

Dendritic cell immunotherapy: mapping the way

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Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system, with the potential to either stimulate or inhibit immune responses. Exploiting the immune-regulatory capacities of dendritic cells holds great promise for the treatment of cancer, autoimmune diseases and the prevention of transplant rejection. Although early clinical trials indicate that DC vaccines can induce immune responses in some cancer patients, careful study design and use of standardized clinical and immunological criteria are needed.

DCs are antigen-presenting cells with the unique ability to take up and process antigens in the peripheral blood and tissues. They subsequently migrate to draining lymph nodes, where they present antigen to resting lymphocytes. Immature DCs are particularly good at antigen ingestion and processing, but for a productive T-cell response they must mature to fully activated DCs, which express high levels of cell-surface major histocompatibility complex (MHC) antigen complexes and costimulatory molecules. DCs of various phenotypes serve as sentinel cells in virtually all tissues—including the peripheral blood, where they are continuously exposed to antigens. Very small numbers of activated DCs are highly efficient at generating immune responses against viruses, other pathogens and endogenous tumors.

A DC vaccine is defined as DCs loaded with antigen, such as a tumor associated antigen. Upon administration into patients, the vaccine is thought to induce an antigen-specific T-cell response against the tumor. The first clinical study of a dendritic cell (DC) vaccine was reported in *Nature Medicine* in 1996 (ref. 1). To date, most DC vaccines have been used to stimulate immune responses, in particular against cancer^{2,3}. At a recent conference in Amsterdam (<http://www.kun.nl/til/dc2003/>), more than 60 different clinical studies in cancer patients were presented (summarized in **Supplementary Table 1** online).

In this perspective we discuss the pitfalls of early DC vaccine trials, the current state of the field and the difficulties that remain to be overcome. Our relative lack of information (Fig. 1) prevents us from designing large studies or including patients with early-stage disease. We put forth a roadmap for standardization and quality control of DC vaccines.

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Generation of DC vaccines

The discovery that myeloid DCs can be easily generated from monocytes or CD34⁺ precursors allowed, for the first time, the procurement of these otherwise scarce cells (less than 0.2% of white blood cells) in considerable numbers⁴. Most clinical studies to date use monocyte-derived DCs. When cultured in the presence of granulocyte-macrophage colony-stimulating factor and interleukin-4, the cells develop into immature DCs over a period of three to five days^{4,5}, and can be further matured by a subsequent one- to two-day culture period using different stimuli. In addition to monocytes, CD34⁺ cells are also used to generate DCs by culture in cytokine mixtures^{6–8}.

Antigen loading of DCs

To be effective as an antigen-presenting cell, the MHC molecules of a DC must be loaded with antigenic cargo. Several techniques have been developed to load human DCs with tumor-associated antigens, the most widely used being incubation of DCs with human leukocyte antigen (HLA) class I- and class II-binding peptides, which can bind directly to MHC molecules on the cell surface (**Supplementary Table 2** online). Alternatively, peptides can be endogenously loaded onto MHC molecules after proteolytic processing of endocytosed tumor lysates or recombinant protein. RNA encoding tumor antigens or derived from tumors can be exploited to produce tumor antigens in DCs themselves. Tumor-derived RNA has the advantage that tumor antigens encoded by uniquely mutated genes are presented, but has the disadvantage that an unknown number of autoantigens are also presented. DCs expressing transgenes encoding tumor antigens may be more specific primers of antitumor immunity⁹.

Presentation of exact HLA class I-binding peptides is suboptimal for two reasons: first, specific CD4⁺ T-cell help to generate antigen-specific T cells is lacking; and second, the half-life of such peptide-MHC complexes is relatively short. Longer peptides, provided that they contain both class I and class II epitopes, could be useful as the next generation of peptide immunogens.

Delivery of *ex vivo*-generated DCs

Most immunotherapeutic agents are administered intravenously, but DCs are frequently administered intradermally or even through direct injection into draining lymph nodes (**Supplementary Table 3** online). Recent studies showed that immature DCs migrate less than mature DCs. In general, less than 5% of intradermally administered mature DCs reach the draining lymph nodes¹⁰, amounting to inefficient homing. An increase in migration would enhance the efficacy of the vaccine and allow the use of blood-derived DCs that cannot be enumerated *in vitro*. Approaches to stimulate DC migration use inflammatory cytokines to condition tissues¹¹ (Fig. 2). Matrix metalloproteinases and Toll-like receptor (TLR) ligands also enhance DC migration¹².

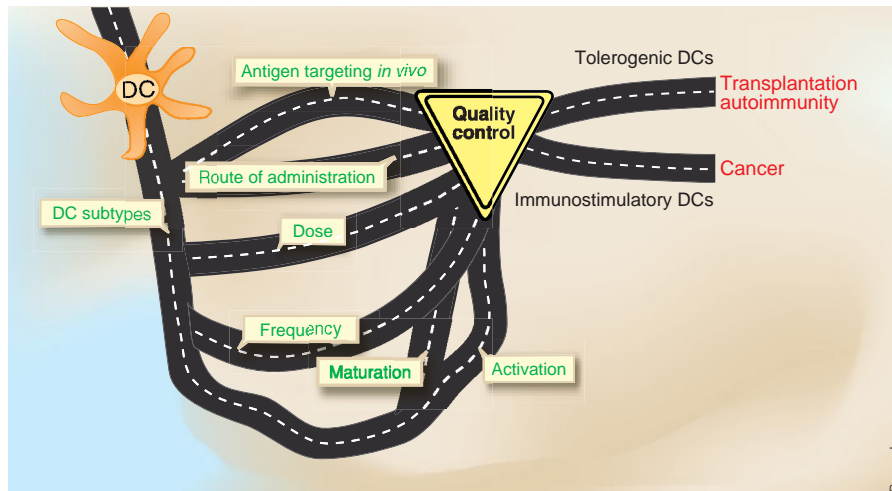


Figure 1 DC immunotherapy: mapping the way. Early clinical trials clearly show the potency of DC therapy, but there are still too many variables preventing its introduction as a standard cancer treatment. There is a strong need for standardization and quality control of this technique. Additional research-driven (pre)clinical studies must be carried out to evaluate the most effective DC subtypes, the optimal conditioning and activation stimuli, the optimal route of administration, and the optimal dose and frequency of DC vaccinations. The generation of immature, tolerogenic DCs suggests the applicability of DC immunotherapy to diseases that require silencing of unwanted immune responses.

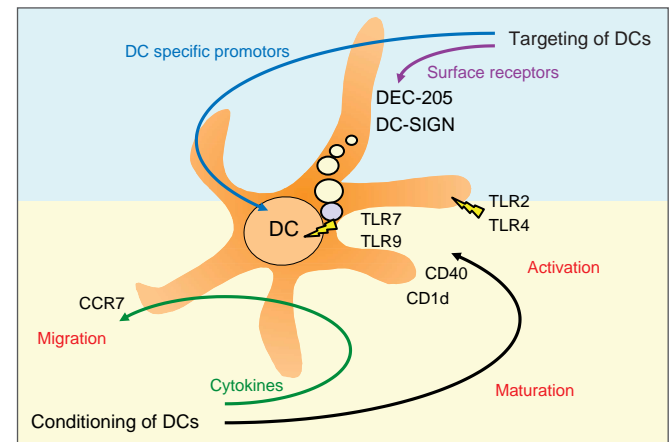
Recent preclinical studies in mice showed that the number of DCs migrating from skin to draining lymph nodes is 100-fold higher than previously estimated, and that these DCs persist for two weeks¹³. These findings, as well as the observation that a DC can interact with 5,000 T cells per hour^{14,15}, may explain how rare antigen-specific T cells can interact with DCs, and how results from preclinical studies can be used in designing more effective DC vaccines.

Direct injection of DCs into lymph nodes, or intralymphatic injection, circumvents the skin migration problem^{10,16}. Although intranodal injection can destroy the architecture of the first node, migration to subsequent nodes follows the physiological pathway through lymph vessels. The efficacy of intranodal compared with intradermal administration remains to be explored in much more detail. Recent preclinical findings indicate that it may be beneficial to combine different routes of administration: depending on the localization of the tumor, intravenous or intradermal vaccination may be preferential for visceral and nonvisceral metastases, respectively. This may relate to the interactions between DCs and T cells in different lymphoid organs, and the subsequent homing patterns of the activated T cells¹⁷.

Ex vivo- compared with in vivo-generated DCs

Given the labor intensity required to generate DCs for each patient, it would be advantageous to be able to directly isolate antigen-loaded DCs from the blood without *in vitro* culture, or to activate and target DCs *in vivo*. FLT-3 ligand is a potential candidate for expanding human DCs *in vivo* without activating them^{18,19}.

Figure 2 DC immunotherapy: exploiting molecular mechanisms. Conditioning of DCs by cytokines, CD40 ligand, TLR ligands or α -galactosylceramide, which binds to CD1d, should be explored to optimize DC maturation and activation, and to improve the migratory capacity of DCs *in vivo*. In addition, *in vivo* targeting of tumor-specific antigens to DCs, by exploiting either DC-specific surface receptors or promoters of DC-specific genes, may eventually replace vaccination with *ex vivo*-generated, antigen-loaded DCs.



In humans, several types of DCs can be distinguished in the blood²⁰. Both myeloid and the lymphoid related plasmacytoid DCs have been isolated from blood, and antitumor responses using these DC subtypes have been reported in animal models^{21,22}. Although blood DCs may not require extensive culture, they must be activated through molecularly defined triggers of DC activation, such as TLRs or CD40 ligand, before reinfusion²³, particularly because nonactivated or improperly activated DCs can cause T-cell tolerance rather than productive T-cell immunity²⁴. The small numbers of blood DCs that can be obtained from peripheral blood may be sufficient for vaccination, but a brief culture period will likely always be required to activate the cells.

Full activation of DCs

Current studies of DC cancer immunotherapy have focused on induction and expansion of tumoricidal CD8⁺ cytotoxic T cells. Early mouse studies showed that in the presence of a genetically defective CD4⁺ T-helper cell response, the CD8⁺ cytotoxic T-cell response could be restored by vaccination with *ex vivo*-activated DCs. It is now known that activated CD4⁺ T-helper cells upregulate CD40 ligand, and that signaling through the CD40 receptor activates DCs²⁵.

Recent studies in mice indicate that DCs also mature through interaction with natural killer T cells. This process is dependent on natural killer T-cell activation with the synthetic glycolipid antigen α -galactosylceramide, presented by CD1d molecules on the DCs²⁶. Microbial ligands, particularly TLR ligands, provide a third pathway to induce cytotoxic T-cell responses. Upon interaction with TLRs intracellularly or at the DC surface (Fig. 2), these ligands exert powerful DC activation signals equivalent to the action of CD40 agonists, and even operate in the absence of CD4⁺ T-cell help²⁷. The immunostimulant keyhole limpet hemocyanin is frequently used in many of the current DC vaccination trials to stimulate CD4⁺ T-cell help. Because optimal results are achieved if CD4⁺ T-cell help is tumor specific²⁵, DCs loaded with tumor-derived MHC class I- and class II-binding peptides have been explored in several vaccination trials²⁸.



Apart from their signaling to DCs, CD4⁺ T-helper cells in their own right exert antitumor effects through cytokine secretion²⁵. Based on early studies in which interleukin-2 may have bypassed the need for T-cell help²⁹, low-dose infusion of interleukin-2 or other cytokines is now being combined with DC vaccination in some instances (Supplementary Table 4 online).

Targeting DCs *in vivo*

Antigen loading and activation of the patient's own DCs *in vivo* would be an ideal scenario. Activation of DCs is essential to induce immunity, as DCs induce tolerance in the absence of acute inflammation and infection³⁰. It may thus be efficacious to target a combination of antigens and activators to DCs. One way to direct activation is by specific targeting of TLR ligands to DCs through DC-specific molecules.

Recently, several DC-associated C-type lectin-like receptors (DEC-205, DC-SIGN) have been shown to rapidly bind and endocytose material. Antigens loaded through these receptors are efficiently channeled into endocytic compartments for loading of MHC class II molecules and stimulation of CD4 T-cell proliferation³¹. Notably, when antigen is complexed to DEC-205, this leads not only to CD4⁺, but also to CD8⁺ T-cell responses³⁰, indicating that cross-presentation mechanisms may guarantee an effective immune response when antigen is recruited to DCs through these receptors.

Using DC immunotherapy to induce tolerance

An increasing number of preclinical studies are focusing on the capacity of immature DCs to induce antigen-specific nonresponsiveness, or tolerance, after antigen capture. DCs in the steady state are immature and can silence immunity in an antigen-specific manner, either by deleting T cells or by expanding regulatory T cells²⁴. Immature DCs incubated with agents such as dexamethasone³², vitamin D³³ and the Rel-B inhibitor Bay 11-7082³⁴ induce peripheral tolerance.

While most clinical DC vaccination studies thus far have been aimed at stimulating immune responses, the finding that immature DCs silence immune responses suggests a potential role for clinical DC applications in management of transplantation, allergy, autoimmunity and chronic inflammatory diseases.

Lessons learned from early patient studies

DC vaccines are safe, with minimal side effects^{7,8,16,28,35–38}. However, several of the initial vaccines used immature rather than mature DCs, which may have affected the immunological and clinical outcome. Comparative studies with immature or mature DCs have shown that only mature DCs stimulate T-cell responses^{16,37,39} and enhance homing to draining lymph nodes, the sites where therapeutic T-cell responses must be initiated¹⁰. The majority of studies investigated the therapeutic effects of DC vaccines in late-stage cancer patients with metastasis. Clinical results were variable, and notably long-lasting clinical responses were observed in several studies. In several patients, clinical responses coincided with a specific cytotoxic T-cell response against the antigens present in the DC vaccine, rather than a nonspecific activation of the immune system (through natural killer T-cell activation or cytokines, for example).

The above observations support a promising role for DC vaccination. Variables that require standardization include the mode of DC preparation, the subtype, maturation and activation status of DCs, the dose and timing interval, the route of administration and the mode of antigen loading (Supplementary Tables 2–6 online). The full potential of these immunostimulatory cells has not yet been exploited, which excludes the introduction of this type of therapy in meaningful phase 3 trials.

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

Several of the early studies published were inadequate in their design and interpretation. Indeed, quality control of the DC vaccines and information on phenotypic differences between DCs of individual patients has been largely lacking in many studies. Rather, the miraculous cure of one patient is highlighted without proper discussion of the potential reasons for treatment failure in other patients. In addition, some early studies used fetal calf serum for DC culture^{40,41}, which we now agree should be replaced by human serum or, preferably, no serum at all⁴² to ensure standardized conditions and no contamination with undefined proteins. Recent negative publicity in newspapers and scientific journals⁴³ has, unfortunately, not promoted the potential beneficial use of DC immunotherapy.

In most DC trials, clinical responses are not reported using specific, well-defined and generally accepted criteria for the codification of tumor responses. These criteria are well described by the commonly used World Health Organization and response criteria in solid tumors (RECIST)⁴⁴ response evaluation schemes in which tumor responses

are measured objectively. Most studies focus on clinical responses without monitoring the relevant immune responses, and thus provide only anecdotal information. Combining clinical and immunological data can result in improved use and ability to monitor the benefits of DC vaccination. When comparing DC vaccination with other forms of therapy, the nature of the DC vaccine requires definition.

DC vaccines and clinical studies must be standardized

In Box 1 we suggest a series of clinical and immunological criteria that should be met when designing clinical studies with DCs (see refs.

8,28,45 for examples). Studies investigating the effects of DC vaccines should include information on the DC maturation and activation status, as well as quality control. In Box 2 we list several phenotypic and functional criteria for immature and mature DCs. Standardization of DC vaccines will allow observations from different clinical studies to be compared⁴⁶. Most important is a precise description of the preparation of the vaccine, the viability of the cells just before administration and the phenotypic analysis (purity and maturity) of each individual DC vaccine preparation. This is the only way for reproducible quality of the vaccine to be guaranteed. These create the basis for DC vaccine release

criteria (Box 2). Additional criteria can be defined that may help validate the vaccine preparation process (Box 2).

Large, randomized clinical studies in which DC therapy is compared with current standard treatments will ultimately be needed to assess clinical efficacy of DC vaccines. Protocols to generate DCs must be simplified to enable preparation of DC vaccines as a blood transfusion product.

The experts at the June 2003 Amsterdam meeting concluded that further preclinical animal studies and small pilot studies in patients must be rigorously performed to determine the standardized treatment for larger patient groups, and that, for reasons of comparison, only one variable at a time should be changed in new protocols. Our relative lack of information (Fig. 1) prevents us from designing large, two-armed studies or including patients with early-stage disease. The collaboration between academia and biotech companies will promote future development of DC immunotherapy.

Mapping the way

Antigen-loaded, *ex vivo* monocyte- or CD34⁺ cell-derived DCs are the current gold standard in DC immunotherapy. Although many variables need defining, we believe the following developments will be implemented shortly:

1. *Ex vivo* culture will be standardized. DCs will be cultured in defined media with cytokine cocktails, DC activators (such as TLR ligands or CD40 ligand), or both. Closed culture systems will preferably be used, which will simplify handling and thus limit the use of expensive and labor-intensive Good Manufacturing Practice (GMP) facilities. Similarly, product endpoints, purity, viability after thawing, and phenotype (surface antigens, cytokine expression and migratory capacity) will be defined (Box 2).

2. Because monovalent specificity against a single antigenic peptide is unlikely to act in patients with a large tumor burden, and in view of the tumor variants that might arise, vaccination against multiple tumor antigens as well as tumor-associated antigens expressed by stromal or endothelial cells in the tumor will be explored. Suitable targets

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.

Release criteria:

Microbiological controls Negative for bacterial and fungal contamination

Purity: >80% as determined by flow cytometry, light scatter or staining with non-DC lineage markers.

Morphology: Immature: loosely adherent, floating, roundish cells with some extensions

Mature: loosely attached, veiled and clustered cells

Phenotype: Monocyte-derived:
Immature: CD14^{-lo}CD83⁻CD80^{-lo}CD86^{lo}MHC class I⁺MHC class II⁺CCR5⁺
Mature: CD83⁺CD80⁺CD86⁺MHC class I⁺MHC class II⁺CCR7⁺

CD34⁺ cell-derived:
Interstitial: CD14⁺CD1a^{+/-}CD83⁺CD80⁺CD86⁺MHC class I⁺MHC class II⁺

Langerhans cells: CD14⁻CD1a⁺CD83⁺CD80⁺CD86⁺MHC class I⁺MHC class II⁺

Viability: >70% as determined by Trypan blue exclusion

Optional validation criteria:

Stability of DC phenotype Determined after one and two days of subsequent culture in medium either without or with cytokines
'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)

Induction of immune response: Mixed lymphocyte reaction: T-cell proliferation at DC/PBMC (peripheral blood mononuclear cell) ratio of 1:20 in at least one donor
Recognition of loaded antigen by T cells, as determined by cytotoxicity assay or cytokine production (especially important when antigen is loaded before freezing)

Antigen-loaded state (Only possible when DCs are loaded with well-defined antigens, such as peptides, proteins or RNA)
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)



may include antigens that the tumor cannot afford to downregulate, particularly those directly implicated in malignant behavior, to minimize the chances of immune escape. For efficacy of tumor-reactive T cells, quality is as important as quantity. Flooding the system with relatively ineffective, low-affinity T cells is unlikely to generate a sustained antitumor response.

3. Immature DCs have tolerogenic potential because they induce regulatory T cells and inhibit immune responses⁴⁷. It is therefore important to remove or prevent the outgrowth of regulatory T cells that might inhibit immune responses in cancer patients. Clinical studies exploiting antibodies to CD25, to remove CD25⁺ regulatory T cells before DC vaccination, have just begun. In similar strategies, antibodies to CTLA-4 have been shown to block regulatory T cells⁴⁸.

4. Other DC subtypes, in particular plasmacytoid DCs, should be considered for therapeutic use. Distinct DC subsets may express different adhesion and homing receptors that directly affect their migratory behavior. Plasmacytoid DCs, for instance, not only prime antigen-specific T cells but also infiltrate tumor tissues²². Their capacity to secrete large amounts of interferon type I may induce maturation of local myeloid DCs, facilitating cross-priming of endocytosed targets²².

Conclusions and future prospects

DC immunotherapy has been introduced in the clinic, and has proven to be feasible, nontoxic^{7,8,16,28,35–38} and effective in some patients, particularly if the DCs have been appropriately matured and activated. Several newly recognized DC functions pertinent to vaccine design are also emerging. *In vivo* activation and targeting of DCs, as well as exploitation of DCs to silence immune responses, will expand the application of DCs to a wide variety of immune-mediated diseases.

The challenge facing scientists and clinicians is to prove efficacy in well-controlled clinical studies and extend the use of DC immunotherapy for treatment of patients with low tumor burden. Standardization of DC vaccines needs to be the focus of ongoing research. Proving efficacy in early stage cancer in which DC-mediated immunotherapy will be most effective will require large, expensive and long-term followup studies. Collaborative efforts between biotech companies, blood banks and academic institutions will enhance the development of DC vaccine immunotherapy. Optimization of this strategy may lead to a therapy of incontrovertible efficacy, mapping the way toward a promising, nontoxic alternative to existing treatments.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests. Published online at <http://www.nature.com/naturemedicine/>

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