stimulated blood lymphocyte cultures from peptide-vaccinated melanoma patients in vitro with melanoma antigens, followed by cloning of the antigen-specific cells and T-cell receptor sequence analysis of the clones. We took another approach by analyzing T cell responses in vaccinated patients from biopsies of delayed type hypersensitivity reactions (DTH) that were performed with the peptide-loaded DC vaccine. In these DTH biopsies we found evidence for functional antigen-specific T cell responses after vaccination. Moreover, the presence of these specific T cells was significantly correlated with a prolonged progression-free survival in metastatic melanoma patients. These data suggest that the success or failure of tumour-specific T cells to migrate towards the DTH site reflects the
potency of the T cells at the site of dis-
disease.

<table>
<thead>
<tr>
<th>DC culture method</th>
<th>Antigens</th>
<th>Ag-loading method</th>
<th>Route</th>
<th>No. pts</th>
<th>Clinical responses</th>
<th>Immunological response</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4/GM-CSF/FCS moDCs</td>
<td>tyrosinase, gp100, MART-1, MAGE-1, MAGE-3 or lysate</td>
<td>peptides/lysate</td>
<td>in</td>
<td>16</td>
<td>2 CR, 2 PR</td>
<td>not tested</td>
<td>49</td>
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<td>MAGE-3A,2, tyrosinase, gp100, MART-1 or lysate</td>
<td>peptides/lysate</td>
<td>in</td>
<td>33</td>
<td>3 PR, 1 MR, 6 SD</td>
<td></td>
<td>93</td>
</tr>
<tr>
<td>IL-4/GM-CSF moDCs</td>
<td>MART-1, gp100</td>
<td>MHG-I peptides</td>
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<td>1 PR</td>
<td>1</td>
<td>85</td>
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<td>IL-4/GM-CSF moDCs</td>
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<td>autologous peptides</td>
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<td>96</td>
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<tr>
<td>IL-4/GM-CSF moDCs</td>
<td>MART-1, gp100, tyrosinase</td>
<td>MHG-I peptides</td>
<td>iv</td>
<td>16</td>
<td>1 CR, 2 PR, 2 MR</td>
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<tr>
<td>IL-4/GM-CSF moDCs</td>
<td>MART-1</td>
<td>MHG-I peptides</td>
<td>id</td>
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<td>1 CR, 1 SD, 1 MR</td>
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<td>IL-4/GM-CSF/FCS moDCs</td>
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<td>in/sc</td>
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<td>1 PR, 1 CR</td>
<td>3</td>
<td>94</td>
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<tr>
<td>IL-4/GM-CSF moDCs</td>
<td>gp100/tyrosinase</td>
<td>MHG-I peptides</td>
<td>iv/id</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>52</td>
</tr>
</tbody>
</table>

Table 1. Results of published DC-vaccination trials with non-matured DCs in metastatic mela-
noma

Results of published DC-vaccination trials in which non-matured DCs were used. Only melanoma pa-
tients with distant metastases and evaluable disease are included. Abbreviations: IL, interleukin; GM-
CSF, granulocyte macrophage-colony stimulating factor; FCS, fetal calf serum; moDCs, monocyte-
derived DCs; sc, subcutaneous; in, intranodal; id, intradermal; iv, intravenous; CR, complete response;
PR, partial response; MR, mixed response; SD, stable disease (≥ 4 months).
1Immunological response is defined as any evidence of melanoma peptide or tumor-specific T cell re-
sponse after vaccination by one or more test methods; the number of patients with an immune reponse
are given. 2After DC-vaccination these patients received treatment with an anti-CTLA4 blocking anti-
body.

In a provocative study Lonchay et al. showed that although frequencies of vaccine-specific T cell may be higher after
vaccinations, this does not necessarily mean that these cells are the true effector
cells that actually cause the tumour regres-
sion168. They found that other tumour-
specific T cells that were already present
prior to vaccination were much more ex-
panded after vaccination than the vaccine-
specific T cells. The current hypothesis is
that vaccine-specific T cells ignite a certain ‘spark’ in the tumour, causing the non-
active tumour-residing specific T cells to
become active and proliferative. Thus, the
expanded specific T cell frequencies that
are observed upon DC vaccination and
 correlate with an improved clinical out-
come may cause tumour regression either
in a direct or in an indirect manner.
7.3 Study design and clinical endpoints in DC vaccination

The study design and clinical endpoints that should be used in therapeutic cancer vaccine studies, were recently defined in a consensus process by the Cancer Vaccine Clinical Trial Working Group, representing academia and pharmaceutical and biotechnology industries with participation from the US Food and Drug Administration. There are several differences between cytostatic drugs and therapeutic vaccines that should be taken into account when designing and evaluating clinical cancer vaccine trials.

Firstly, conventional RECIST criteria may not always be the most appropriate endpoint to decide which DC vaccine will be the most optimal vaccine to be tested in large clinical trials. Because vaccine-induced immune responses will take some time before they become clinically apparent, initial minor progression could be acceptable. In fact, adequate immune induction could theoretically first induce enlargement of tumour lesions through T cell infiltration and local inflammation. For these reasons, it may be proper to deviate from standard RECIST criteria, provided that the new criteria are pre-defined and clearly described, as proposed before.

Whether functional imaging has a role in the monitoring of clinical responses in cancer vaccine trials remains to be determined. It has been shown that upon DC vaccination regional non-tumourous lymph nodes become FDG-positive on positron emission tomography-scanning, reflecting immune activation rather than tumour progression.

A second major difference between conventional cytostatic therapy and DC vaccination is that the highest dose is not necessarily the most effective one. And because DC vaccines have an almost negligible toxicity, conventional dose-escalating phase I trials with toxicity as the primary endpoint would not result in the selection of the optimal dose to be used in further clinical testing. This problem has been circumvented by performing dose-escalation studies with immune response as the primary endpoint rather than toxicity (see for example ref. 15).

Another difference is that in patients with advanced disease, the patient group in which phase I trials are usually performed, immunotherapy will probably have little chance of being successful because of immunosuppressive mechanisms at the tumour site. For these reasons, the conventional phase I-II-III trial paradigm that is applied in the field of cytostatic therapy, may not be the most optimal trial design for DC vaccination.

The Cancer Vaccine Clinical Trial Working Group consensus recently proposed a two-step development in clinical trial design in cancer vaccination: proof-of-principle trials and efficacy trials. The following criteria were proposed for proof-of-principle trials. They should include a minimum of 20 patients in a homogenous, well-defined population. The disease should not be rapidly progressive in order to allow the vaccines adequate time to induce immunological activity. Study objectives should include determination of dose and schedule and demonstration of immunological activity as proof-of-principle. Immunological activity is demonstrated if determined in 2 separate, established and reproducible assays at 2 consecutive follow-up time points after the baseline assessment.

Efficacy trials then formally establish clinical benefit, either directly or through a surrogate endpoint. In contrast with the field
of conventional cytostatic therapies, it is promoted that these trials are randomized. We believe that these guidelines are of great practical use and could help the field forward.

Table 2. Results of published DC-vaccination trials in which DCs were used that had received some form of maturation signals. Only melanoma patients with distant metastases and evaluable disease are included. Abbreviations: IL, interleukin; GM-CSF, granulocyte macrophage-colony stimulating factor; MCM, monocyte-conditioned medium; TNFα, tumor necrosis factor alpha; PGE2, prostaglandin-E2; moDCs, monocye-derived DCs; Flt3L, Flt3-Ligand; SCF, stem cell factor; IFN, interferon; sc, subcutaneous; in, intranodal; id, intradermal; iv, intravenous; il, intralymphatic; CR, complete response; PR, partial response; MR, mixed response; SD, stable disease (> 4 months).

<table>
<thead>
<tr>
<th>DC culture method</th>
<th>Antigens</th>
<th>Ag-loading method</th>
<th>Route</th>
<th>No. pts</th>
<th>Clinical responses</th>
<th>Immune responses</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4/GM-CSF/MCM/</td>
<td>MAGE-3</td>
<td>MHC I peptides</td>
<td>sc, id, iv</td>
<td>13</td>
<td>6 MR</td>
<td>9</td>
<td>56</td>
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<tr>
<td>TNFα/ moDCs</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>IL-4/GM-CSF/IL-1β/IL-6/TNFα/PGE2 moDCs</td>
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<td>MHC I+II peptides</td>
<td>sc</td>
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<td>12</td>
<td>57</td>
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<td>IL-4/GM-CSF/MCM/</td>
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<td>MHCI peptides</td>
<td>sc, iv</td>
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<td>1 SD</td>
<td>8</td>
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<td>TNFα moDCs</td>
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<td>3 PR</td>
<td>13</td>
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<td>MHCI peptides</td>
<td>id, iv</td>
<td>10</td>
<td>1 PR, 1 MR, 3 SD</td>
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<td>TNFα +PGE2 moDCs</td>
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<td>MHCI peptides</td>
<td>sc</td>
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<td>4 CR, 4 PR, 3 SD</td>
<td>17</td>
<td>15, 82, 83</td>
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<td>CD34+ DCs</td>
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<td>IL-4/GM-CSF/TNFα/</td>
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<td>MHCI peptides</td>
<td>iv</td>
<td>14</td>
<td>1 PR, 6 SD</td>
<td>1</td>
<td>18</td>
</tr>
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<td>IL-3/IL-6/SCF CD34+ DCs</td>
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<tr>
<td>Flt3L/GM-CSF/TNFα</td>
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<td>MHCI peptides</td>
<td>sc</td>
<td>20</td>
<td>1 SD</td>
<td>7</td>
<td>17</td>
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<tr>
<td>/IFNα CD40L moDCs</td>
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<td></td>
</tr>
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<td>IL-4/GM-CSF/TNFα/</td>
<td>Allogeneic lyase</td>
<td>Allogeneic lyase</td>
<td>sc</td>
<td>20</td>
<td>1 CR, 1 PR</td>
<td>2</td>
<td>61</td>
</tr>
<tr>
<td>CD40L moDCs + sc IL-2</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>IL-4/GM-CSF/IL-1β/IL-6/TNFα/PGE2 moDCs</td>
<td>Autologous tumor RNA</td>
<td>RNA-electroporation</td>
<td>id or in</td>
<td>21</td>
<td>1 MR</td>
<td>9</td>
<td>110, 111</td>
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<tr>
<td>IFN/IL-3/polyLC moDCs</td>
<td>NA17, MAGE-3</td>
<td>MHCI +II peptides</td>
<td>sc, id</td>
<td>4</td>
<td>1 SD</td>
<td>1</td>
<td>84</td>
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<td>MHC I+II peptides</td>
<td>sc</td>
<td>53</td>
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<td>87</td>
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<td>Allogeneic lyase</td>
<td>in, sc, id</td>
<td>10</td>
<td>1 SD</td>
<td>3</td>
<td>98</td>
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<td>GM-CSF/IL-13/Ribonuclease/IFNγ</td>
<td>MART-1, ± NA17</td>
<td>MHCI+II peptides</td>
<td>il then in</td>
<td>14</td>
<td>2 SD</td>
<td>5</td>
<td>133</td>
</tr>
</tbody>
</table>
1 In a follow-up report\(^2\), all complete responders were still free of disease with a median of 5 years.

2 This is a multi-institutional, randomized phase III trial. In the other treatment-arm 55 patients were treated with DTIC, in which 3 PR and 10 SD were seen. No differences in overall or progression-free survival were observed.

And although in some instances it may be sufficient to include smaller numbers of patients (for example in trials investigating DC migration in vivo), we believe that adherence to these guidelines should be encouraged.

7.4 DC Companies

The preparation of a standard high-quality DC vaccine that meets Good Manufacturing Practice/Good Laboratory Practice (GMP/GLP), and clinical grade criteria is a laborious and costly process. Dedicated GMP/GLP cleanrooms are needed. Culturing a DC vaccine for one patient takes at least two persons two to three full working days. Even more costly are the culture media and cytokines that need to be toxin-free, clinical grade materials. Together with the fact that the vaccines are patient-specific, ‘tailor-made’, these issues gave rise to the long-held belief that companies would not be interested in DC vaccination. However, at the moment several companies are trying to get approval from official regulatory authorities. One company-made DC vaccine is Sipuleucel-T, which is made of antigen-presenting cells that are collected by two density centrifugation steps, pulsed with recombinant prostatic acid phosphatase fused to GM-CSF\(^3\). A placebo-controlled phase III trial in hormone-refractory metastatic cancer patients was not able to show improvement in time to progression, the primary endpoint, although overall survival was prolonged from 21.4 to 25.9 months\(^4\). Initially an FDA Advisory Committee recommended to approve Sipuleucel-T, but the FDA decided to await further proof of efficacy. This should come from an ongoing phase III trial with overall survival as primary endpoint of which the first interim analysis is planned in 2008\(^5\). Other firms are seeking approval by the European and US regulatory authorities for vaccines targeted at lymphoma, sarcoma, glioblastoma and acute myelogenous leukaemia\(^6\).

7.5 Combination therapies

There is a need to test combinations with more cytotoxic therapies, given the low frequency of clinical responses in patients with advanced disease upon DC vaccination alone. Although initially chemotherapy was believed to be detrimental to T cell-directed immunotherapy because most chemotherapy regimens have a myelosuppressive effect, now more evidence is accumulating that some forms of chemotherapy may not harm T cell responses and may in fact have a synergistic effect together with immunotherapeutic approaches (reviewed in ref. 176). It is tempting to speculate on the possibility of tumour debulking by chemotherapy, combined with immune surveillance and immune memory induction by vaccination therapy to prevent relapses. The same applies to radiotherapy and targeted therapy. Another interesting approach involves in situ tumor destruction by cryo- or radiofrequency ablation in combination with immune activation, including injection of DCs\(^7\). Clinical data are lacking, but trials combining chemotherapy and DC vaccination are in progress. As discussed above, also combination therapy with anti-CTLA4 and DC vaccination may have a synergistic effect. It is currently under study whether DC vaccination
can enhance the graft-versus-tumour effect of stem cell transplantation and donor lymphocyte infusions in haematological malignancies. Adoptive T cell transfer following non-myeloablative but lymphodepleting chemotherapy showed impressive clinical results in advanced melanoma patients. Adoptive T cell transfer generates a high, but short peak of antigen-specific T cells, whereas DC vaccination induces T cell responses more gradually that endure longer, providing a rationale to combine the two treatment modalities. In preclinical models DC vaccination indeed boosted and sustained anti-tumour T cell responses after adoptive T cell transfer. Trials in the near future will have to answer the question whether DC vaccination can add to the efficacy of these other anti-cancer treatment modalities.

8 Conclusion and future prospects

DC vaccination has shown to be feasible and safe. Immunological responses are frequently observed. Clinical responses have been reported, but the incidence is low. Exciting new insights arise from preclinical studies, some of which are currently being applied in clinical studies. For example, depletion of suppressor T cells combined with DC vaccination may enhance the immunogenicity of the vaccine, as has been shown in prostate cancer patients. Also blockade of the inhibitory T cell molecule CTLA-4 by monoclonal antibodies could enhance the immunogenicity of DC vaccines. Combination treatment with chemotherapy, radiotherapy or other tumour ablative treatments needs to be further investigated. Trials with TLR-ligand activated DCs are eagerly awaited. Although these novel treatment strategies are now entering clinical studies, the pace of in vitro and animal research is inevitably faster than that of clinical research. For this reason a lot of crucial questions regarding the optimal DC vaccine for clinical use remain unanswered to date. These questions concern the optimal methods for culture, maturation and antigen-loading, route of administration, subsets of DCs and effects of suppressor T cell-depletion or blockade of other inhibitory pathways. Significant progress is only to be expected from well-designed, properly conducted and comparative studies with biological endpoints.

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Summary and future prospects
SUMMARY

In chapter 1 we describe the basic immunobiology of dendritic cells (DCs) in the context of anti-tumor immunity. The principle of DC vaccination is introduced and we address several relevant questions on this topic. We emphasize the importance of careful study design, the use of standardized clinical and immunological criteria for response evaluation, and adequate quality control for DC vaccine preparation and administration.

In chapter 2 the results are reported of a clinical study in metastatic melanoma patients who were vaccinated with either immature or mature DCs, loaded with melanoma-associated antigenic peptides and the control protein keyhole limpet hemocyanin (KLH). Patients that were vaccinated with mature DCs showed a robust T and B cell response against KLH upon vaccination, indicating that a de novo immune response was induced. In patients who were vaccinated with immature DCs, these responses were absent or very weak. In addition, only in patients who were vaccinated with mature DC, delayed type hypersensitivity (DTH) reactions against the vaccine were observed after treatment. Importantly, tumor antigen-specific T cell responses were only observed in patients that were vaccinated with mature DCs, and the presence of these T cells correlated with a clinical benefit.

DCs can be administered in several manners, such as intravenously, intradermally or directly into lymph nodes under ultrasound guidance. For the latter method to be successful, the accurate delivery into the lymph nodes is of utmost importance. In chapter 3 we describe a novel method of tracking DCs upon vaccination in lymph nodes in patients using magnetic resonance imaging (MRI).

Dendritic cells were labeled with $^{111}$In-oxine and superparamagnetic iron oxide particles separately and coinjected in a lymph node to be resected. This provided a unique opportunity not only to obtain MRI scans at 3 Tesla before surgery, but also to generate high-resolution magnetic resonance images at 7 Tesla of individual resected lymph nodes and to correlate the results with scintigraphy and (immuno)histopathology. This is the first report of effective tracking of ex vivo-labeled therapeutic cells in humans by MRI. We demonstrate that MRI is at least as sensitive as scintigraphic imaging for detecting DC migration in vivo. However, MRI in contrast to scintigraphy is able to verify the anatomic location of injected DCs. Subsequent migration upon injection was only observed when DCs were correctly injected into the lymph node, demonstrating the importance of accurate administration of this cellular therapy. Inadequate delivery may explain why only a limited proportion of patients is responding in ongoing clinical trials of intranodally injected DC vaccines.

Most DC vaccination protocols use peptide fragments of tumor-associated antigens. In vitro and animal studies have shown that the immunogenicity of peptides can be improved by modification of the amino acid sequence, resulting in enhanced MHC class I binding affinity and stability of peptide-MHC complexes. When used to load on DCs, this would result in enhanced immune responses upon vaccination. In chapter 4 we tested this hypothesis based on preclinical evidence in a clinical study. Metastatic melanoma patients were vaccinated with mature DC pulsed with either wildtype or modified
gp100 peptides. We observed functional T cell responses against the vaccine antigens in 2 of 15 patients vaccinated with wild-type gp100-loaded DCs, versus 1 of 12 patients vaccinated with modified peptide-loaded DCs. The 3 patients with functional T cell responses also had the best clinical response, with long-term (>8 years) complete responses in two patients. These data show that the use of modified as compared to wild-type gp100 peptides for DC loading does not enhance tumor-specific immune responses.

Most studies with DC-based vaccines have been performed in melanoma patients. In chapter 5 we report the results of a clinical study in colorectal cancer patients. Patients with resectable liver metastases were vaccinated with carcinoembryonic antigen (CEA) peptide-loaded DCs. CEA-specific T cell reactivity was monitored in peripheral blood, biopsies of vaccination sites and post-treatment DTH skin tests and, when available, also in resected abdominal lymph nodes and tumor tissue. We observed high numbers of functional CEA-specific T cells in post-treatment DTH biopsies in 7 out of 10 patients. These responses were not found in biopsies of first vaccination sites, indicating a de novo T cell induction or at least a strong potentiation by the vaccine. In addition, CEA-specific T cells were detected in a resected lymph node in one patient. Thus, vaccination with CEA-peptide loaded mature DCs induced potent CEA-specific T cell responses in advanced colorectal cancer patients.

An alternative method to load antigen on DCs consists of transfection of mRNA encoding the antigen. Theoretically this has several advantages including presentation of more antigenic epitopes, longer antigen presentation and availability for all patients independent of HLA-type. In chapter 6 we determined the optimal conditions for mRNA-electroporation of DCs for clinical application in relation to different maturation cocktails. This technique resulted in a rapid and long-enduring expression of tumor antigens and accurate T cell activation in vitro. However, activation of DCs with polyinosinic-polycytidylic acid [poly(I:C)], which is often used in maturation protocols, resulted in a considerably decreased antigen expression. A possible explanation for this observation lies in the fact that poly(I:C) is a synthetic double-stranded RNA and culturing of DCs with this virus-associated molecular pattern results in the induction of IFN-induced genes and innate anti-viral effector molecules and consequently in decreased translation of the mRNA encoding the antigen. Thus, electroporation of mature DCs with tumor antigen-encoding mRNA is attractive for use in DC vaccination protocols in cancer patients, but protein expression should be tested for each maturation cocktail.

In chapter 7 these data were put to the test in a clinical study comparing the immunogenicity of DCs pulsed with CEA-peptide (as described in chapter 5) with DCs electroporated with CEA-mRNA in colorectal cancer patients. CEA peptide-specific T cells were detected in 8 out of 11 patients in the peptide group, but in none of the 5 patients in the RNA group. These data demonstrate that CEA-mRNA transfection as antigen loading strategy offers no benefit as compared to peptide loading, at least in this model.

Next we explored the feasibility of the combination of DC vaccination with cytotoxic chemotherapy. The theoretical ad-
vantage of this combination is that it would allow tumor antigen release combined with the recently reported induction of immunogenic tumor cell death\textsuperscript{1-3}. However, the direct effect of cytotoxic anti-cancer drugs on DC function and their effect on immune inhibitory pathways that are present in the tumor microenvironment are largely unknown. We investigated the effect of cytotoxic drugs on DC function and on tumor cell immunogenicity, and the results are reported in chapter 8. In addition to their well-known direct cytotoxic effects, we demonstrate that platinum-based anti-cancer drugs modulate immuno-suppressive pathways by acting both at the level of the tumor as well as on cells of the immune system. We also detail a novel mechanism underlying the action of these chemotherapeutics: that platinum compounds dephosphorylate Signal Transducer and Activator of Transcription 6 (STAT6) resulting in downregulation of the T cell inhibitory molecule Programmed Death Receptor-Ligand 2 (PD-L2), which is ultimately responsible for the observed effects. In line with these in vitro findings, we observed in a retrospective study that patients with STAT6-expressing head and neck cancer displayed significantly enhanced recurrence-free survival upon treatment with cisplatin-based chemoradiation compared to patients with STAT6-negative tumors, demonstrating the clinical relevance of platinum-induced STAT6 modulation. These findings demonstrate that platinum compounds not only directly kill tumor cells but also enhance T cell stimulation by DCs. At the same time, tumor cells are also sensitized to lysis by cytotoxic T cells through inactivation of STAT6/PD-L2 pathway. This novel action of platinum compounds may extend their therapeutic application and provides a rationale for their use in combination with other immuno-stimulatory compounds to increase the clinical efficacy of cancer treatment.

To test whether antigen-specific T cell induction can still occur during treatment with oxaliplatin-based chemotherapy, we performed a small pilot study in 8 stage III colon cancer patients, of which the results are described in chapter 9. These patients received the same CEA-specific DC vaccine as described in chapter 5, in addition to standard adjuvant treatment with oxaliplatin and capecitabine. In 4 out of 7 patients functional CEA-specific T cell responses were observed. In addition, we demonstrated an enhanced non-specific T cell reactivity upon oxaliplatin administration. KLH-specific T cell responses remained unaffected by chemotherapy, whereas B cell responses were diminished. These results demonstrate that oxaliplatin-based chemotherapy does not interfere with tumor antigen-specific vaccination and even may enhance T cell-mediated immune responses, and they strongly support further testing of the combined use of immunotherapeutic approaches with oxaliplatin-based chemotherapy.

In chapter 10 we review the current status of the field of DC vaccination, focusing on melanoma.

**FUTURE PROSPECTS**

More than a decade has passed since the first clinical DC vaccination studies were reported\textsuperscript{4,5}. Since then, numerous clinical and preclinical studies have been performed. Although much progress has been made as described in the previous chapter, unfortunately the majority of patients have
no clinical benefit from DC vaccination. So what are we doing wrong? There is more than one answer to this question.

DC activation
Most studies used cytokines to mature the DCs, but we know now that full maturation using pathogen-associated molecular patterns to activate the DCs is crucial for the induction of robust, functional antitumor T cells. Many novel experimental compounds are currently being developed that can be used as potent DC-activating stimuli.

Immune escape
Tumors use numerous mechanisms to escape from the immune system. Even if very potent T cells are induced by DC vaccines, clinical efficacy will be limited if these inhibitory pathways are not overcome. Important therapeutic possibilities include CTLA-4 blocking antibodies or antibodies blocking the PD-1/PD-L pathway. In a recent study treatment with an anti-CTLA4 antibody resulted in a clinical benefit in metastatic melanoma patients, demonstrating that the concept of blocking inhibitory pathways is indeed a promising strategy. Studies investigating the combination of vaccination therapy with these antibodies are currently ongoing and the results are eagerly awaited. Recently it has been shown that melanoma stem cells may not express tumor antigens, which could result in immune escape despite robust vaccine-induced antitumor immune responses. A surface-marker for these stem cells has been discovered, possibly allowing new therapeutic approaches.

Tumor stage
Most studies have tested the immunological and clinical efficacy of DC vaccination in end-stage cancer patients. In these patients, the immune system is often suppressed, either systemically or locally in the tumor microenvironment. In these patients with gross tumor volume it is questionable whether DC vaccination, which needs some time to result in proper T cell activation, is able to induce clinical responses. Rather, it will often be ‘too little, too late’. Patients with limited tumor burden, such as in the adjuvant setting, may provide a better platform to test the efficacy of DC vaccines.

Combination therapies
The above-mentioned mechanisms of immune escape and the rationale for prior tumor debulking provide strong arguments to combine DC-based therapy with other treatment modalities. A promising example is platinum-based chemotherapy, since it provides tumor debulking (antigen release), immunogenic tumor cell death, DC activation and disarmament of immune-suppressive mechanisms at the tumor site (as described in chapter 8).

So what have we learned? DC vaccination is able to induce tumor-specific immune responses. Importantly, the presence of these vaccine-induced immune responses correlates with the induction of clinical responses in cancer patients. Several aspects of DC vaccination, such as activation, culturing, administration and antigen-loading have been optimized. And finally, DC vaccination can be combined with chemotherapy without compromising its immunogenicity.

Where should we go from here? The use of fully TLR-activated DCs appears most promising, as well as the combination of DCs with treatment modalities that block immune suppression and/or chemotherapeutics that allow T cell induction, but at
the same time break immunosuppressive networks at the tumor microenvironment. Furthermore, the combination of (platinum-based) chemotherapy with immune activating signals could obviate the need of the administration of ex vivo cultured DCs: in this concept the chemotherapy-induced cytotoxicity provides antigen release, immunogenic cell death, reduction of immune suppression at the tumor site and down-regulation of inhibitory molecules on the DCs; the immune stimuli provide full T cell and DC activation. There are several good candidate compounds that have recently entered the clinic (some of which still in a experimental phase), such as anti-CTLA4, CD40-ligand, PD-1 blocking antibodies and Toll-like receptor triggers8,14,15. Therefore, the road to clinical studies and hence clinical application may be short. Hopefully, these treatments will result in better treatment options for cancer patients in the near future.

REFERENCES

Nederlandse samenvatting
Dankwoord
Curriculum Vitae
List of publications
Vaak wordt kanker voorgesteld als een vijand die we de oorlog moeten verklaren. Als je die vergelijking door zou trekken zou je het afweersysteem kunnen zien als het verdedigende leger. De soldaten van dat leger zijn de zgn. T cellen, de cellen die in staat zijn kankercellen te doden. Echter, T cellen zijn van nature niet in staat de kankercellen op te ruimen. Daarvoor moeten ze eerst gealarmeerd worden. Daar komen dendritische cellen (DCs) bij kijken. Dat zijn cellen die door het hele lichaam zwerven op zoek naar indringers. Hoewel ze zelf niet in staat zijn tumorcellen te doden, kunnen ze wel stukjes van dode kankercellen opnemen. Dode kankercellen zijn altijd ruim aanwezig in tumoren. Zie het als een slagveld waar de zwarte helmen van de vijand op de grond liggen en de DCs pikken die op (figuur 1).

DCs zijn dus aanvankelijk de verkenners en nadat ze de vijand hebben gezien, promoveren ze snel tot generaal. Nadat ze geactiveerd zijn, kunnen T cellen zichzelf vermenigvuldigen tot wel 10.000 keer, ze verlaten de lymfeklier en verspreiden zich door het hele lijf. Als ze nu een kankercel tegenkomen die de antigenen op zijn oppervlakte heeft waartegen de T cel is geactiveerd (‘draagt een zwarte helm’), zal de T cel dit herkennen en de kankercel doodmaken (figuur 3).
kweken in de aanwezigheid van groefactoren. Vervolgens kunnen ze beladen worden met de tumor-antigenen en teruggegeven worden aan de patiënt door injectie. De DCs moeten dan de lymfeklieren in de patiënt bereiken om daar de T cellen te activeren tegen de tumor-antigenen. De geactiveerde T cellen moeten dan de klieren verlaten, zich verspreiden door het lijf, de tumor infiltreren en de kankercellen herkennen en uiteindelijk doden. Het doel van deze techniek is dus heel specifiek het afweersysteem te alarmeren en te activeren tegen de kankercellen, zodat deze opgeruimd worden.

In hoofdstuk 1 beschrijven wij de achtergronden van DC-vaccinatie, de resultaten van klinische studies in kankerpatiënten en de moeilijkheden die zich voordoen bij het onderzoek van deze behandelmethode.

Het activatie signaal dat DCs geven als ze antigenen presenteren aan de T cellen, wordt gegeven door co-stimulatie-moleculen op het oppervlakte van de cel (in figuur 2 het waarschuwingsbord). Voor goede T celactivatie is dat signaal cruciaal. Als DCs door het lichaam zwerven, zonder de aanwezigheid van een ‘gevaar-signaal’, zijn ze in een immature staat. In het immature stadium bezitten ze de juiste eigenschappen om antigenen op te nemen en te verwerken. In de aanwezigheid van een gevaar-signal worden de DCs mature (denk aan de vergelijking van net: de immature DCs zijn uitstekende verkenners, bij maturatie worden ze als het ware gepromoveerd tot generaal en delen ze de lakens uit, bepalen wie wanneer en hoe wordt aangevallen). De gevaars-signalen die leiden tot maturatie kunnen bepaalde herkenningsmoleculen zijn van bacteriën of virussen, maar ook immunologische signaalmoleculen die vrijkomen in een tumormilieu. Door deze maturatie-signalen veranderen DCs in cellen die met name goed zijn in snelle migratie naar lymfeklieren en het presenteren van antigenen. Daarnaast zetten ze hun costimulatie-moleculen in hoge mate op hun oppervlak. Dit leidt ertoe dat DCs in mature staat alle eigenschappen bezitten om T cellen goed te kunnen activeren. Het was niet bekend welk type DC het best gebruikt kon worden om kankerpatiënten te behandelen met DC vaccinatie. In de meeste studies die werden verricht werden immature DCs gebruikt.

In hoofdstuk 2 beschrijven we een klinische studie waarbij wij patiënten met uitgezaaid melanoom (een zeer kwaadaardige vorm van huidkanker) behandelde met immature of mature DCs die waren beladen met tumor-antigenen. We vonden dat alleen bij patiënten die waren behandeld met mature DCs er een sterke afweerreactie tot stand was gekomen tegen de antigenen in het vaccin. Bovendien vonden we alleen bij patiënten die waren behandeld met mature DCs een stabilisatie van het ziekteproces, danwel een afname van de uitzaaing. Uit deze studie blijkt dat voor vaccinatie van kankerpatiënten mature DCs gebruikt dienen te worden, geen immature.

DCs kunnen op verschillende manieren toegediend worden: onder de huid, via de bloedbaan, of via directe injectie in lymfeklieren. Bij de laatste methode vonden we eerder dat na injectie de verdere verdeling naar overige klieren buitengewoon wisselvallig was. Om meer inzicht te krijgen in de accuratesse van injectie, testten wij de mogelijkheid van het labelen van DCs met ijzerbolletjes. Het betreft minuscule ijzerbolletjes waar een dun laagje suiker omheen zit. Deze bolletjes zijn geregistreerd als contrastvloeistof bij MRI-scans.
hoofdstuk 3 beschrijven we de resultaten van deze studie. We vonden dat DCs deze bolletjes uitstekend konden internaliseren zonder aantasting van hun immunologische eigenschappen. Na injectie in melanoompatiënten konden we de lokatie van de DCs goed zien met behulp van MRI scans. Zo vonden we ook dat in sommige patiënten de DCs naast de lymfeklieren werden geïnjecteerd ondanks dat injectie onder geleide van een echo-apparaat werd verricht. En het was juist bij deze patiënten dat we geen verdere verspreiding naar volgende klieren konden zien.

Deze bevindingen laten het belang van adequate toediening van DCs zien. Daarnaast blijkt uit deze studie voor het eerst dat cellen uitstekend te volgen zijn na injectie in patiënten met behulp van ijzerpartikels en MRI. Deze resultaten kunnen ook van belang zijn voor andere terreinen van onderzoek waarbij cellen op een accurate wijze moeten worden toegediend op exact de juiste anatomische locatie, zoals bijvoorbeeld bij stamcelonderzoek.

In hoofdstuk 4 beschrijven we een studie waarbij we patiënten behandelden met DCs met daarop twee verschillende vormen van hetzelfde antigeen (de ‘zwarte helm’). De ene helft van de patiënten kreeg DCs beladen met normale melanoom-eiwitfragmenten (peptiden), de andere helft kreeg DCs beladen met gedomificeerde peptiden. De modificatie bestond eruit dat een aminozuur van het peptide was veranderd, waardoor het beter bindt aan zgn. HLA-moleculen op de DC (dat zijn moleculen die het antigeen op het oppervlak van de DC presenteren; zie ze als de hand die de zwarte helm vasthoudt). Dat lijkt er toe dat er meer peptiden op de DC blijven plakken en daardoor beter aangeboden worden aan de T cellen. Daarnaast is uit kweekbuisonderzoek gebleken dat de T-celreactiviteit daarbij hoger is dan bij ‘gewone’ peptiden. Of dat in patiënten na toediening ook zo zou zijn was niet bekend. Om die reden hebben wij dit onderzocht in patiënten met gemetastaseerd melanoom. We vonden dat zowel in de groep patiënten die behandeld waren met DCs beladen met gewone peptiden, als in de patiënten bij wie de peptide waren gedomificeerd afweeractivatie optrad tegen de melanoomatigenen. Ook waren er in beide groepen klinisch relevante afnames te zien van de melanoomactiviteit bij enkele patiënten, bij 1 patiënt was er zelfs een volledige verdwijning van melanoomuitzaaiingen. Echter, er was geen duidelijke toename in de afweeractivatie te zien bij de patiënten die waren behandeld met de gedomificeerde peptiden. Uit deze studie bleek derhalve geen duidelijke meerwaarde van het gebruik van gedomificeerde peptiden voor DC vaccinatie.

Veruit de meeste DC vaccinatie studies zijn verricht in patiënten met gemetastaseerd melanoom. Of deze methode ook potentieel zou kunnen hebben in andere tumortypes was onvoldoende bekend. Om die reden hebben we de mogelijkheid onderzocht in darmkankerpatiënten (hoofdstuk 5). Dit deden wij bij patiënten met leveruitzaaiingen van darmkanker die zouden kunnen worden verwijderd door de chirurg. Vooraf vaccineerden wij de patiënten. Ten eerste was dit omdat op het moment van de studie deze groep patiënten niet standaard chemotherapie kreeg en wij op dat moment meenden dat chemotherapie mogelijk slechter zou zijn voor het afweersysteem (hierover later meer). En ten tweede konden we zo goed de afweerreactie beijken na vaccinatie in tumorweefsel zelf en in klieren in de buik, omdat die toch zouden worden verwijderd. Als antigeen om de DCs te beladen gebruikten we CEA.
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is een eiwit dat eigenlijk altijd als herkenningsmoelcuul op darmtumoren voorkomt. We konden in de meeste van de 10 patiënten die we vacineerden een zeer duidelijke T cel afweerreactie vinden tegen CEA na vaccinatie.

Het is ontzettend lastig om in bloed antigeen-specifieke T cellen aan te tonen, dat wil zeggen de T cellen die alleen een bepaald eiwit van interesse herkennen, in dit geval CEA. Uit andere studies is bekend dat als 1 op de miljoen T cellen specifiek tumorantigeen kan herkennen na vaccinatie, dit al genoeg kan zijn om een klinisch relevante afname te bereiken. Je kunt je voorstellen dat het heel lastig is om die ene op de miljoen T cellen te vinden. Om deze reden gebruikten we een speciale methode om specifieke T cellen op te sporen in de patiënt na vaccinatie: we injecteerden een klein beetje DC vaccin in de huid van patiënten reeds nadat ze de vaccinatiebehandeling hadden afgerond. Lokaal in de huid ontstaat dan wat ontsteking, waardoor T cellen aangetrokken worden. Als daar een CEA-specifieke T cel bijzit wordt die geactiveerd (omdat de DCs zijn beladen met CEA) en gaat delen. Lokaal krijg je dan dus veel CEA specifieke T cellen. Na 48 uur nemen we van dat stuk huid een kleine biopsie, wat we daarna op kweek zetten. Wij testten in hoofdstuk 6 de techniek van electroporatie. Hierbij breng je gedurende een aantal milliseconden DCs een kleine schok toe, waardoor de celwand zich heel kort opent. Bijgevoegd RNA kan dan naar binnen gaan. We tonen aan dat met deze techniek het mogelijk is om goede eiwitproductie te krijgen van de relevante tumorantigenen (zowel melanoom als darmkanker-antigenen) en we laten zien dat de
DCs T cellen specifiek tegen deze antige­
nen kunnen activeren. Wel vonden we dat
bij verschillende maturatie-methoden er
verschillende uitkomsten waren.

In hoofdstuk 7 kijken we vervolgens in
patiënten of vaccinatie met DCs die ge­
electroporeerdo zijn met RNA onderdaad
ook beter zijn dan DCs die zijn beladen
met peptiden. We deden dit in darmkan­
kernpatiënten met hetzelfde stadium als in
hoofdstuk 4, dus met leveruitzaaiingen die
verwijdert konden worden. We vaccineer­
den deze patiënten met DCs die waren ge­
electroporeerd met CEA-RNA. De immu­
nologische resultaten vergeleken we met
de resultaten van de eerdere studie, waarbij
we patiënten vaccineerden met CEA-pep­
tide beladen DCs. Door een moeizame pa­
tiënteninclusie hebben we helaas slechts 5
patiënten op deze manier kunnen behan­
delen. In die 5 patiënten vonden wij geen
teken van CEA-specifieke T cel reactvi­
teit na vaccinatie. Hoewel op basis van dit
kleine aantal patiënten beperkte conclusies
getrokken kunnen worden, lijkt CEA-elec­
troporatie niet tot betere resultaten te lei­
den dan CEA-peptide als methode van an­
tigeenbelading van DCs voor vaccinatie.

In hoofdstuk 8 beschrijven we onderzoek
naar het effect van chemotherapie op DC
functie. Als DC vaccinatie gecombineerd
zou kunnen worden met chemotherapie
zou dit wellicht de klinische effectiviteit
kunnen verbeteren. We kweekten DCs in
de aanwezigheid van een breed scala aan
chemotherapeutica en gebruikten vervol­
gens deze DCs om in kweekbuisjes T cel­
ten te activeren. We vonden dat DCs die
waren gekweekt in de aanwezigheid van
platinum-bevattende chemotherapie een
sterk toegenomen potentie hadden om T
cellen te stimuleren. Het betreft de chemo­
therapeutica oxaliplatin, carboplatin en
cisplatin die bij zeer veel verschillende
vormen van kanker dagelijks gebruikt
worden in de kliniek. We vonden dat onder
invloed van platinum-chemotherapie het
molecuul PD-L2 op het oppervlak van de
DC naar binnen werd geklapt. PD-L2 is
een remmend eiwit dat normaal gesproken
er voor zorgt dat T cel activatie niet te veel
‘doorschiet’ en is dus van belang bij het in
toom houden van afweerreacties. Doordat
PD-L2 als het ware naar binnen klap on­
der invloed van platinum-chemotherapie,
resulteert dat in een verhoogde T celactiva­
tie. We hebben nog verder gekeken waar­
om de hoeveelheid PD-L2 op de DC ver­
mindert door platinum-chemotherapie en
dit bleek te berusten op inactivatie van het
eiwit STAT6 dat de hoeveelheid PD-L2 op
de cel reguleert.

Nu is het zo dat niet alleen DCs PD-L2 op
hun oppervlak hebben, maar tumorcellen
ook. Ze gebruiken dit molecuul als verde­
digingsschild tegen T cellen (figuur 4).

**Figuur 4**

We vonden dat platinum-chemotherapie
ook in tumorcellen PD-L2 naar beneden
brengt als gevolg van inactivatie van
STAT6 en daarmee een verdedigingsme­
chanisme van de kankercellen afpakt (fi­
guur 5). Onze hypothese was dat platinum­
chemotherapie niet alleen werkt door di­
rect kankercellen dood te maken, maar ook
door het immuunsysteem te activeren en
tumorcellen te ‘ontwapenen’ tegen het af­
weersysteem. Om dit te testen hebben wij gekeken naar STAT6 aanwezigheid in tumoren van patiënten met mond-keelkanker die werden behandeld met cisplatin en bestraling. We vonden inderdaad dat patiënten die STAT6 in hun tumor hadden, een beter reactie lieten zien op de cisplatin therapie, hetgeen impliceert dat het immunologische effect van platinum-chemotherapie ook van klinisch belang is.

Figuur 5

Platinum-chemotherapie leidt dus tot verhoogde DC activatie en ‘ontwapening’ van kancercellen. Om te kijken of het gecombineerd kan worden met immuuntherapie is het van belang om te weten wat er gebeurt met de T cellen tijdens de chemotherapie. In hoofdstuk 9 onderzoeken we dit door darmkankerpatiënten te vaccineren met CEA-beladen DCs in combinatie met oxaliplatin chemotherapie. Het betrof patiënten die darmkanker hadden zonder uitzaaiingen op afstand, maar wel in lokale klierstations. Dat betekent dat ze een hoge kans hebben op het krijgen van uitzaaiingen op afstand, maar wel in lokale klierstations. Dat betekent dat ze een hoge kans hebben op het krijgen van uitzaaiingen op afstand, maar wel in lokale klierstations. Dat betekent dat ze een hoge kans hebben op het krijgen van uitzaaiingen op afstand, maar wel in lokale klierstations. Dat betekent dat ze een hoge kans hebben op het krijgen van uitzaaiingen op afstand, maar wel in lokale klierstations. 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DANKWOORD

Wetenschap bedrijven is hard werk, onzekere uitkomsten, nieuwe ideeën verzinnen, data interpreteren en herinterpreteren, protocollen maken, experimenten herhalen en nog eens herhalen, vroeg-opstaan-laat-naar-bed, laat-opstaan-vroeg-naar-bed (in het weekend dan), afwachten, rennen en vliegen, stilstaan, wanhoop, eureka en heel veel samenwerken. Boven alles is wetenschap bedrijven gewoonweg onzettend spannend!

Bij translationeel onderzoek, zoals dat ook beschreven is in dit proefschrift, ligt de nadruk bij uitstek zeer sterk op (multi-disciplinair) samenwerken.

Ik wil dan ook iedereen die heeft bijgedragen aan het onderzoek van de afgelopen jaren vanuit de grond van mijn hart bedanken voor de leerzame, uitdagende en zeer plezierige tijd.

Speciaal wil ik iedereen die mij heeft begeleid bij het leren van onderzoek doen bedanken, het was toch niet eenvoudig om van een doktertje met twee linker handen een nette pipeteur annex klinisch onderzoeker te maken.

Bedankt allemaal!

Joost
Curriculum Vitae (nederlands)


Voor dit onderzoek ontving hij diverse prijzen, waaronder een *Van Walree-beurs* van de Koninklijke Nederlandse Akademie van Wetenschappen, een reisbeurs van de Nederlandse Vereniging van Immunologie en een *Leopoldina Fellowship for Excellent Cancer Research* van de Duitse Academie van Wetenschappen.

In 2006 ontving hij een subsidie van de Stichting Sascha Swarttouw-Hijmans om het onderzoek te verrijken zoals dat is beschreven in hoofdstuk 8 en 9. In 2009 ontving hij een subsidie van de Stichting Vanderes, voor vervolgonderzoek van dit werk. Hiermee kon hij een post-doc aanstellen, die hij thans begeleidt.

In 2009 begon hij binnen de opleiding interne geneeskunde met het aandachtsgebied Medische Oncologie (opleider prof. Winette van der Graaf). Op 1 november 2010 is zijn opleiding afgerond en zal hij werkzaam zijn als staflid bij de afdeling Medische Oncologie van het Radboud UMC.

In 2010 ontving hij een vierjarig *fellowship translationeel kankeronderzoek* van KWF/Kankerbestrijding voor vervolg van het onderzoek zoals dat is beschreven in hoofdstuk 8 en 9. Een deel van dit onderzoek zal hij verrichten in het lab van prof. Richard Lake en prof. Bruce Robinson aan de *University of Western Australia* te Perth, Australië.

Joost is getrouwd met Dorit en heeft drie heel eigenwijze kinderen Wim, Luuk en Merel.
Curriculum Vitae (english)

Willem Joost Lesterhuis, born in Leeuwarden on the third of May 1975, grew up in Nes, on the island Ameland. Already early in life his urge to publish became apparent with a publication in the amongst peers highly-cited journal Donald Duck. After moving to the mainland, he went to secondary school in Oosterwolde, Friesland. He started medical school in 1994 at the Free University in Amsterdam. He followed additional courses in immunology. In 2001 he graduated cum laude as a medical doctor.

Driven by fascination of the immune system he moved to Nijmegen in 2001 to do research at the departments of Tumor Immunology and Medical Oncology of the Radboud University Nijmegen Medical Centre, under supervision of prof. Kees Punt, prof. Gosse Adema, dr. Jolanda de Vries and prof. Carl Figdor. Mainly thanks to the persuasiveness of the late prof. Pieter de Mulder, he took on the challenge of applying to The Netherlands Organization for Health Research and Development for an AGIKO scholarship (junior doctor in training to become clinical researcher). He was rewarded one in 2002 and could use it to combine residency training in Internal Medicine at the Radboud University Nijmegen Medical Centre (tutors prof. Jos van der Meer, prof. Paul Stuyl, dr. Jacqueline de Graaf and later dr. Chantal Bleeker-Rovers) with the research that is described in this thesis.

For this research he received several awards, including a Van Walree Scholarship from the Royal Dutch Academy of Science, a travel grant from the Dutch Society of Immunology and a Leopoldina Fellowship for Excellent Cancer Research from the German Academy of Science.

In 2006 he obtained funding from the Sascha Swarttouw-Hijmans Foundation to do the research as described in chapters 8 and 9. In 2009 he was awarded a grant by the Vanderes Foundation to follow up on that research. With this fund he was able to appoint a post-doc, who he is now supervising.

In 2009-2010 he was trainee in Medical Oncology (tutor prof. Winette van der Graaf). He will complete his training on the 1st of November 2010, after which he will be a member of staff in the department of the Medical Oncology in the Radboud University Nijmegen Medical Centre.

In 2010 he received a 4-year fellowship for translational cancer research from the Dutch Cancer Society to further follow up on the research that is described in chapters 8 and 9. He will perform part of this research in the lab of prof. Richard Lake and prof. Bruce Robinson at the University of Western Australia, Perth, Australia.

Joost is married to Dorit and has three rascally kids Wim, Luuk and Merel.
LIST OF PUBLICATIONS


