Review Article

Dendritic cell therapy in melanoma

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Abstract: Dendritic cell (DC) vaccines are cancer vaccines used currently as melanoma therapies. They act as adjuvants initiating the immune responses, but not only as they can also have effector activities redirecting cytotoxic CD8$^+$ T cells against melanoma. Ex vivo preparation of monocyte derived DCs has been implemented to produce large numbers of DCs for clinical therapy, highlighting the necessity of activate DC s to produce Th1 cytokines, especially TNF-a and IL-12 with potent anti-tumour actions. Several clinical trials both in the European Union and USA are open currently using DC vaccines, alone or in combination with other immunotherapies. The type of antigen is also an active area of investigation involving tumour antigens and bacterial epitopes, both providing good responses. Bacterial epitopes presented the advantage versus tumour antigens that they can prepare the vaccination site as they induce innate and specific immune responses as they are potent recall antigens that expand cytotoxic responses.

Keywords: Dendritic cells (DCs); melanoma; Listeria; vaccines

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Introduction

Dendritic cells (DCs) are central cells of the immune system at the interphase of innate and specific immunity, the latter also known as adaptive immunity. Therefore, they stimulate naive T cells to initiate the immune responses, but also, they interact with and influence the responses of other innate immune cells, such as macrophages or natural killer cells (NK). To initiate the immune responses, DC cells encounter and degrade antigens to small molecules called epitopes. Next, these epitopes bind to either class I major histocompatibility molecules (MHC-I) or class II MHC (MHC-II) to present them to CD8$^+$ T cells (MHC-I) or to CD4$^+$ T cells (MHC-II) by interaction with the T cell receptor. This whole process of antigen processing and presentation gives a first signal to T cells (signal 1). This antigen specific signal 1 in T cells is not sufficient for T cell activation. T cell activation requires that CD28 molecules of T cells, recognizes the co-stimulatory molecules CD80 (B-7.1) or CD86 (B-7.2) in DC (signal 2). Signals 1 and 2 induce the production of IL-2 by T cells and as a feedback mechanism, the expression in these cells of two molecules, the α chain of IL-2 receptor (CD25) and CD40L, the ligand of CD40. The system CD40/CD40L amplifies antigen presentation and signal 2. The binding of IL-2 to its receptor (CD25/CD122) activates mTOR, the target for rapamycin (signal 3) that results in T cell clonal proliferation and generation of effector cells such as cytotoxic T cells (1). There are two negative regulators of this overall process in T cells: (I) CTLA-4 in T cells that, if present, binds to CD80 or CD86 and stops the activation of T cells, avoiding over-activation of the immune system and (II) the cell death program antigen 1 or PD-1 also in T cells that, if present, binds to their ligands in DC, PD-L1 or PD-L2 molecules, blocking the system (*Figure 1*).

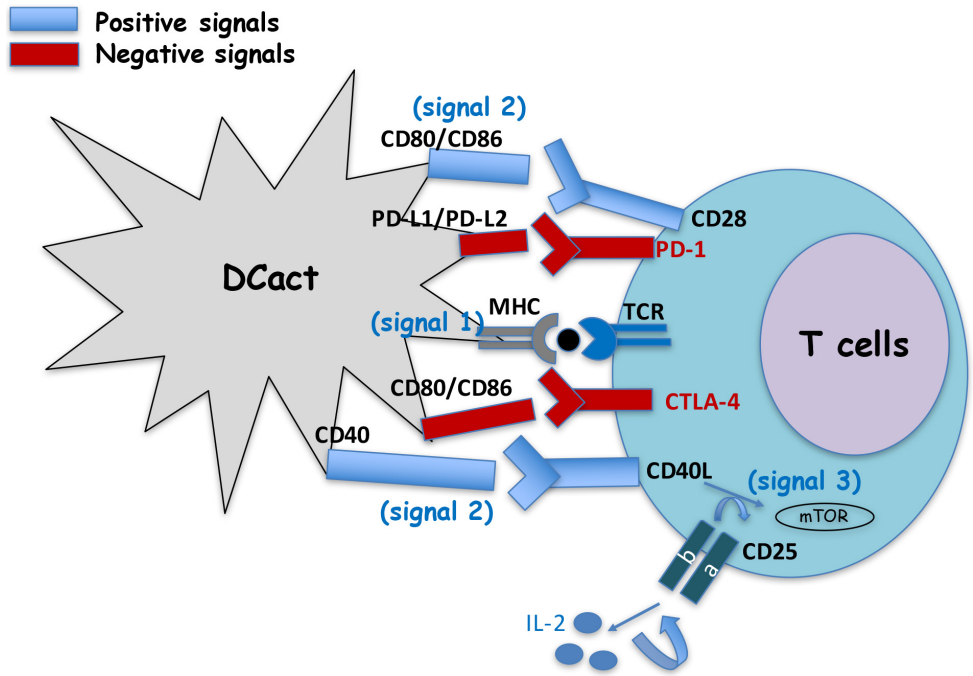

Figure 1 Activation of T cells by dendritic cells. The figure describes the three signals required to activate T cells and the molecules involved (positive signals), as well as the negative signals downregulating the system.

The function of DC *in vivo* is the surveillance of the organism to phagocyte and destroy intruders such as pathogens or tumour cells. They can act in two processes of the immune responses, in the priming phase as well as in the effector phase. In the priming phase, DC recognize molecular patterns on pathogens or in tumour cells using different Toll-like receptors (TLR) that activates signalling pathways that remodel cell surface molecules and produce different types of cytokines. Cell surface molecules suffering deep remodelling are those involved in antigen presentation, MHC-I, MHC-II molecules and co-stimulatory molecules, CD40, CD80 and CD86. In this regard, mature DC of any source, either plasmacytoid DC or monocyte derived DC (MoDC), show significant expression of MHC-I or MHC-II molecules but low or absent expression of co-stimulatory molecules. While activated MoDC present high expression of molecules involved in antigen presentation, MHC-I, MHC-II and high expression of all or some co-stimulatory molecules, CD40, CD80 or CD86. Also mature or activated MoDC produce different types of cytokines. The cytokines produced by DC depend on the signals they receive from the environment, being these cytokines classified as Th1 cytokines/chemokines with a pro-inflammatory pattern, TNF-α, MCP-1, IL-1β, IFN-α/β and IL-12p40/IL-12p70 or Th2 cytokines with predominant anti-inflammatory profiles as IL-6, IL-10 or TGF-β (*Figure 2*). However, the MoDC cytokine profile in each situation varies and the levels of each one of these Th1 or Th2 patterns determine immune-stimulation or immune-suppression profile, being in general Th1 profiles of MoDC considered immune stimulatory and Th2 profiles, immune suppressor. Mature MoDC in general, produce a mixture of Th1 and Th2 cytokines, while activated MoDC produced almost exclusively Th1 cytokines.

Next, MoDC can also act in the effector phase of the immune response, either $CD4^+$ or $CD8^+$ T cells, that are usually IFN-γ producers. The type of predominant effector cell would depend on the pathogen or tumour cell. In general, pathogens activate both $CD4^+$ and $CD8^+$ effector T cells that destroy the pathogens either by induction of neutralizing antibodies or activating cytotoxic T cells that lysed those cells infected with the pathogens. In the case of tumour cells, $CD8^+$ T cells are mainly stimulated by DC as well as NK cells in order they kill directly the tumour cells (2).

Vaccines

Cancer vaccines are expected to activate the immune system

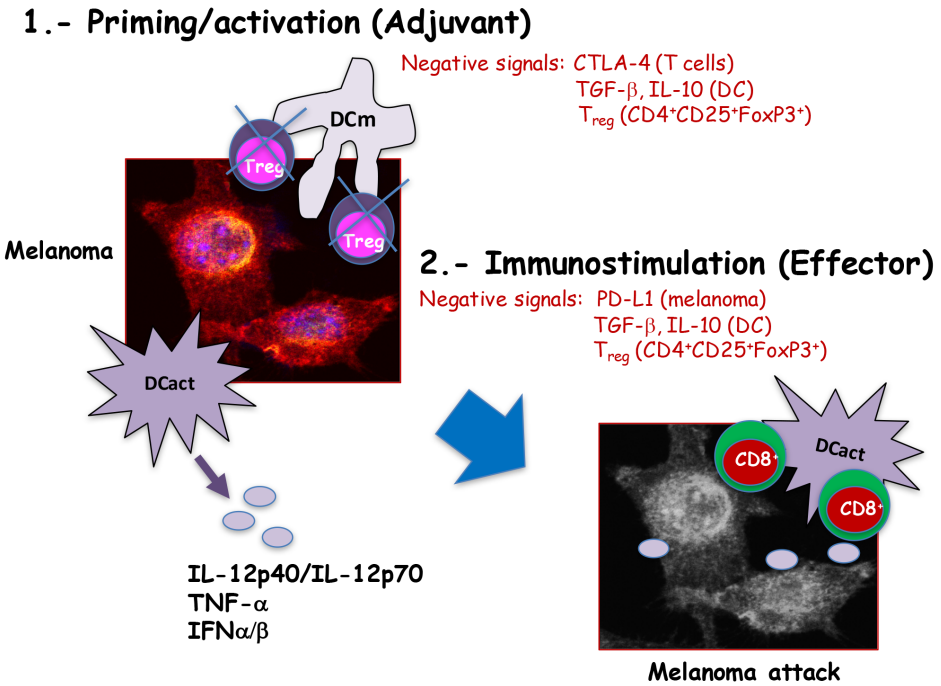

Figure 2 Preparation of monocyte derived dendritic cells (MoDC) for therapies and differentiation between mature and activated dendritic cells: markers and cytokines. The figure describes briefly the procedure to produce MoDC and the markers and cytokines that differentiate mature and activated MoDC or DC, in general.

as adjuvants and deliver antigens to T cells. Cancer vaccines function different than prophylactic vaccines as they are expected to expand only cytotoxic $CD8^+$ T cells to cause tumour regression. It is suitable that cancer vaccines also decrease the number or activating responses of tumour immunosuppressor signals of T regulatory (T_{reg}) cells or myeloid derived suppressor cells (MDSC). The different types of cancer vaccines approved by FDA are autologous, peptide, dendritic or vector-based vaccines.

Autologous vaccines are composed of tumour cells extracted from each patient. They may contain tumour-associated antigens (TAA) of each tumour but whole cells are not different than normal cells and therefore, the response they generate might not be specific enough to cause impact in the tumour size. For this reason, autologous vaccines are modified to improve their immunogenicity.

A variation of autologous vaccines is allogenic whole tumour cell vaccines expressing GM-CSF and irradiated (GVAX) (1). This approach uses a tumour cell line that expresses a plurality of shared tumour antigens such as tyrosinase, gp100, MART-1/melan-A, and MAGE-A3 and genetically modified to secrete GM-CSF, a potent adjuvant that induces DC maturation. Peptide vaccines target the above-mentioned tumour antigens but identifying those peptides unique to cancer cells. While generating specific immune responses, they do not implement survival.

Vector-based vaccines, either bacterial or viruses induce specific as well as substantial immune responses that affect clinical results. They are based in the assumption that the primary function of the immune system is to protect against foreign pathogens. However, they present several disadvantages, as the immune responses against the vector are restricted and they cause pathogenesis or be mutagenized. In this regard, attenuated mutants of *Listeria monocytogenes* either deficient in two virulence factors ActA and Internalin B, known as LADD mutants (Aduro Biotech) (3,4), or deficient in a partial sequence of the main virulence factor listeriolysin O (LLO) (Advaxis) (5), are constructed to express different tumour antigens, such as prostate PSV or mesothelin (CRS-207) alone or in combination with GVAX (6), supposed a great expectation as these bacteria induced not only strong innate immune responses but also cytotoxic immune responses. However, recently CRS-207 in combination with GVAX failed in trials against pancreatic cancer, getting patients worse and develop symptoms associated with the bacterial pathogen.

Dendritic vaccines are potent antigen-presenting cells with high capacity to induce T cell immune responses through eliciting pro-inflammatory cytokine responses and stimulating cytotoxic T cells. However, tumours are immunosuppressive environments and might damp the effectiveness of DC. DC are in very low frequencies in the peripheral blood and tissues and therefore, they should be expanded either *in vivo* or *ex vivo* in order to use them as therapies. The first developing approach using DC vaccines consisted on infusing immature DC that collected antigens *in vivo*. Next, MoDC were harvested from patients and generated at large scale to yield sufficient numbers of cells for clinical application. Next, MoDC were pulsed *ex vivo* with TAA, tumour lysates, whole tumour cells, tumour RNA or apoptotic tumour cells or bacteria derived reagents to induce MoDC maturation. Mature MoDC are re-infused back in patients with the purpose to induce tumour-specific immune responses.

Current *ex vivo* expansion of MoDC is performed after incubation with GM-CSF and IL-4 to induce full maturation of DC. The procedure implies first Ficoll isolation of leukocytes, $CD45^+$ cells. Second, monocytes are selected as $CD14^+$ positive cells in MACS columns (MACS^{TM}, Myltenyi) to provide large numbers of mature MoDC. Mature MoDC are next activated with different agents such as bacteria as *Mycobacterium* BCG strain (BCG) or *Listeria monocytogenes (Listeria)* that signal through TLR-2 or TLR-4 or bacterial oligonucleotides (ODN) with CpG motifs (*Figure 2*). Trials with MoDC have used indistinctively either mature or activated DC, but they produce different cytokines that can generate different immune responses. Therefore, it is important DC vaccines produce Th1 cytokines with tumorigenic abilities, such as TNF-α or IL-12p40/IL-12p70, otherwise they would not induce cytotoxic immune responses and be ineffective as anti-tumour therapies. In this regard, *ex vivo* mature MoDC obtained from patients with malignancies produced predominantly Th2 phenotypes (7). However, if they are activated with adjuvants such as *Listeria* peptides in nanoparticle platforms, MoDC of these patients shifted their Th2 immune responses towards Th1 profiles (8).

DC can also be activated *in vivo* requiring the use of non-specific adjuvants such as GM-CSF, CpG, stimulating cytokines as IFN-α/β, STING-activating cyclic dinucleotides (9,10), bacteria as BCG or *Listeria* or bacterial products. In this regard, IFN-α is a common adjuvant therapy applied in melanoma patients after surgery to prevent or delay tumour relapses (11-13). Bacterial toxins can also present anti-tumour activity such as LLO, a pore-forming cytotoxin of *Listeria* and high capacity to expand non-specific immune responses, binds to TLR-2 costimulatory molecules as CD14 and act as potent adjuvant (14).

DC vaccines in melanoma therapy

DC vaccines have been used to activate the immune system in autologous and allogenic vaccination strategies using peptides or tumour lysates (15-18). However, the response rates using DC vaccines have been usually low ranging from 10% to 20%, although they are the only immunotherapy so far that increases patient survival rates. DC vaccines can stimulate the immune system to respond to one specific antigen or carbohydrate moiety or to elicit an immune response against multiple tumour antigens, incorporating either allogenic whole cells, autologous tumour cells, recombinant proteins or tumour lysates. Moreover, DC vaccines are safe immunotherapies compared with other adjuvants as IFN-α, or immune check point inhibitors that cause immunological adverse events and have low number of responders.

In general, the success of DC vaccines against melanoma is explained by their participation in two stages of the induction of immune responses (*Figure 3*). First, as adjuvants they act in the priming stage of the immune responses, decreasing the activation of T_{reg} cells localized in the tumour environment. Moreover, as mentioned before, activated DC vaccines produce large amounts of TNF-α, IFN-α/β and of IL-12p40/IL-12p70 cytokines with anti-neoplastic abilities. These pro-inflammatory cytokines act as feedback mechanisms, activating cytotoxic $CD8^+$ T cells. Second, as immune stimulators in the effector phase of the immune responses, activated DC interacted efficiently with cytotoxic $CD8^+$ T cells and expanded melanoma specific $CD8^+$ T cells. Finally, melanoma specific $CD8^+$ T cells can attack melanoma and benefit tumour regression.

Since the approval in 2010 of the first DC vaccine for prostate cancer, sipuleucel-T (Provenge), this early design has improved currently (19) and DC vaccines have raised a huge focus in melanoma treatment. In this regard, trials for human leukocyte antigen (HLA)-$A2^+$ advanced melanomas have been conducted (20,21), presenting significant clinical responses, albeit the number of patients benefiting of these approaches were small. DC vaccines embedded in matrices with thrombin and fibrinogen were used to increase host resistance of patients with melanoma, because they activated

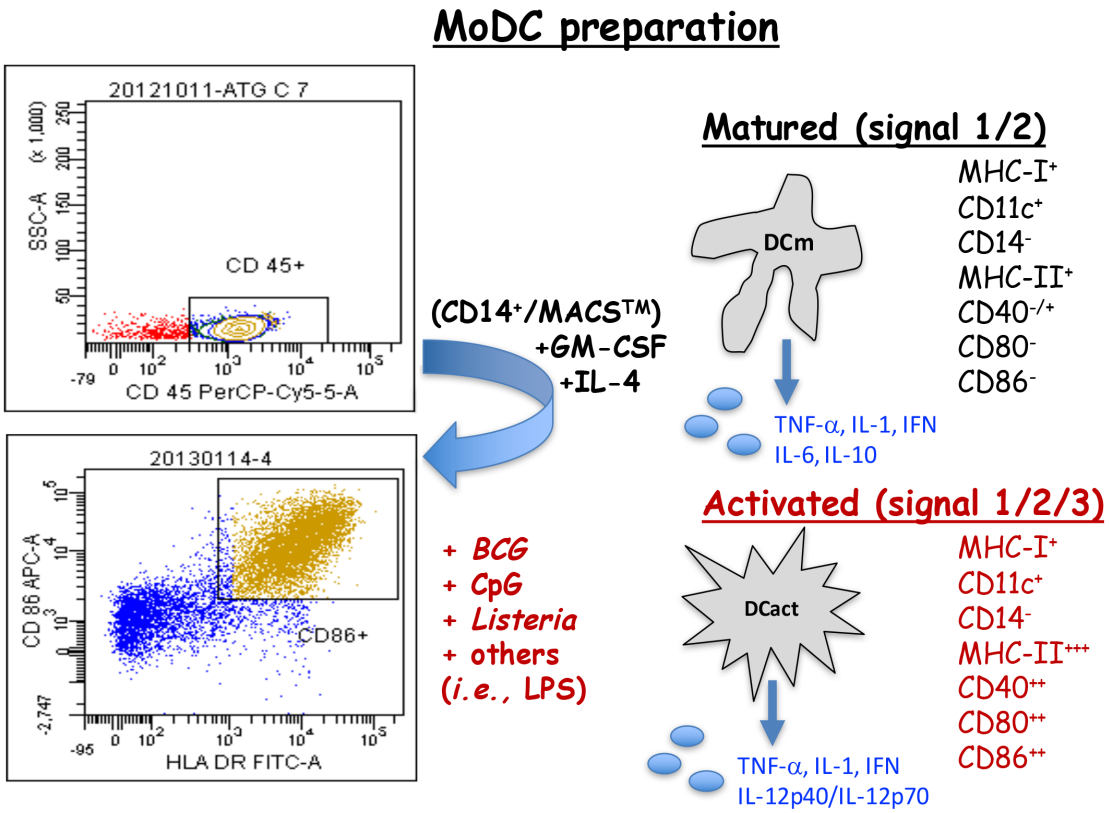

Figure 3 Sites of action of dendritic cell vaccines against melanoma. The figure shows real melanoma cells and the two sites of action of dendritic cell vaccines in the activation of the immune response, at the priming stage also known as adjuvant effect and at the effector stage also called immunostimulation. The figure also shows the negative signals involved in both stages.

DC and induced high production of IFN-γ. This approach was also used in experimental models of melanoma and observed great reduction of tumour responses. In fact, mice presented 65% of tumour remission and developed effective immune responses. These DC matrices were valid approaches that interacted with immune cells, including lymphocytes (22). One of the first trials using MoDC pulsed with GM-CSF and IL-4, evaluated sixteen patients of melanoma treated with autologous MoDC pulsed with a cocktail of specific MART-1, MAGE-1, MAGE-3, gp100 or tyrosinase peptides to fit into especial class I HLA molecules of the patients. This trial also included KLH as an antigen that induced non-specific $CD4^+$ T cells to interact with $CD8^+$ T cells and DC vaccination was in lymph nodes non-involved in the tumour. Five out of sixteen patients presented tumour regression and two completed responses that lasted over fifteen months (15).

Later on, similar approaches were used with a similar peptide cocktail of melanoma antigens, MART-1, Melan-A, gp100 or tyrosinase to load MoDC in cancer vaccines in phase I and phase II trials (20,21). In fact, peptides, $tyrosinase_{386-406}$, MART-1/$Melan\text{-}A_{51-73}$ or $gp100_{44-59}$ showed high induction of immune responses. Twenty-three patients out of thirty-seven showed antibody and T cell responses, two patients antibody response only and five showed T cell response only.

Electroporation of DC with mRNA encoding different activating markers such as CD40L, CD70 and a constitutive active form of TLR-4, referred as TriMix (23) constituted a potent DC-based cellular therapy.

Other approaches to activate DC have been the use of whole bacteria such as attenuated *Mycobacterium BCG*, *Salmonella* or *Listeria monocytogenes* as non-specific adjuvants that efficiently activate DC (3). In fact, their use is based in the first reports of immunotherapy by William Coley that presented the utility of bacteria and bacterial products in the treatment of cancer using *Staphylococcus pyogenes* to treat his patients. Nowadays,

the use of attenuated *Mycobacterium* strains as BCG is a frontline treatment in several melanoma trials (24,25) as well as other tumours as bladder cancer. However, the action of BCG implies non-specific immunostimulation of the immune responses and not direct killing of cancer cells. A different action supposes the use of *Listeria* against melanoma. This rare human bacterial pathogen induced apoptosis of melanoma cells, even at very low doses and expands $CD8^+$ T cell cytotoxic responses, benefiting also the expansion of melanoma specific cytotoxic responses (26). Moreover, DC loaded with $LLO_{91–99}$ peptides can act as recall antigens that pre-condition the vaccination sites and induced anti-tumour immune responses (27). This effect appeared similar to the action reported by the potent recall antigen and bacterial toxin, tetanus toxoid loaded into DC vaccines, that induced effect responses against glioblastoma (28).

Another highly active area is the use of biomaterials or nanoparticles to improve DC and T cell function at various tissues for the adjuvant treatment of melanoma (29-32). The use of biomaterials and nanomaterials implies solving several issues with adjuvants as the short half-lives of antigens plus adjuvants or the loss of viability upon *in vivo* transplantation of activated cell products. Therefore, biomaterials are used to extend the duration of the immune responses as well as controlling antigen localization in tissues or targeting to DC cells. The use of scaffolds of macroporous poly(lactide-co-glycolide) (PLG) matrices can regulate the trafficking and activation of DC in the tissues (33). These matrices can be implanted *in vivo* and potentiate the immune responses. They are fabricated with GM-CSF, a CpG ODN and tumour lysate. When they were implanted in experimental mice, they recruited DC in a melanoma model and increase the cytotoxic responses, causing melanoma regression (34). Other types of materials are gold glyconanoparticles that bear carbohydrates in their structures and therefore are targeted to DC and other antigen presenting cells as macrophages (29,35).

DC loaded with peptides of bacterial toxins able to elicit anti-tumour immune responses as 91–99 LLO peptide, are able to act as efficient adjuvants and induce strong innate and cytotoxic responses, expanding melanoma specific cytotoxic responses and preventing melanoma growth, dissemination and formation of metastasis (27). $LLO_{91–99}$ peptide loaded into DC is able to induce effector $CD8^+$ T cells that localize in the tumour, inducing apoptosis of melanoma. These DC vaccines also decrease T_{reg} in the tumours and induce NK cells, therefore, promoting anti-melanoma immune responses, but also increase the positive signals between DC and T cells and control tumour growth and dissemination.

Trials with DC vaccines for melanoma

One of the great advantages of using DC vaccines for melanoma therapy is their safety and potent ability to combine with other immunotherapies as immunological check point inhibitors, either in patients or in experimental models (36-40).

The current trials with DC vaccines for melanoma have been approved by FDA in USA as well as by the European agency of the medicaments and they are compiled in *Table 1*. Some of these trials involved DC vaccines and other strategies as tumour infiltrating lymphocytes (trial 2006-005238-19 in Spain) or uses electroporated DC with mRNA (trial 2011-001410-33 in Belgium) or combination with other adjuvants as pegylated IFN-α (trial 2005-002636-97 in Great Britain) or the use of the TriMix DC formula combined with ipilimumab (trial 2010-023058-35 in Belgium). Some the trials are for advanced melanoma, others for early stages melanoma IIA, IIB, IIIB, IIIC and others for uveal melanoma. In all cases, DC vaccines are prepared *ex vivo* using mature MoDC. A large amount of trials haw requirements of HLA-A2 expression as well as other melanoma specific antigens such as gp100 or tyrosinase (*Table 1*).

Conclusions

DC cell vaccines because of their safety and dual action into the two arms of the immune responses, the priming and effector stages are versatile immunotherapies that can be used to stimulate the immune system to fight against melanoma, both early stages and metastatic melanoma and even uveal melanoma. They also accept combination with all types of immunotherapies currently been used for melanoma, immunological check point inhibitors as ipilimumab or adjuvant therapies as pegylated IFN-a. Their high induction of TH1 cytokines as TNF-a and IL-12p40/IL-12p70 and activation of anti-melanoma cytotoxic $CD8^+$ T cells are their best features to be used as adjuvants, with specific antigens or with polyvalent vaccines. In this regard, bacterial epitopes loaded into DC as $LLO_{91–99}$ peptides are also emerging as potent anti-melanoma treatments.

Table 1 Trials in European Union and United States of America involving dendritic cells

Trials with dendritic cells (number, sponsor, country code and ID)	Title	Starting date	Medical condition
2007-007847-28 (Universitätsklinikum Erlangen-DE) (DERMA-ER-DC08)	A multicenter, randomized, two-armed, open-label phase III study to evaluate the adjuvant vaccination with tumor RNA-loaded autologous dendritic cells versus observation of patients with resected mel…..	2011-09-16	Uveal melanoma
2015-005322-19 (Radboud University Nijmegen Medical Centre-NL) (MIND-DC)	A randomized, double-blind, placebo-controlled phase III study to evaluate active immunization in adjuvant therapy of patients with stage IIIB and IIIC melanoma with natural dendritic cells pulsed...	2016-01-21	Melanoma with regional lymph nodes metastases (stage III)
2012-001410-41 (Istituto Scientifico Romagnolo per lo studio e la cura dei tumori-IT) (IRST172.02)	Vaccination with autologous dendritic cells loaded with autologous tumor lysate or homogenate combined with immunomodulating radiotherapy and/or preleukapheresis IFN-alfa in patients with metastati...	2013-05-30	Metastatic melanoma
2009-015737-73 (Radboud University Nijmegen Medical Centre-NL) (2009-015737-73)	Single-step antigen loading and TLR activation of dendritic cells by mRNA electroporation for vaccination in stage III and IV melanoma patients	2010-02-04	Melanoma patients expressing gp100 and tyrosinase
2008-001973-14 (Radboud University Nijmegen Medical Centre-NL) (KUN2006-3699)	TLR ligand matured dendritic cell vaccination in melanoma patients: the key towards a more potent immune induction?	2009-04-27	HLA-A2.1 positive melanoma patients expressing gp100 and tyrosinase
2008-001974-33 (Radboud University Nijmegen Medical Centre-NL) (08/014)	mRNA-transfected dendritic cell vaccination in high risk uveal melanoma patients	2009-04-14	HLA-A2.1 positive patients at high risk of uveal melanoma, expressing gp100 and tyrosinase
2010-020228-23 (Radboud University Nijmegen Medical Centre-NL) (NL32381.000.10)	Immunochemotherapy: do platin-based chemotherapeutics enhance dendritic cell vaccine efficacy in melanoma patients?	2010-12-21	Melanoma patients expressing gp100 and tyrosinase
2010-023058-35 (UZ Brussel-BE) (UZB-VUB-10-001)	A two-stage phase II study of autologous TriMix-DC therapeutic vaccine in combination with ipilimumab in patients with previously treated unresectable stage III or IV melanoma	2011-01-04	Previously treated unresectable stage III or IV melanoma
2005-002636-97 (Immuno-Designed Molecules-GB) (DC-MEL-203)	An open-label, phase II study of matured dendritic cells pulsed ex vivo with 3 melanoma cell line lysates (IDD-3) with or without peginterferon alpha-2b (PegIFN-a 2b) in patients with resected sta...	2005-09-19	Patients with cutaneous melanoma (stage IIc, IIIb, IIIc)
2011-001410-33 (UZ Brussel-BE) (UZB-VUB-11-01)	Randomized phase II clinical trial on mRNA electroporated autologous dendritic cells for stage III/IV melanoma in patients who are free from measurable tumor lesions following the local treatment o...	2016-03-10	Stage III/IV melanoma free of measurable tumour lesions
2011-001474-25 (Klinik für Dermatologie, Venerologie und Allergologie; Charité-Universitätsmedizin Berlin-DE) (SVM9122)	A single-centre, single arm, open-label, exploratory trial of interleukin-2 administered subcutaneously as neo-adjuvant treatment prior to sentinel lymph node biopsy(SLNB)/ complete lymph node disse...	2012-01-02	Melanoma patients stage III
2006-005238-19 (Instituto Cientifico y Tecnologico de Navarra-ES)	Phase II study with immunotherapy with dendritic cells and tumour infiltrating lymphocytes in solid tumours	2007-07-26	Melanoma, renal carcinoma, hepatocarcinoma
NCT01753089 (Dana Farber Harvard Center-USA) (12-306, NCI-2013-01799, NCT01753089)	Dendritic cell activating scaffold in melanoma, immunotherapy (vaccine), dendritic cell (phase I)	2012-12-17	Melanoma with recurrent stage IIIC or with stage IV
NCT02334735 (Icahn School of Medicine at Mount Sinai-USA)	A comparison of matured dendritic cells and montanide® in study subjects with high risk of melanoma recurrence (phase II)	2015-01-06 (recruiting)	Melanoma stages IIB-IV

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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