

Dendritic cell vaccines in melanoma: From promise to proof?

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Abstract

Dendritic cells (DC) 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 DC are used in clinical studies, while from preclinical studies it is evident that DC that are activated by pathogen-associated molecules are much more potent T cell activators. For sake of easy accessibility monocyte-derived DC are often used, but are these cells also the most potent type of DC? 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.

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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 DC 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.

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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 studies [1].

Dendritic cells (DC) are crucial as the sentinels of the immune system. It has been proposed that when the tumour reaches a certain size and causes damage to the surrounding tissues with release of products by the microenvironment, local DC 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’ DC; appropriately activated and loaded with tumour antigen. The underlying principle is that DC are the most potent antigen presenting cells of the immune system that play a central role in the induction-phase of antigen-specific immunity. DC 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 DC 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 DC (Tables 1 and 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. DC subsets

Two distinct categories of DC exist: plasmacytoid DC and conventional or ‘myeloid’ DC [7]. Plasmacytoid DC 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 dif-

Table 1
Results of published DC-vaccination trials with non-matured DC in metastatic melanoma

DC culture method	Antigens	Ag-loading method	Route	No. pts	Clinical responses	Immunological response ^a	Reference
IL-4/GM-CSF/FCS moDC	Tyrosinase, gp100, MART-1, MAGE-1, MAGE-3 or lysate	Peptides/lysate	in	16	2 CR, 2 PR	Not tested	[49]
IL-4/GM-CSF/FCS moDC	MAGE-3A.2, tyrosinase, gp100, MART-1 or lysate	Peptides/lysate	in	33	3 PR, 1MR, 6 SD	13	[93]
IL-4/GM-CSF moDC	MART-1, gp100	MHC-I peptides	iv	10	1 PR	1	[85]
IL-4/GM-CSF moDC	Autologous peptides	Autologous peptides	id	22	1 CR, 2 PR	Not tested	[96]
IL-4/GM-CSF moDC	Lysate	Lysate	id	14	1 PR, 1 SD	4	[91]
IL-4/GM-CSF moDC	MART-1, gp100, tyrosinase	MHC-I peptides	iv	16	1 CR, 2 PR, 2 MR	5	[76]
IL-4/GM-CSF moDC	MART-1	MHC-I peptides	id	4	1 CR ^b , 1 SD ^b , 1 MR	4	[86]
IL-4/GM-CSF/FCS moDC	Lysate	Lysate	in/sc	4	1 PR, 1 CR	3	[94]
IL-4/GM-CSF moDC	gp100/tyrosinase	MHC-I peptides	iv/id	9	–	–	[52]

Results of published DC-vaccination trials in which non-matured DC were used, only melanoma patients with *distant metastases and evaluable disease* are included. **Abbreviations:** IL, interleukin; GM-CSF, granulocyte macrophage-colony stimulating factor; FCS, fetal calf serum; moDC, monocyte-derived DC; sc, subcutaneous; in, intranodal; id, intradermal; iv, intravenous; CR, complete response; PR, partial response; MR, mixed response; SD, stable disease (≥ 4 months).

^a Immunological response is defined as any evidence of melanoma peptide or tumour-specific T cell response after vaccination by one or more test methods; the number of patients with an immune response are given.

^b After DC-vaccination these patients received treatment with an anti-CTLA4 blocking antibody.

Table 2
Results of published DC-vaccination trials with matured DC

DC culture method	Antigens	Ag-loading method	Route	No. pts	Clinical responses	Immune responses	Reference
IL-4/GM-CSF/MCM/TNF- α /moDC	MAGE-3	MHC I peptides	sc, id, iv	13	6 MR	9	[56]
IL-4/GM-CSF/IL-1b/IL-6/TNF- α /PGE ₂ moDC	MAGE-3, MAGE-1, tyrosinase, MAGE-4, MAGE-10, gp100, MART-1	MHC I+II peptides	sc	19	1 CR, 4 SD	12	[57]
IL-4/GM-CSF/MCM/TNF- α moDC	MAGE-3	MHCI peptides	sc, iv	8	1 SD	8	[59]
GM-CSF/Calcium-ionophore/IL-2/IL-12 moDC	MART-1, gp100, tyrosinase,	MHC I peptides	in, id, iv	27	3 PR	13	[78]
IL-4/GM-CSF/MCM/TNF- α +PGE2 moDC	gp100, tyrosinase	MHC I peptides	id, iv	10	1 PR, 1 MR, 3 SD,	2	[52]
Flt3L/GM-CSF/TNF α CD34+ DC	MART-1, MAGE-3, gp100, tyrosinase	MHC I peptides	sc	18	4 CR ^a , 4 PR, 3 SD	17	[15,82,83]
IL-4/GM-CSF/TNF α /IL-3/IL-6/SCF CD34+ DC	MART-1, gp100, tyrosinase, MAGE-1, MAGE-3	MHC I peptides	iv	14	1 PR, 6 SD	1	[18]
Flt3L/GM-CSF/TNF α /IFN α CD34+ DC	MART-1, MAGE-3, gp100, tyrosinase	MHC I peptides	sc	20	1 SD	7	[17]
IL-4/GM-CSF/TNF- α /CD40L moDC	Allogeneic lysate	Allogeneic lysate	sc	20	1 CR, 1 PR	2	[61]
IL-4/GM-CSF/IL-1 β /IL-6/TNF- α /PGE ₂ moDC	Autologous tumour RNA	RNA-electroporation	id or in	21	1 MR	9	[110,111]
IL-4/GM-CSF/TNF α moDC \pm sc IL-2	Allogeneic lysate	Lysate	id	15	6 SD	9	[99]
IFN β /IL-3/polyI:C moDC	NA17, MAGE-3	MHCI +II peptides	sc, id	4	1 SD	1	[84]
IL-4/GM-CSF/IL-1 β /IL-6/TNF- α /PGE ₂ moDC	MAGE-1, MAGE-3, tyrosinase, gp100, MART-1	MHCI +II peptides	sc	53	2 PR, 8 SD ^b	Not given	[87]
GM-CSF/IL-13 moDC	Allogeneic lysate	Allogeneic lysate	in, sc, id	10	1 SD	3	[98]
GM-CSF/IL-13/Ribomunyl/IFN γ	MART-1, \pm NA17	MHCI+II peptides	il then in	14	2 SD	5	[133]

Results of published DC-vaccination trials in which DC 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; TNF α , tumour necrosis factor alpha; PGE₂, prostaglandin-E₂; moDC, monocyte-derived DC; 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).

^a In a follow-up report Ref. [82], all complete responders were still free of disease with a median of 5 years.

^b 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.

ferentiate into DC that are capable of activating naïve T cells against allo-antigens [9] and exogenous antigens [10]. In the context of cancer, plasmacytoid DC 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 DC or ‘myeloid’ DC [12] can be further divided into migratory DC, which actively sample the peripheral tissues and migrate to draining lymph node to present antigens to T cells, and lymphoid-tissue-resident DC which captivate local (foreign and self-) antigens and present it to local T cells [13]. Examples of lymphoid-tissue-resident DC are splenic and thymic DC. Migratory DC derive from both CD34+ precursor cells and monocytes. Monocytes can differentiate into DC 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 DC.

Recently, it was proposed to recognize a third distinct group of conventional DC, ‘inflammatory’ DC: cells that are not present in the steady state, but that appear under the influence of inflammation or infection [13].

3. DC subsets in clinical trials

Monocyte-derived DC have been used in most vaccination studies because of the relative ease with which large quantities of cells can be obtained. Usually, one leukapheresis is enough to obtain approximately $100\text{--}150 \times 10^6$ cells. Yields are much lower with CD34+ selection and repeated leukaphereses are often necessary to obtain enough cells [15]. The same accounts for circulating blood DC, which can be obtained from peripheral blood after in vivo Flt-3L expansion and negative selection ex vivo [16].

Banchereau et al. have reported impressive immunological and clinical results using Flt3-ligand expanded CD34+ DC [15]. In other studies with CD34+ DC, using different maturation and vaccination regimes, clinical responses were observed less frequently [17,18]. The same variable outcomes are seen in studies with monocyte-derived DC (Tables 1 and 2, and section on clinical results). These differences may well be more 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 [7], and that perhaps it is beneficial even to combine different subsets of DCs, as has been done in the above mentioned studies [15].

4. Maturation

The term ‘maturation’ refers to the phenotypical and functional reaction of DC upon encountering danger signals. Maturation can be induced by pro-inflammatory cytokines such as IL-1 β or IL-6, by interaction with T cells and by interaction with pathogens. DC can detect pathogens through pattern recognition receptors such as Toll-like receptors (TLRs) [19]. 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) [20].

In the past 10 years, the term ‘mature’ DC has generally been used to describe T (helper 1) cell stimulatory DC. Immature DC were considered to be primarily involved in the recognition and uptake of antigen. Upon receiving maturation signals these immature DC would then change their chemokine receptor repertoire and upregulate their co-stimulatory molecules, thus acquiring the phenotype of mature DC that are capable of migration to the lymph nodes and activation of T cells. In the absence of maturation signals, DC would not upregulate their co-stimulatory molecules and would thus remain energy- 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 reference [21]). For instance, in the steady state, that is in the absence of ‘danger’, immature DC have been shown to be capable of circulating through the tissues and lymphoid organs, encountering and capturing both self-antigens and innocuous environmental antigens [22]. It is suggested that through this mechanism immature DC play a critical role in the continuous induction of peripheral tolerance, thereby preventing both auto-immunity and hyperreactivity [23]. In addition, although the expression of co-stimulatory molecules is one of the phenotypic markers of mature DC, the induction of a tolerogenic immune response depends on the presence of these molecules as well [24]. In a chimeric murine model it was shown that although cytokine-matured DC demonstrated upregulation of co-stimulatory molecules and induction of T cell proliferation, the activated T cells did not fully develop into IFN γ -producing effector cells [25]. Only when the DC were activated through Toll-like receptors, also referred to as ‘licensed’ DC [26], the induced antigen-specific T cells were able to fully develop into effector cells. The ‘licensing’ of DC is not only restricted to pathogen-derived signals, since activation by bystander T helper cells may also suffice [27,28].

Another level of complexity is added by the timing and duration of the maturation signal. For example, murine bone marrow-derived DC that are exposed to lipopolysaccharide for 48 h have the same expression of costimulatory molecules CD80, CD86 and CD40 as compared to DC that are exposed to lipopolysaccharide for 8 h and also have a comparable migratory capacity and allostimulatory potential. However, the longer matured DC exhibit a decreased IL-12 production potential correlating with low antigen-specific T cell responses after vaccination in mice [29,30]. 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 DC results in efficient antigen-presentation and subsequent T cell activation [31]. 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 DC in vitro [32] and in vivo [33]. For example, a combination of triggering via TLR3 and TLR 7 leads to a 10–100-fold higher IL-12 production, when compared to DC that have been activated by one of the respective TLRs alone [32]. 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) [34]. 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 [32,35,36]. However, migratory capacity is somewhat hampered in these cells as compared to cytokine-matured DC, which can be restored by co-culturing the DC in PGE2 [36].

If DC with similar expression of co-stimulatory molecules can exert entirely different functions, what then determines the nature of the induced T cell response? The ability of DC to produce IL-12, TNF- α and IL-6 is thought to be important for the induction of robust T cell responses and to bypass suppressor T cell-induced tolerance [37–39]. In addition, several factors such as IL-10, vitamin-D3 and cortico-steroids can skew the DC into a more suppressive T cell type of inducer [40]. However, it still remains poorly understood which mechanisms truly determine the nature of the T cell response as instructed by the DC.

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 DC’, but that there are in fact a lot of different types of mature DC 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 DC 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 DC 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 [41] and natural killer T (NKT) cells [42] can eradicate cancer. NKT cells and DC activate each other communally. DC activate NKT cells by presentation of exogenous (microbial) and endogenous glycolipid antigens, thereby inducing IFN γ release and CD40-ligand upregulation by the NKT cells [43,44]. Reciprocally, NKT cells activate DC via CD40-CD40L interaction. This in turn causes the DC 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, marine sponge-derived glycolipid α -galactosylceramide (α GalCer) has been studied in murine and human studies. A single injection of α GalCer combined with a model antigen induced strong CD4+ and CD8+ T cell responses, through activation of DC in a TLR-independent but NKT cell-dependent manner [45]. The results were confirmed using tumour vaccines instead of a model antigen [46,47]. Thus far, one phase I dose-escalation study with clinical-grade α GalCer (KRN7000) has been carried out in 24 patients with solid tumours, in which no clinical responses were observed [48].

5. Maturation of DC in clinical trials

In early clinical trials only immature DC were used [49,50]. Some of these DC might have been ‘semi-mature’ due to the addition of fetal calf serum in the culture medium [49,51]. Although objective clinical remissions were observed in these studies, there is now strong evidence that immature DC should no longer be used in clinical practice. In a comparative study in metastatic melanoma patients cytokine-matured, peptide-pulsed DC were superior to immature DC, 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 DC showed a strong T cell and B cell response against KLH [52]. 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 [23,53,54].

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 TNF α , with any of the following cytokines in any combination: IL-1 β , IL-6, prostaglandin-E2, or the supernatant of activated autologous monocytes (Monocytes Conditioned Medium) [55–59]. There is some evidence that culturing DC with IL-15 may lead to a type of mature DC that induces stronger Th1 effector type of immune responses [60], however no comparative stud-

ies have been reported yet. Lastly, CD40-ligation has been used as a method of activation of DC in a clinical setting [61,62].

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 DC were used in cancer patients. In vitro data are promising [63], but clinical results with TLR-matured DC are eagerly awaited.

6. Antigen-processing and presentation

Intracellular endogenous antigens usually are presented in MHC class I, whereas exogenous antigens are usually presented in class II by antigen-presenting cells (Fig. 1A) [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 [65,66] are also presented in MHC class I to cytotoxic T cells, a process referred to as cross presentation (Fig. 1A) [67,68]. Thus, tumour antigens derived from necrotic or apoptotic tumour cells can be presented by the DC 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 [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 [74]. The same authors show in another study that apoptotic tumour cells induce immune responses in a TLR-4 dependent manner via secretion of HMBG1 [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.

7. DC antigen-loading in clinical trials

In most clinical DC vaccination studies synthetic MHC class I-binding peptides have been used [15,17,18,49, 52,56,76–86], with class II-binding peptides being added in some (Fig. 1B) [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 [88,89]. However, no clinical trial has yet been performed in which MHC class I antigen-loaded DC are compared to the combination of both MHC classes I and II antigen-loaded DC.

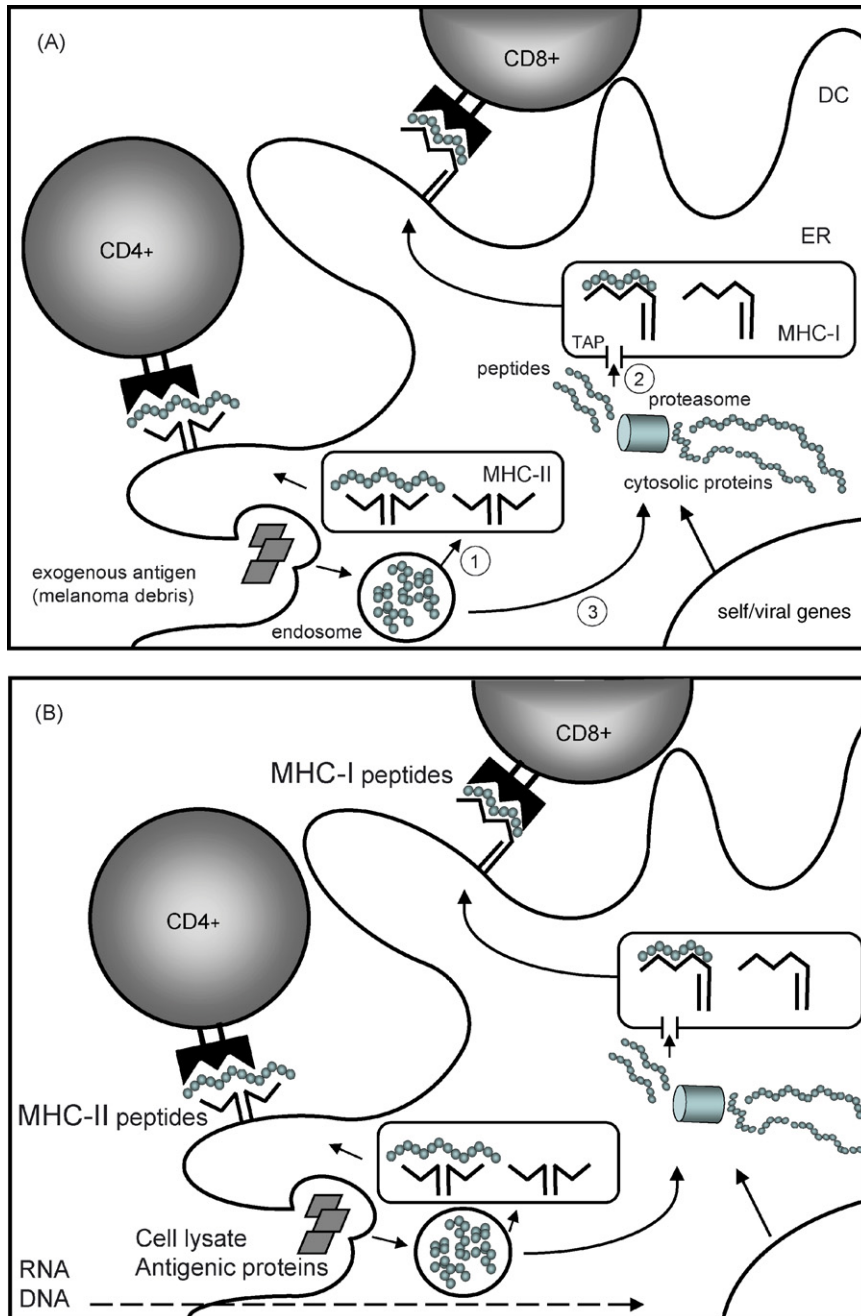


Fig. 1. (A) Antigens can be processed by the DC through three major pathways. Exogenous antigens will be internalized by the DC and enter the endocytic pathway in which they are targeted to lysosome-related MHC class II-rich compartments. In these compartments the antigens are degraded and loaded onto 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 endoplasmic 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 endoplasmic reticulum and transferred to the cell surface (route 2 in the figure), where it is presented to CD8+ cytotoxic T cells. Lastly, DC 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) Melanoma antigens can be loaded onto the DC for antigen presentation either by transfection with DNA or RNA encoding melanoma antigens, or pulsing with tumour lysate, antigenic proteins or peptides.

Instead of HLA-binding peptides, whole antigenic proteins can be used. The DC 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 [90].

Autologous [49,91–96] or allogeneic [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 classes 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 DC [61].

Transfection of DC with RNA concerns a novel antigen-loading technique [100], with either tumour-derived RNA [101,102] or synthetic RNA encoding specific melanoma-associated antigens being used [103]. A benefit of this technique lies in the presentation of several MHC class I epitopes and sometimes also MHC class II epitopes, depending on the presence of an endosomal targeting sequence [104]. Also, it may lead to a more prolonged presentation of the antigen as compared to peptide-loading which appears to be short-lived [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 [103,106–109]. Furthermore, anti-tumour T cell responses and some evidence for clinical activity have been reported in patients vaccinated with DC electroporated with tumour-derived RNA [110,111].

In a study with 33 metastatic melanoma patients vaccination with autologous tumour lysate-loaded DC resulted in a slightly higher response rate compared to peptide-pulsed DC (3 versus no partial remissions, respectively) [93]. Equal immunogenicity was demonstrated for peptide- and RNA-pulsed DC in colorectal cancer patients [112]. However, immature DC were used in both studies. Mature DC 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 DC *in vivo* would obviate the need for laborious *ex vivo* culturing protocols [113]. Although this appears to be feasible through DC specific molecules such as the C-type lectins DC-SIGN [114] and DEC-205 [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 DC by delivering danger signals *in situ*, which can be combined with chemokine treatment in order to increase the number of intratumoural DC [116]. In addition, local tumour-destructing therapies can induce antigen-release *in situ*, for example by using radiotherapy [117], chemotherapy [118] or radiofrequency ablation [119]. The immunogenicity of these methods may then be further enhanced by the local delivery of DC activating signals [120,121].

8. Migration

DC 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 DC 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 [122,123]. CCR7 guides the DC 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 (Fig. 2A) [124]. Expression of CCR7 is upregulated upon DC maturation, resulting in an enhanced migratory capacity of matured DC as compared to immature DC [73]. In addition, inflammatory signals such as prostaglandin-E2 are needed to further sensitize CCR7 to its ligands [125,126]. These findings are of importance for DC-based vaccination, since they show that it may be beneficial to culture DC in the presence of prostaglandin-E2 in order to get a good migratory capacity after vaccination, even though prostaglandin-E2 has been described to skew DC under some circumstances towards a Th2 type of immune response [40].

Interestingly, in a murine model DC migration could be increased up to 10-fold after pretreatment of the injection site with TNF α or unloaded DC, due to the upregulation of CCR7-ligand CCL21 in lymphatic endothelial cells, resulting in a superior magnitude and quality of the T cell response [123].

9. Route of DC administration in clinical trials

For the effective induction of immunity it is obligatory for the DC 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 responses [127]. As different routes lead to different sites of accumulation of the vaccinated DC, these issues are of importance when considering the route of delivery of the DC (Fig. 2B). Murine models have shown that after intravenous injection the majority of DC accumulate in the spleen and to a lesser extent in the lungs, kidneys and liver, while hardly any DC end up in peripheral lymph nodes [128].

In vivo studies in cancer patients have shown that after intradermal injection approximately 2–4% of the DC migrate to draining lymph nodes [129]. There is now convincing evidence from several independent human studies that migration after subcutaneous injection is much lower compared with intradermal injection [84,129–131].

Intranodal injection results in a much higher amount of DC accumulating in lymph nodes, not only in the injected node, but also in subsequent draining nodes [129]. Migration to these subsequent nodes via the physiological pathway

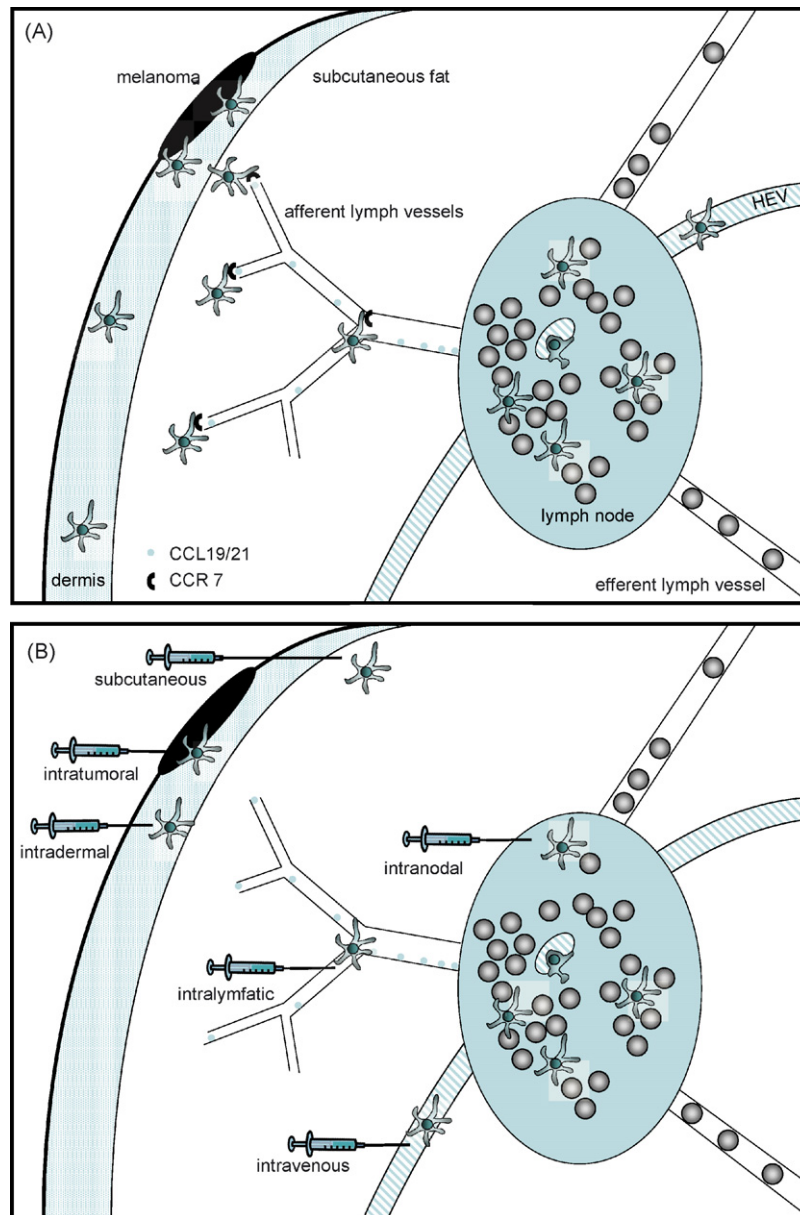


Fig. 2. (A) DC that reside in the periphery migrate to draining lymph nodes through afferent lymphatic vessels following chemotactic gradients of CCL19 and CCL21. These chemokines are expressed by lymphatic endothelial cells and lymph node-residing cells. Blood DC enter the lymph nodes through high endothelial venules (HEV). After interaction with DC, 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, intratumoural, intralymphatic, intranodal and intravenous.

through lymph vessels can be as much as 80%. However, the magnitude of DC migration that is observed after intranodal injection is more variable than after intradermal vaccination [129]. Intranodal injections are usually administered under ultrasound guidance. Supposedly, this would result in an assured accurate delivery, which was the reason for some studies to prefer this route of administration [54]. However, using paramagnetic beads-labeled DC that can be tracked in patients after injection by MR imaging, we recently found that intranodal injection is complicated by inaccurate delivery: in a considerable proportion of patients the DC were

misinjected, in the perinodal fat [132]. Inaccurate injection correlated with absent migration to remote lymph nodes. These results explain the variability in migratory outcome after intranodal administration and underscore the importance of accurate delivery [133]. Recently, Lesimple et al. showed that intralymphatic delivery into a lymph vessel in the dorsum of the foot is also feasible [134].

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 response 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 injection [78]. Another study in advanced melanoma patients showed no benefit for intranodal vaccination as compared to intradermal vaccination: of 22 evaluable patients a positive DTH reaction against the vaccine was detected in 7/10 intradermal vaccinated patients and in 3/12 intranodal vaccinated patients [135]. Mullins et al. compared vaccination with DC 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 [136]. Antigen-specific T cell responses were observed regardless of the route of delivery, although IFN γ production after antigen stimulation *in vitro* was only demonstrated in the intradermally and intralymphatically 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 DC was shown to be essential for immune responses against visceral melanoma metastases, whereas for subcutaneous vaccination this was true for non-visceral metastases [137]. These findings indicate that the combination of different routes of administration may be beneficial to target different tumour locations in the entire body [138].

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.

10. 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 DC (as discussed above), the suppressive activity of so-called regulatory T cells (Treg) 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 at 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 [139,140]. Treg 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 [139]. Treg function is controlled by cytokines, cross-talk with antigen-presenting cells, but also by direct interaction with pathogens via TLRs [141]. The mechanisms by which Treg exert their suppressive function are not completely elucidated, but it may happen in a cell–cell contact dependent manner via camp as second messenger [142] as well as through cytokines such as IL-10 or TGF β [140]. The clin-

ical importance of Treg in cancer has been demonstrated by Curiel et al. [143]. A high number of Treg in tumours of ovarian cancer patients correlated with poor survival. In other tumour types, these data have been confirmed [144–146]. 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 [147,148]. 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 [148]. 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) [149].

The ligands of CTLA-4 are the DC co-stimulatory 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 [149,150].

PD-1 is another inhibitory T cell surface molecule that upon binding to its ligands PD-L1 and PD-L2 induces anergy or tolerance [149]. Interestingly, PD-L1 and 2 are expressed by several types of cancer, which may allow the tumour to escape from immune surveillance [151,152]. This phenomenon can be reversed in mice by treatment with an anti-PD-L1 antibody [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 [154].

11. 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 diphtheria toxin coupled to IL-2, resulting in enhanced antigen-specific T cell responses after DC vaccination [155]. Denileukin diftitox as a single agent has shown promising immunological and clinical results in phase I and II studies in ovarian cancer [185]. In melanoma however, thus far the data are conflicting, with one study failing to show any efficient Treg depletion [156], while another preliminary study showed tumour regression in two patients [186]. Suntharalingam et al. found enhanced antigenic immune responses after peptide vacci-

nation and a significant reduction in Treg frequencies in melanoma patients after denileukin diftitox [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 TGN1412 again demonstrated the enormous potency of drugs that manipulate the co-stimulatory pathways [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 [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 [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 [163], however clinical trials that are specifically designed to answer this question have not been published.

12. 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 [49,50,56]. Since then numerous small studies have been performed, especially in melanoma patients (Tables 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 DC 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 DC are compared with studies in which matured DC have been used (Tables 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 DC 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 [87]. How can this disappointing

result be explained? As reported by the authors themselves, the DC 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 DC. Others have pointed at 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 [90]. 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.

13. 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 [15,165], but not in all [93]. 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 [166]. 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 Coullie 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 [166]. We took another approach by analyzing T cell responses in vaccinated patients from biopsies of delayed type hypersensitivity reactions that were performed with the peptide-loaded DC vaccine [165,167]. 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 [165]. 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 disease.

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 regression [168]. They found that other tumour-specific T cells

that were already present prior to vaccination were much more expanded 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 outcome may cause tumour regression either in a direct or in an indirect manner.

14. 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 [169]. 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 [169]. 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 [4]. 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 [170].

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 [15]).

Another difference is that in patients with advanced disease, the patient group in which normally phase I trials are performed, immunotherapy will probably have little chances 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 [169]. The following criteria were proposed for proof-of-principle trials. They should include a minimum of 20 or more 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 two separate, established and reproducible assays at two consecutive follow-up time points after the baseline assessment. Efficacy trials then formally establish clinical benefit, either directly or through a surrogate endpoint.

We believe that these guidelines are of great practical use and could help the field forward. 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.

15. 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 [171].

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 [172]. 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 [173]. 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 [171,174]. Other firms are seeking approval by the European and US regula-

tory authorities for vaccines targeted at lymphoma, sarcoma, glioblastoma and acute myelogenous leukaemia [171].

16. 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 [175] and may in fact have a synergistic effect together with immunotherapeutic approaches (reviewed in Ref. [176]) [75,118]. 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 [177] and targeted therapy [178]. Another interesting approach involves in situ tumour destruction by cryo- or radiofrequency ablation in combination with immune activation, including injection of DC [119–121,179]. However, clinical data are lacking, 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 [180]. Adoptive T cell transfer following non-myeloablative but lymphodepleting chemotherapy showed impressive clinical results in advanced melanoma patients [181,182]. 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 [183], 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 [183,184]. 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.

17. 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 [155]. Also blockade of the inhibitory T cell molecule CTLA-4 by monoclonal antibodies could enhance the immunogenicity of DC vaccines

[162]. Combination treatment with chemotherapy, radiotherapy or other tumour ablative treatments needs to be further investigated. Trials with TLR-ligand activated DC 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 DC 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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