REVIEW

Special Focus: Viral-immunotherapies and vaccines in melanoma

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Melanoma Management

Dendritic cell vaccines for melanoma: past, present and future

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Practice points

Past & present

During the past two decades, clinical investigation has established:

- Dendritic cell (DC) products can be reliably manufactured using peripheral blood mononuclear cells.
- DC products can be safely administered by a variety of routes, doses and schedules in patients with metastatic melanoma.
- Various DC products to a variety of tumor-associated antigens have induced and/or enhanced immune responses in most patients.
- Objective tumor regressions have been documented in association with DC–vaccine therapy, but infrequently.
- DC–vaccine therapy appears to be associated with enhanced long-term survival, but this needs to be confirmed with large randomized clinical trials with long follow-up.

Future perspective

While there are ways to improve on DC manufacturing, recent advances in tumor immunology and genomics suggest that DC therapy will be most improved by:

- Focusing on autologous tumor antigens, including antigens associated with each patient's cancer-initiating cells, rather than allogeneic cell lines or cocktails of melanoma antigens that are shared among many patients.
- Using combination therapy with checkpoint inhibitors that make the host's immune system more receptive to making and sustaining antitumor immune responses.

Administering dendritic cells (DC) loaded with tumor-associated antigens (TAA) *ex vivo* is a promising strategy for therapeutic vaccines in advanced melanoma. To date the induction of immune responses to specific TAA has been more impressive than clinical benefit because of TAA limitations, suboptimal DC and possibly immune-checkpoint inhibition. Various products, antigen-loading techniques, treatment schedules, routes of administration and adjunctive agents continue to be explored. Biologic heterogeneity suggests autologous tumor as the optimal TAA source to induce immune responses to the entire repertoire of unique patient-specific neoantigens. Many questions remain regarding the optimal preparation of DC and strategies for antigen loading. Effective DC vaccines should result in additive or synergistic effects when combined with checkpoint inhibitors.

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KEYWORDS

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Dendritic cells (DC) have been the focus of intense clinical investigation in cancer for the past 20 years. Injection or infusion of DC loaded with tumor-associated antigens (TAA) *ex vivo* has been embraced as a popular strategy for therapeutic vaccines in an effort to induce or enhance antitumor immune responses in cancer patients, especially those with metastatic melanoma. This review summarizes the history of DC investigation in cancer immunotherapy, the basics of DC cellular biology and physiologic function that are important for understanding various strategies used to manufacture DC products, the results of clinical trials in melanoma patients and recent changes in perspective that are driving current clinical trials with DC vaccines.

Historical milestones for DC in melanoma

In 1868, the German pathologist Paul Langerhans described cells in the skin, which for years have borne his name. In 1973, Ralph Steinman and Zanvil Cohn discovered related cells in the spleen and various lymphoid tissues of mice, and called them ‘dendritic cells’ because of their thin tree branch-like extensions (from the Greek word *dendron* meaning tree) [1]. In the fall of 2011, Steinman was awarded the Nobel Prize in Medicine for his body of work related to the function of DC and their critical role in initiating and enhancing immune responses.

In 1994, methods for differentiating DC from peripheral blood mononuclear cells (PBMC) using granulocyte-macrophage colony-stimulating factor (GM-CSF) were described [2]. This made it feasible and practical to derive large quantities of DC for clinical investigation. Two years later was the first report of a clinical trial testing a DC vaccine product. In this trial, DC were pulsed with patient-specific idiotype protein from follicular lymphoma patients [3]. Cellular anti-idiotype responses were detected in all four patients, antitumor effects in three and objective tumor regressions in two. In 1998, there was the first clinical report of antitumor effects in melanoma patients [4]. Tumor responses were reported in five of 16 melanoma patients treated with DC derived from PBMC and pulsed with a cocktail of HLA-restricted melanoma-associated peptides or lysates of autologous tumor. In 2000, methodology was described for producing and cryopreserving DC that retained biologic activity [5]. This important advance eliminated the need to perform

repeated apheresis procedures, and made patient scheduling for treatment more flexible. In 2002, a DC/tumor cell hybrid fusion product, sometimes referred to as a dendritoma, was used in the treatment of melanoma patients [6]. In this formulation, the hybrid was created by using polyethylene glycol to fuse autologous DC with cells derived from fresh autologous tumor. In 2006, 19 patients were treated with DC that had been transfected with melanoma mRNA via electroporation [7]. In that same year, there were reports of clinical benefit using DC loaded with TAA from allogeneic cell lines [8,9], as well as by subcutaneous (SC) injections of DC loaded with TAA from self-renewing tumor cells from short-term autologous cell lines [10]; and a 108-patient randomized trial in patients with measurable metastatic melanoma showed similar clinical benefit for SC injections of DC pulsed with peptide antigens compared with intravenous (IV) dacarbazine chemotherapy [11]. In 2010, sipuleucel-T, a DC-enriched leukocyte product in which peripheral blood leukocytes were cultured with a fusion protein of prostatic acid phosphatase and GM-CSF, was approved for the treatment of hormone-refractory prostate cancer based on 4.1-month improvement (19%) in median overall survival [12]. This product was administered IV every 2 weeks for 6 weeks with a new product manufactured each time from fresh leukocytes obtained by leukapheresis. In 2012, a randomized trial in patients with metastatic melanoma showed a striking survival benefit, 72 versus 31% at 2 years, for SC injections of DC loaded with TAA compared with injections of irradiated tumor cells [13]. In this trial, the source of TAA were self-renewing cells derived from short-term autologous tumor cell lines, and both DC and tumor cell products were administered in GM-CSF.

DC: physiology, antigen processing & presentation

The interest in applying DC in cancer immunotherapy stems from its position at the evolutionary interface of innate and adaptive immunity. DC are derived from hematopoietic stem cells and are a member of the macrophage family. DC are the most potent of antigen presenting cells in terms of their ability to initiate and sustain the process of adaptive immunity, which includes features of antigen specificity, antibody-mediated humoral immunity, antigen-specific cellular immunity and memory [14–17]. DC can activate

both memory and naive T and B cells and stimulate natural killer and other cells associated with innate immunity [18]. There is great interest in using *ex vivo*-generated DC as the delivery system for antigen-based cancer vaccines rather than just injecting the antigens themselves and relying on uptake and presentation of TAA by endogenous DC.

• Cell physiology

DC are characterized as immature or mature, which are important for distinct, but related, biologic functions [14–16]. There are also intermediary and reversible, transition states sometimes referred to as activated or semimature. Immature states are associated with immune tolerance, mature states with immune stimulation and the semimature states can be immune suppressing or immune enhancing depending on other signals [19]. Immature DC typically do not have dendrites and are primed for antigen loading rather than antigen presentation. Immature DC reside in peripheral tissues where they sample cells and debris for molecules that reflect ‘pathogen- or damage-associated molecular patterns’ that encourage endocytosis. DC recognize pathogen-associated molecular pattern or damage-associated molecular pattern on apoptotic or necrotic cells using membrane receptors, such as Toll-like receptors and cytosol receptors such as the NOD-like receptors. Soluble and membrane-bound matter are internalized by phagocytosis and pinocytosis, which activate DC and initiate a maturation process that can be completed in less than 24 h. During this maturation transition, DC lose their endocytosis function and convert to a state optimized for antigen presentation to other immune cells. This includes upregulation of the costimulatory molecules CD80, CD86 and CD40, and increased expression of CD83. Upregulation of these costimulatory molecules is necessary for immune activation, otherwise DC remain in an immature or semimature state that can induce immune tolerance to the immunogen [19]. Activated DC upregulate the chemokine receptor CCR7 that induces DC migration to lymph nodes via the lymphatics or blood stream and inhibits apoptosis. During migration, DC undergo further maturation. Once DC reach lymph nodes, mature DC communicate via their dendrites where antigen is presented to T lymphocytes via the T-cell receptor (TCR), in association with interactions between costimulatory molecules. This highly specialized

communication between DC and T lymphocytes is controlled by various tissue-bound molecules and cytokines. For instance, in order to effectively recognize antigen via the TCR, CD86 (B7) on DC must interface with CD28 on T-cells, but this process can be competitively inhibited by cytotoxic T-lymphocyte antigen-4 (CTLA-4) binding to CD86. For DC activation and expansion of antigen-specific CD8⁺ T-cells, there appear to be at least three critical steps: first, the binding of antigenic peptide presented by DC to the TCR on T lymphocytes (signal 1), the interaction of costimulatory molecules CD80/CD86 (B7 molecules) on DC and CD28 on T lymphocytes (signal 2), and then release of IL-12p70 by DC that leads to a cascade of other cytokines (signal 3) [20,21]. Other well-documented interactions include CD40 on DC with CD154 (CD40 ligand) on T-cells, and CD58 on DC with CD2 on T-cells. DC also can express programmed death molecule-1 (PD-1) and its ligand (PD-L1), which are important in the inhibition of immune responses.

For clinical use, immature DC most often have been derived from PBMC by incubation with IL-4 and GM-CSF over 6 days, although related methods have produced effective DC in 48 h [22]. Other cytokines such as IL-13 or IL-15 have been used instead of IL-4. Subsequent antigen loading via endocytosis is associated with maturation. Work with HLA-restricted melanoma peptide antigens suggested that chemical induction of complete maturation was necessary to optimize induction of antigen-specific immune responses by DC that had not been activated by natural chemicals associated with phagocytosis. For instance, in one small study mature DC pulsed with such peptides were twice as likely to produce antigen-specific responses than immature DC pulsed with peptides [23], and in a randomized trial immature DC pulsed with HLA-restricted peptides were less immunogenic than the same peptides injected with GM-CSF [24]. Maturation of immature DC can be achieved *in vitro* by incubation with a variety of inflammatory cytokines such as TNF- α , IFN- γ , CD40 ligand and IL-6 [19]. However, such chemically induced maturation is not necessary if appropriate signals are being released by dying cells. In fact, maturation prior to antigen loading is counter-productive; activated, mature antigen-loaded DC secrete IL-12, and especially the IL-12p70 subunit. DC are programmed to survive for only a few days. From a feasibility

perspective, it is important that mononuclear cells, immature DC and activated, mature antigen-loaded DC, all can be cryopreserved with retention of biological function.

• Antigen presentation

DC process and present antigens in the context of MHC molecules that facilitate distinction between self and nonself [15,16,25]. DC process and present internal (endogenous) and external (exogenous) proteins differently. Internal proteins are processed via endosomes known as proteasomes where proteins are broken down into eight to ten amino acid sequences that are transported to the endoplasmic reticulum where MHC class I (MHC I) molecules are assembled with beta-2 microglobulin. The peptide–MHC1 complex is then transported to the cell surface. This process facilitates immune recognition of abnormal intracellular proteins that result from viruses or mutated self-antigens. From an evolutionary perspective, it appears that MHC I expression evolved primarily to activate CD8⁺ cytotoxic T lymphocytes (CTL) to kill virally infected cells, but this process also enables recognition and destruction of defective cells or autologous cancer cells that express unique antigens that result from mutations. This process leads to activation of type-1 helper T-cell (Th1) helper cells that secrete IL-2 and IFN- γ that further activate CTL. CD8⁺ CTL are believed to be the most critical effector cells for an immune-mediated antitumor response.

In contrast to the process for internal antigen presentation, DC process external proteins, which are obtained by phagocytosis or pinocytosis, in enzyme-laden lysosomes or phagosomes where they are broken down into sequences of 8–25 amino acids that are assembled with MHC class II (MHC II) molecules and transported to the cell surface. From an evolutionary perspective, it appears that MHC II expression evolved to induce and activate type-2 helper T-cell (Th2) responses and B-cell responses that are useful for eradicating bacteria and parasites. Th2 lymphocytes secrete a variety of cytokines including IL-4, IL-5, IL-6 and IL-10 and activate B cells to produce antibodies to facilitate antibody-dependent, cell-mediated cytotoxicity and opsonization of microbes to facilitate phagocytosis. Th2 responses are not believed to be as crucial as Th1 responses in the induction of antigen-specific CTL, but they play an important facilitating role [26].

MHC II presentation can also lead to activation of Th17 cells, so named because they secrete IL-17 [27]. The cytokines IL-6 and TGF- β generate Th17 cells, and IL-23 is important for their expansion [28]. Th17 cells appear to be especially important in protecting mucosal barriers from pathogens, are implicated in graft-versus-host disease and their dysregulation has been implicated in some autoimmune diseases. However, in a study of 200 ovarian cancer samples, the presence of large numbers of Th17 cells was associated with a more favorable survival [29]. In that study, Th17 cells and IL-17 also were correlated with the presence of Th1-associated cytokines (IL-2 and IFN- γ) in the tumor microenvironment. In a melanoma mouse model, adoptive cell therapy with Th17 cells produced better anti-tumor effects than Th1 cells [30]. In patients with metastatic melanoma, three weekly injections of DC loaded with autologous TAA was associated with increased IL-17 serum levels, which were associated with improved survival [31].

The classical concepts regarding protein processing and antigen presentation for endogenous versus exogenous proteins have led many investigators to focus on strategies that increase the probability that TAA are processed and presented as endogenous proteins rather relying on the natural process of endocytosis [32]. This has included electroporation of peptides, tumor lysates and mRNA. However, the Th1 response is not limited to external proteins. The process of ‘antigen cross-presentation’ allows antigens derived from external proteins to be phagocytosed by DC, but still end up being presented in the context of MHC I [33]. One way in which this happens is when MHC1 molecules and proteins are processed together in phagosomes. Cells dying in apoptosis digest their own DNA by enzymes in their endosomes (autophagy) where they are already in close association with MHC I [34]. Thus, phagocytosis of a tumor cell and its membrane products can result in presentation of TAA via both MHC II and MHC I, both of which are important for an optimal immune response [26]. There also appear to be other mechanisms that transport exogenous peptides directly to the proteasomes where they can be united with MHC I molecules.

• MHC I & II

As noted in the previous section, recognition of self-MHC I and II molecules is crucial for the communication that results in DC-induced

immune responses. HLA are the MHC proteins of humans. Most HLA genes are encoded on chromosome 6, but beta-2 microglobulin is encoded on chromosome 15 [35]. There are numerous normal genetic variants of MHC molecules. MHC I (A, B, C) help present peptides derived from intracellular proteins via proteasomes while MHC II (DP, DM, DOA, DOB, DQ and DR) present antigens derived from extracellular antigens via phagosomes. Each human has three HLA types and four isoforms of DP, DQ and DR, and two isoforms of DRB3, DRB4 or DRG5). Unfortunately, because of mutations, cancer cells sometimes have low or no expression of these MHC molecules. This is one of the evolutionary interactions between the immune system and malignant cells that results in 'immune editing' out of tumor cells that did express MHC I and II, but were then eliminated by CTL, leaving only MHC-deficient tumor cells to survive [36]. There also may be mutations affecting DC function that favor survival of tumor cells with certain antigens.

DC vaccines in melanoma

Many of the published studies with therapeutic antimelanoma DC vaccines are summarized in [Table 1](#) [4,6,8,9,11,13,37–53]. Several important variables are included there, such as specifics of each DC product, antigen sources, co-administered adjuvants, routes and schedules of administration, the patient population and measures of

therapeutic efficacy. Not included are the specifics of DC generation and TAA loading, nor the quantity or viability of DC delivered per dose and/or cumulatively. Most studies have utilized doses ranging from 1 to 30 million DC, and have administered multiple doses. Summaries of immune responses are not included because of space limitations, but as a generalization, all studies showed evidence of induction or enhancement of immune responses to known TAA, but these were not predictive of clinical benefit. The study sizes are small, ranging from only 10 to 54 patients, and many were designed to test primarily for immune response rather than clinical benefit. All of these were open-label trials, and only two represent arms from randomized trials with a control arm [11,13]. Most of the studies were conducted at single institutions rather than multiple centers. Most of the patients had distant metastatic disease (stage IV), but several trials also included small numbers of patients with regionally recurrent and/or regionally advanced unresectable disease (stage III). This is an important distinction because patients with recurrent stage III disease have a better prognosis than those with distant metastatic disease [54].

Details regarding prior therapies are not available for many of the studies summarized in [Table 1](#) but may be important in terms of immune inhibitory or enhancing effects. Most of these trials were conducted in an era before

Table 1. Clinical trials of dendritic cells in patients with advanced melanoma.

Study	Year	Stage	Pts	Antigens	Adjuvant	Route	Efficacy	Ref.
Nestle <i>et al.</i>	1998	IV	16	gp100, Melan-A and Tyrosinase or autologous tumor lysate	KLH	IN weekly × 4	3/12 OR 1 CR 2/4 PR 1 CR	[4]
Thurner <i>et al.</i>	1999	IV	13	Mage-3A1	Tetanus toxoid or tuberculin	SC & ID q2wk × 3, then q2wk IV × 2	0/13 OR, CR of certain lesions	[37]
Banchereau <i>et al.</i>	2001	IV	18	gp100, Melan-A, tyrosinase, and Mage3	Influenza matrix protein and KLH	SC q2wk × 4	0/18 OR	[38]
Krause <i>et al.</i>	2002	IV	17	Autologous tumor	Allogeneic DC dendritoma	SC q4wks	1/17 OR	[6]
Hersey <i>et al.</i>	2004	IV	14 19	gp100, Melan-A and Tyrosinase or Autologous tumor	KLH	IN weekly × 4, then q2wk × 2, then q4wk × 2	0/14 OR 3/19 OR	[39]
Haenssle <i>et al.</i>	2004	IV	11	Autologous tumor	Allogeneic DC Dendritoma LD IL-2	ID or SC q4wks	0/11 OR	[40]

CR = Complete response, DC = Dendritic cells, GM-CSF = Granulocyte macrophage colony stimulating factor, HD = High dose, HLA = Human lymphocyte antigen, IFN- α = Interferon alpha, ID = Intradermal, IN = Intranodal, IV = Intravenous, IL-2 = Interleukin-2, KLH = Keyhole limpet hemocyanin, LD = Low dose, med = Median, mos = Months, NED = No evidence of disease, OR = Objective response, OS = Overall survival, Pts = Number of patients, SC = Subcutaneous, wks = Weeks.

Study	Year	Stage	Pts	Antigens	Adjuvant	Route	Efficacy	Ref.
Trefzeret <i>et al.</i>	2004	IV (n = 17) III (n = 3)	20	Autologous tumor	Allogeneic DC dendritoma	ID q4wks	1/20 OR Med OS 16 mos	[41]
Banchereau <i>et al.</i>	2005	IV	22	gp100, Melan-A, Tyrosinase, Mage-3	Influenza matrix protein ± KLH	SC q2wk × 4	0/22 OR Med OS	[42]
Palucka <i>et al.</i>	2006	IV	20	Allogeneic tumor cell line lysate		SC q4wk × 8	2/20 OR Med OS 22-mos	[8]
Salcedo <i>et al.</i>	2006	IV & III	15	Allogeneic tumor cell line lysate	Hepatitis B protein and/or tetanus toxoid	SC, ID and i.n.q4wk × 4	1/15 OR	[9]
Wei <i>et al.</i>	2006	IV	10	Autologous tumor	Autologous DC Dendritoma LD IL-2	SC q 3 mos	1/10, CR	[43]
Schadendorf <i>et al.</i>	2006	IV	53	gp100, Melan-A, tyrosinase, Mage-1 and Mage-3		SC q2wk × 5, then q4wk	2/53 OR Med OS 9.3 mos 2-yr OS <20%	[11]
Hersey <i>et al.</i>	2008	IV	18 16	gp100, Melan-A, Tyrosinase, Mage-3 or autologous tumor	KLH ± LD IL-2 KLH ± LD IL-2	IN weekly × 4, then q2wk × 2, then q4wk × 2	2/9 OR + IL-2 1/9 OR no IL-2 Med OS 18-mos 0/16 OR Med OS 18-mos	[44]
Redman <i>et al.</i>	2008	IV	24	Autologous tumor	KLH LD or HD IL-2	ID q2wks × 3	0/24 OR	[45]
Dillman <i>et al.</i>	2009	IV (n = 39) III (n = 15)	54	Autologous tumor cell line	GM-CSF	SC weekly × 3, then monthly × 5	0/15 OR 73% 2-yr OS 54% 5-yr OS	[46]
Lopez <i>et al.</i>	2009	IV (n = 43) III (n = 7)	50	Allogeneic tumor cell line lysates	Alum or KLH ± LD IL-2	ID q10days × 2, then q 20 days × 2	Med OS 15 mos 5-yr OS 12% (for stage IV)	[47]
Ribas <i>et al.</i> [	2010	IV (n = 27 III (n = 11)	38	Allogeneic tumor cell line lysates		ID & SC q2wk × 6, then q6wk × 2	3/33 1 CR	48]
Ridolfi <i>et al.</i>	2011	IV	27	Autologous tumor lysate	KLH LD IL-2	ID or SC q15days × 2, then q4wk × 4	8/27 OR, 2 CR Med OS 16-mos	[49]
Lesterhuis <i>et al.</i>	2011	IV	27	gp100 Tyrosinase	KLH	IV and ID q2wk × 3	2/27 OR 2 CR	[50]
Oshita <i>et al.</i>	2012	IV	24	Gp100, tyrosinase, Melan-A, Mage-A2, Mage-A3	KLH	SC weekly × 4, then 2 wk later, then q4wk × 5	1/24 OR	[51]
Dillman <i>et al.</i>	2012	IV (n = 15) III (n = 3)	18	Autologous tumor cell line	GM-CSF	SC weekly × 3, then monthly × 5	2-yr OS 72% 1 delayed CR	[13]
Wilgenhof <i>et al.</i>	2015	IV (n = 14) III (n = 1)	15	mRNA for Gp100, tyrosinase, Melan-A, Mage-A2, Mage-A3 and HLA II	± IFN-α	ID and IV q2wk × 4 then at week 16	4/15 RR 2 CR	[52]
Wilgenhof <i>et al.</i>	2015	Resected NED III and IV	30	mRNA for Gp100, tyrosinase, Melan-A, MAGE-A2, Mage-A3 and HLA II	± IFN-α	ID q2wk × 6	70% 4-yr OS	[53]

CR = Complete response, DC = Dendritic cells, GM-CSF = Granulocyte macrophage colony stimulating factor, HD = High dose, HLA = Human lymphocyte antigen, IFN-α = Interferon alpha, ID = Intradermal, IN = Intranodal, IV = Intravenous, IL-2 = Interleukin-2, KLH = Keyhole limpet hemocyanin, LD = Low dose, med = Median, mos = Months, NED = No evidence of disease, OR = Objective response, OS = Overall survival, Pts = Number of patients, SC = Subcutaneous, wks = Weeks.

effective BRAF/MEK inhibitors and immune-checkpoint inhibitors were in widespread use. In many trials, DC products were co-administered with Keyhole Lymphocyte Hemocyanin, bacterial toxins or cytokines such as IL-2, IFN- α and GM-CSF. The routes and schedules of administration were highly variable. Study sizes are so small and response rates so low that it is entirely unclear whether there is a preferred strategy for DC-based therapy. In general, demonstration of immune changes was common, but objective responses were uncommon, and responses were most prevalent in patients who had only cutaneous metastatic disease at the time of treatment. Several authors commented on the surprising long-term survival of many patients, but in the absence of contemporary control groups, the prolonged survival may reflect underlying tumor biology rather than effects of the vaccines.

Key issues for DC vaccines

• DC-TAA as opposed to TAA

It is now known that infiltrating cells such as Tregs and myeloid-derived suppressor cells (MDSC) and cytokines secreted by these and inflammatory cells, create an environment that is hostile to inducing and sustaining an effective antitumor immune response [55,56]. Theoretical advantages for using *ex vivo* generated DC to present TAA include the loading of TAA outside the tumor microenvironment, and the ability to produce and administer large numbers of antigen-loaded DC. Both of these goals appear to be achieved by a variety of DC-manufacturing methodologies. In general, trials in which TAA were administered as cells or peptides, which would rely on TAA loading by endogenous DC, have yielded disappointing results, both in terms of immune responses and clinical benefit [57–59]. As to comparative trials of putative therapeutic vaccines, there is one randomized trial in melanoma patients that demonstrated a 22-month increase in median survival and more than a doubling of 2-year survival in association with injection of DC loaded with autologous TAA compared with the injection of irradiated autologous tumor cells expressing such antigens [13].

• Dose response

The unit doses of DC administered have ranged from 1 to 30 million cells and total doses from 5 to 250 million cells. It has generally been assumed that exposure to large quantities of immunogen

would increase the likelihood of eliciting or enhancing an immune response. Reasons for not giving higher doses include minor risks and discomforts to patients that accompany collecting PBMC, practical-manufacturing limitations related to cell numbers, time and cost of DC production, and patient considerations in terms of injections and anatomical sites of repeated administration. There is a wide variation in doses administered among patients, but there is little variation among cryopreserved doses for individual patients [13,46]. Within these single and cumulative dose ranges, there have been no claims of a dose-response relationship, either in terms of immune response or anticancer efficacy, but good prospective comparisons have not been performed, and will be confounded by biologic heterogeneity in terms of cancer cells and immune cells.

• Schedule of administration

As can be seen in [Table 1](#), a variety of schedules have been used to administer DC to melanoma patients. There is no evidence that one schedule is better than any other in terms of immune responses or clinical outcomes. It is also unclear whether there is an advantage to repeated vaccinations, but all studies have used multiple administrations based on standard immunization practices.

• Route of administration

DC have been injected, SC, intradermally (ID), intranodally (IN) and IV and by combinations of these routes. Based on the trials summarized in [Table 1](#), it is not clear that there is a route that is more likely to be associated with clinical benefit. It is known that DC communication with T-cells takes place in lymph nodes, but studies suggest that it is not necessary to use IN or intralymphatic injections, which are technically challenging, in order to induce a desired immune response [7,60]. Mouse studies suggested that SC or ID routes were associated with better DC migration to lymph nodes than the IV route [61], and with better antitumor effects [62]. The IV route is associated with greater toxicity because of the release of cytokines by dying cells and perhaps by DC/immune cell interactions taking place in the blood stream. For these reasons, most trials have utilized SC or ID injections, which are technically less challenging, and are known to be effective routes for vaccinations against infectious diseases. However, ID

has practical limitations in terms of volume that can be injected, which can necessitate multiple injections sites in order to deliver a prescribed dose. Multiple ID injections are time consuming, inconvenient and can result in extravasation of product. For this reason, some investigators who believe that ID may be more effective, have used a combination of ID and SC or ID and IV to deliver a higher dose of DC.

Limited human studies using radiolabeled DC suggest that only a small percentage (2–4%) of injected DC actually reach a regional lymph node, yet there is evidence that this is sufficient to induce an immune response [63]. There are some data suggesting that ID may be somewhat better than SC in terms of DC migration to lymph nodes, but the immune response and clinical significance of this is not clear [64]. One study reported that reducing ID doses to about 5 million DC per injection was associated with a 3% uptake in lymph nodes compared with 0% for 15 million cells, perhaps because of decreased viability associated with cell concentration and the pressures associated with injection [65]. One caveat is that radiolabeling cells in and of itself is associated with decreased cell viability, and these studies did not utilize cytokine adjuvants which might have enhanced the viability and longevity of DC, and may have enhanced maturation which is also associated with better migration.

• Adjuvants

The benefits of co-administering agents with DC is unclear but as can be seen in [Table 1](#), most studies have administered DC with agents expected to have immune enhancing or inflammatory effects, and/or DC-maturation-enhancing effects. There has been no systematic comparison of adjuvants that were co-administered or admixed with DC at the time of administration and such trials would require large numbers of patients because of biologic heterogeneity. Animal studies suggest that cytokines can enhance maturation and migration of DC into lymph nodes [66]. IL-2 has immune-enhancing effects on CTL that are induced by vaccination, but this cytokine also stimulates proliferation of inhibitory Tregs and is quite toxic when given in high doses. As shown in [Table 1](#), there is no evidence that the addition of IL-2 to sustain CTL resulted in better antitumor effects [44,45]. GM-CSF, which has been used extensively in melanoma patients, is important in DC production and may help preserve viability and induce

additional maturation following injection [67]. IFN- α has a variety of effects on various components of the immune system and is approved for the adjuvant treatment of high-risk, early-stage melanoma [68]. There is evidence that IFN- α can be an important signal to drive CD8⁺ cells that have just been activated by DC [19]. These cytokines are all US FDA approved and therefore readily available for clinical use, but none is approved specifically for use as an adjuvant with a vaccine. Other cytokines that are currently investigational, such as IL-12, IL-15 or IL-18 and IL-12p70, might also be considered [20]. The value, if any, of nonspecific immune stimulation by Keyhole Lymphocyte Hemocyanin, microbial cell wall components or other adjuvants such as monatinide or Freund's adjuvant is unclear [69].

• DC sources & subtypes

There are at least three DC subtypes, including monocyte, myeloid and plasmacytic [32]. The frequency of DC in blood is quite low, which is why the focus has been on isolation of precursors and differentiation of precursors into DC. Some early trials derived DC from G-CSF-mobilized CD34⁺ hematopoietic stem cells [38], but most melanoma trials have utilized DC derived from PBMC collected by apheresis without cytokine mobilization. Such DC seem to function well in terms of antigen loading and induction of TAA-specific CTL immune responses. Clinical investigation has repeatedly documented induction of new and/or enhancement of existing immune responses to TAA in most patients treated with various monocyte-derived DC products. The failure to elicit immune responses in all patients is probably more related to individual patient immune systems rather than lack of potency of DC products. Nevertheless, some investigators are examining other subsets of cells, such as plasmacytoid-derived DC as opposed to myeloid-derived DC, or other naturally occurring DC to see if they could create an improved DC product [70,71]. Certain subsets, such as CD141⁺ cells, may provide an advantage in terms of cross-presentation of TAA associated with necrotic cells [72].

The ability to utilize cryopreserved DC derived from PBMC has allowed use of a single leukapheresis procedure to manufacture multiple doses for each patient. Such procedures are commonly used to collect hematopoietic stem cells for transplants and are associated with minimal risk to patients, although central venous access

is sometimes required [73]. However, it might be less expensive and more efficient to derive and expand DC from a smaller volume of blood that can be drawn into a syringe. Relying on existing circulating DC without *ex vivo* expansion could shorten the time to DC product by a few days. The minimal number of DC needed to induce an immune response *in vivo* is unknown and would not be easy to determine because of biologic heterogeneity. However, relative differences may be predicted by *in vitro* assays of biological potency. Most studies have focused on autologous rather than allogeneic DC, the exception being some of the hybrid or dendritoma constructs [6,39,40]. It is possible that genetic engineering could result in development of an off-the-shelf allogeneic DC product that would make blood collection unnecessary; however, for the foreseeable future, it seems that autologous DC will continue to be preferred.

• Tumor-associated antigens

The most important characteristic of any vaccine is its antigens. Inducing immune responses to irrelevant antigens, or to TAA already well recognized by the immune system, are unlikely to be associated with clinical benefit. As reviewed elsewhere, most melanoma vaccine trials have focused on a handful of peptide antigens that are HLA-2A-restricted, or allogeneic cell lines that expressed well-characterized antigens [56,74]. Such studies were crucial for demonstrating definitively that immune responses could be induced or enhanced in cancer patients. However, because of biologic heterogeneity, and the limitations associated with inducing an immune response to only a handful of TAA, or to foreign cells, it is unlikely that such approaches will yield therapeutic success. Among trials utilizing injections of peptides rather than presentation via DC, one large trial showed no benefit with or without GM-CSF [59], while another trial suggested that addition of gp100 increased objective response rates and progression-free survival in patients healthy enough to be treated with high dose IL-2, but had no impact on long-term survival [75]. Because of manufacturing convenience, use of peptide antigens is still attractive whether it be pulsing in culture, or loading via electroporation of peptides or their mRNA [76]. Although large randomized trials utilizing melanoma TAA including gp100, Melan-A/Mart1 and Mage-3 were negative [58], there is still the possibility that certain cancer/testis antigens such

as NY-ESO-1 will be effective targets in melanoma [77]. To simplify manufacturing, allogeneic tumor cell lines have been a popular source of melanoma TAA for many years but have disappointed in clinical trials [56,57]. However, for feasibility reasons, there continues to be interest in DC loaded with TAA derived from allogeneic tumor cell lines [78].

It is now evident that most endogenous anticancer immune responses are directed to unique, patient-specific, neoantigens rather than to common antigens (mutated or not) that are expressed by melanoma cells from many patients [79,80]. Although it has been more than 20 years since it was suggested that the ideal source of TAA should be a patient's own cancer cells [81], it is only within the past few years that this concept has been widely accepted [56,82–84]. We now know that melanoma cells typically contain hundreds to thousands of mutations that can result in patient-specific TAA that can be excellent targets for immunotherapy [85–87]. For this reason, despite the disadvantages from manufacturing and commercialization perspectives, there is increasing interest in utilizing autologous tumors as the source of TAA for therapeutic vaccines. An important question is whether biopsies or resected tumor specimens, or even circulating tumor cells could be sufficient for this purpose, and whether there is a significant advantage for using autologous tumor cell lines as the source of TAA. A major challenge for trials that have attempted to use melanoma samples themselves as the source of TAA is the difficulty in assuring sufficient numbers of malignant cells from which to derive TAA in an established manufacturing process [57,88]. Autologous tumor cell lines not only provide a means to a uniform number of cancer cells but they also contain TAA that may be limited to each patient's tumor stem cells and early progenitor cells, also known as cancer-initiating cells [89–92]. Such a process is associated with additional manufacturing and commercialization costs and challenges, but is theoretically appealing. The effectiveness of vaccination with cancer stem cells has been demonstrated in animal models [93–95], and two Phase II trials in metastatic melanoma, testing patient-specific vaccines consisting of DC loaded with melanoma TAA from autologous tumor cell lines, were associated with 2-year overall survival rates of 72 and 73% [13,46].

• Antigen sources & loading

It is known that antigen presentation is enhanced in association with DC maturation. Although some investigators have used methods that assured maturation of DC prior to antigen loading, others have shown that antigen loading itself is enhanced during the maturation process following phagocytosis. Thus, the desired maturity may differ depending on how DC acquire the antigen. This may explain why similar response rates were observed whether using DC that were considered immature because additional cytokines were not added to the cell culture to induce maturation [39], as opposed to DC that were matured by the addition of cytokines [44]. When ‘immature’ DC were used, objective tumor responses were noted only for the products that were made with autologous tumor [39], which required phagocytosis, but not for peptides, which would have only required pinocytosis. In contrast, when they used ‘mature’ DC products [44], objective tumor responses were limited to peptide-loaded products, and there were no responses when autologous tumor was the source of antigen. It is not clear that there is an advantage to maturing DC with TNF- α , or other cytokines, but if there is, it may be limited to peptide antigens, and maturation should be reserved until after antigen has been loaded.

Because of the importance of MHC I presentation for inducing a Th1 response, there are unresolved issues relevant to methods of antigen loading. As DC therapy was emerging, various melanoma-associated antigens recognized by tumor-infiltrating lymphocytes were being identified. This included gp100, tyrosinase, Melan-A/Mart-1, Mage-1, Mage-3, etc. For this reason, early DC trials mostly focused on these HLA-A2-restricted antigens in the approximately 50% of patients with such an HLA allotype, and autologous tumor or allogeneic cell lines were used as the antigen source for patients who were HLA-A2 negative. Some were concerned that phagocytosis would only lead to MHC II presentation and Th2 responses, which led to strategies to manufacture TAA intracellularly with mRNA to encourage MHC I presentation. However, perhaps because of cross-presentation, Th1 responses have been documented consistently with TAA loading via phagocytosis. Furthermore, it is now accepted that a simultaneous Th2 response induced by MHC II presentation is critical to a robust CTL

response, perhaps because of cytokine release and/or enhancement of a weak immune response rather than induction of a totally new response. Intracellular cross-presentation of antigen suggests that any means by which DC are loaded may result in effective antigen loading [33]. There is some evidence that efficiency of antigen loading and potency is enhanced by electroporation, and this process may offer advantages over co-incubation with whole tumor cells or with tumor cell lysate [96–99]. An alternative to transfection with mRNA to assure processing of antigens as if they were endogenous, is the creation of hybrids of DC with tumor cells, also known as dendritomas [6,43,100].

At this time, clinical data are too limited to conclude that there is a preferred source of TAA for DC loading. In Table 1, If one adds up the purported objective responses, the response rate is 11/201 (5.5%) for the nine reports in which autologous DC were loaded with HLA-2 restricted peptides [4,11,37–39,42,44,50,51], and 22/231 (9.5%) for the 13 studies in which autologous DC were loaded with antigens from allogeneic or autologous tumor cells or their lysates [4,6,8,9,39–41,43–46,48,49] ($p = 0.11$ by Chi-square test). The response rate was 14/115 (12.2%) for the seven studies that used autologous DC and autologous tumor cells or lysate as the antigen source [14,39,43–46,49] compared with 8/116 (6.9%) for the six studies that used either allogeneic DC or allogeneic tumors as the antigen source ($p = 0.17$) [6,8,9,40,41,48], 6/68 (8.8%) for the three studies that used autologous DC loaded with TAA from allogeneic tumor cell lines ($p = 0.48$), and 11/201 (5.4%) for the nine studies that utilized peptide antigens ($p = 0.034$). Based on the above, it appears that the best clinical results to date have been achieved with autologous DC loaded with TAA from autologous tumor cells. The one study that used mRNA to express common melanoma antigens intracellularly reported an objective response rate of 4/15 in patients with measurable disease [53]. In that study, the monocyte-derived DC were electroporated with mRNA encoding a CD40 ligand, a Toll-like receptor-4, CD70, fusion proteins of an HLA class II targeting signal (DC-LAMP) and one of the one of four full-length melanoma antigens among gp100, tyrosinase, Mage-1 or Mage 3 based on antigen expression in each patient’s tumor. This preparation is now referred to as the TriMix-DC therapy [99].

• Product manufacturing

Large-scale manufacturing of DC vaccine products is necessary for multicenter clinical trials and potential commercialization. For multicenter trials, this requires multistep logistics for the handling, processing, storing and shipping of intermediary materials, such as PBMC and autologous tumor, as well as the storage, shipping and administration of the final DC product [11,48]. Currently, most approaches require two intermediate products (antigen or tumor cells and autologous DC) before production of the final product (antigen-loaded DC). The ability to cryopreserve the final DC products has been essential for the conduct of most clinical trials reported to date. However, the only FDA-approved DC-based therapy (sipuleucil-T for prostate cancer) does not use cryopreserved cells [12]. A course of sipuleucil-T treatment plans for three apheresis procedures (one every 2 weeks) to manufacture a new product each time, which necessitates quality control and quality assurance release for each product. The ability to cryopreserve monocytes, DC and antigen-loaded DC that retain biological activity means that repeated PBMC collections and cell cultures are not necessary, which is a big advantage to keep down the costs of commercial manufacturing and complexity of treatment [5,102].

• Patient selection

There was a time when it was generally believed that any vaccine approach could only work in the least immune-compromised patients, and that vaccine trials should be limited to patients with minimal or no tumor burden. For this reason, trials have usually been limited to patients who have a good performance status and are not incapacitated by their cancer. At this time, there is no evidence that DC vaccines provide any benefit to patients who are bed-ridden with progressing cancer that has proven refractory to all other therapies. However, immune responses to specific TAA have been demonstrated repeatedly in patients with a good performance status, regardless of prior therapy, regardless of tumor burden and regardless of whether they exhibit delayed-type hypersensitivity reactions to common cutaneous recall antigens. Although theoretically the greatest benefit may be in patients who have a minimal tumor burden, clinical benefit also has been documented in patients with widespread disease. From a clinical trial perspective, the fastest way to glean positive

information is from clinical trials that enroll patients with measurable disease and advanced disease. Surrogate markers for objective tumor response and/or prolonged survival could enable a faster interpretation of clinical trials, but none have been validated. As noted previously, most trials of DC therapeutic products have demonstrated induction or enhancement of anti-TAA immune responses, but these do not necessarily predict a measurable clinical benefit.

Conclusion

Several hundred melanoma patients have been treated with DC vaccines. Work in the past 20 years has convincingly shown that DC vaccine products usually do induce or enhance immune responses to TAA, but that such immune responses are not sufficient in and of themselves to assure regression of established tumor or to prolong survival. Some of these disappointing clinical results may relate to the products themselves, and especially the TAA being presented, but we also know that the immune system of each patient and the biology of each patient's cancer are important determinants of whether induced or enhanced immune responses can result in clinical benefit. However, several immunotherapy products have been associated with improved overall survival despite lack of a high objective response rate and or a significant impact on progression-free survival [103]. Further clinical investigation of DC vaccines is warranted.

Future perspective

Going forward, we can anticipate emphasis on the following: first, efforts to produce more active DC, typically by transfection with mRNA for agents that activate and mature DC; second, different methods of antigen loading, such as electroporation of tumor lysates or mRNA with the goal of making the processes more efficient while retaining or increasing the potency of DC with both MHC I and MHC II presentations that enhance both Th1 and Th2 response; third, increasing focus on autologous tumor antigens based on the recent confirmation that most melanoma patients have hundreds to thousands of nonsynonymous mutations that can result in patient-specific neoantigens; and fourth, approaches that combine DC vaccines with immune-checkpoint inhibitors. In terms of these approaches, while there may be some improvements in DC themselves and adjuvants,

the change that is most likely to yield significant benefit is increasing focus on DC products that take advantage of autologous tumor as the source of TAA.

We now know that most patients do make immune responses against their cancers, although those responses may be suppressed by immune-checkpoint mechanisms [104–106], which can often be overcome with anticheckpoint monoclonal antibody therapy [107–109]. However, in many patients, there is still an opportunity to induce new immune responses or enhance existing weak immune responses, by a patient-specific vaccine strategy [74,84]. It is unlikely that trials focusing on elimination of Tregs using monoclonal antibodies, other T-cell-targeted therapies or low-dose cyclophosphamide, or even toxic precondition regimens such as lymphoablative chemotherapy with agents such as high-dose cyclophosphamide and fludarabine, will provide as much benefit as combining DC vaccines with checkpoint inhibitors [110,111].

The patient-specific autologous tumor approach for personalizing DC vaccines maximizes the potential for a polyclonal T-cell response to TAA and assures the potential recognition of the entire repertoire of neoantigens that are unique to any given patient. Sources of autologous TAA include lysates from autologous tumor, mRNA from autologous tumor and mRNA, lysates or cells from autologous tumor cell lines. The autologous cell line strategy may provide the additional benefit of targeting TAA that are only expressed on tumor cells, and especially tumor stem cells and early progenitor cells, which are present in only small quantities in whole tumor samples [90]. Peptide-based DC products will continue to be tested because they are the easiest from a product development perspective and lend themselves readily to assays to detect antigen-specific immune responses, however, increasingly the DC products that appear most promising are those that utilize autologous tumor antigens [112].

There will also be efforts to load DC with personalized TAA cocktails based on genomic or phenotypic testing of each individual patient. Unfortunately, the latter approach, although appealing as a means to apply personalized medicine, requires a stockpile of different antigens, extensive testing and does not take into account the uniqueness of epitopes associated with neoantigens. An alternative approach is to generate the mRNA for all identified mutations

that are predicted to be immunogenic through the combination of genomic analysis, statistical probability of antigenicity of nonsynonymous mutations and synthesis of mRNA for antigen loading of DC. Such an approach has been used to produce patient-specific DC vaccines to selected neoantigens, and shown to induce new antitumor immune responses and to enhance weak existing immune responses [84]. A caveat is that such predictive models need further optimization, and at this time it is not practical to manufacture mRNA from hundreds of mutations in each patient.

The repeated observations of delayed responses, disease stabilization and long-term survival in DC-treated patients suggest that effective immune responses have emerged in many patients. The observation that DC often induce an immune response without clinical benefit raises the possibility that checkpoint inhibitors may have blunted the immune responses, although it is also possible in the tumor microenvironment immunosuppressive Tregs and MDSC have inhibited local tumor responses. For these reasons, in patients with advanced melanoma, we can anticipate clinical trials in which patients receive anticheckpoint therapy in conjunction with DC vaccines presenting TAA that are patient-specific (autologous) or personalized. It is possible that clinical benefit may be even greater when such approaches are combined with agents that target Tregs and/or MDSC [110,111].

Once effective TAA-presenting DC have been established, it is likely that the best clinical results will be associated with combination therapy. Animal models have shown that combinations of vaccines and checkpoint inhibitors produce better anticancer effects than either treatment alone [113,114]. The major recent advance in cancer immunotherapy is the clinical application of monoclonal antibodies to CTLA-4, PD-1 and PD-L1 for the treatment of cancer, especially melanoma-, Ipilimumab-, nivolumab-, pembrolizumab- and atelizumab-block endogenous suppression of new and existing anticancer immune responses. However, they only can work if TAA are being presented and an immune response has already been mounted or is in the process of being induced. Despite their tremendous impact in the clinic, it is evident that there is still an unmet need based on observations that these agents do not benefit all patients, and additional antitumor immune responses can be

induced, or weak antitumor immune responses enhanced [74].

Given the current state of such knowledge, a preferred strategy may be to give DC vaccines in combination with anti-CTLA antibodies to facilitate the initial immune response by suppressing CTLA-4 inhibition of CD28–CD80 costimulatory molecules, followed by, or concurrent with anti-PD-1 or anti-PD-L1 antibodies to overcome the suppressive effects of PD-1/PD-L1 interaction between activated T-cells and tumor cells. Such trials can be conducted using the standard doses and schedules currently approved for the checkpoint inhibitors in patients with a good performance status, who have minimal or extensive metastatic disease. An example of a combination therapy is recently reported trial that combined transfection with mRNA to enhance and sustain DC function with transfection of peptide antigens and treatment with the anti-CTLA-4 antibody ipilimumab [115]. The overall response rate was 38%, including eight complete and seven partial responses with a 51% 6-month progression-free survival rate. This is much higher than response rates observed with either modality alone. At the moment, there are no validated tumor markers to guide patient selection for

such combination therapies. It is also feasible to combine DC vaccines with targeted therapies such as combinations of BRAF and MEK inhibitors, but the best results should be with checkpoint inhibitors.

A continuing challenge will be that there are so many DC and antigen variables that could be addressed, that it will be difficult to perform comparative trials to establish the superiority of one approach over all others. Rather, it is more likely that biotechnology companies will develop a specific product and proceed with clinical trial designs that include active control or placebo arms that could lead to regulatory approval of a specific product rather than answering a specific question related to optimization of the DC vaccine product.

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