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Enhancement of dendritic cells as vaccines for cancer

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Abstract

Dendritic cells are the most potent antigen-presenting cells known; owing to their ability to stimulate antigen-specific cytolytic and memory T-cell responses, their use as cancer vaccines is rapidly increasing. While clinical trials provide evidence that dendritic cells vaccines are safe and elicit immunological responses in most patients, few complete tumor remissions have been reported and further technological advances are required. An effective dendritic cell vaccine must possess and maintain several characteristics: it must migrate to lymph nodes, have a mature, Th1-polarizing phenotype expressed stably after infusion and present antigen for sufficient time to produce a T-cell response capable of eliminating a tumor. While dendritic cells are readily matured *ex vivo*, their phenotype and fate after infusion are rarely evaluable; therefore, strategies to ensure that dendritic cells access lymphoid tissues and retain an immunostimulatory phenotype are required. In order to best exploit dendritic cells as vaccines, they may require genetic modification and combination with other strategies including adoptive T-cell transfer, inhibition of regulatory T cells or modulation of inflammatory pathways.

Keywords

cancer immunotherapy; dendritic cell; genetic modification; immunology; tumor immunity; vaccine

Dendritic cells & stimulation of the immune response

Dendritic cells (DCs) are the most potent antigen-presenting cells in the body; they function as sentinels of the immune system, constantly sampling the microenvironment for ‘danger signals’, which include inflammation, damaged tissue and pathogens. DCs robustly activate antigen-specific T cells that can eliminate not only pathogen-infected cells but also tumor cells. For this reason, there is great interest in the optimization of DCs as cancer vaccines. This article will focus specifically on the use of DCs as vaccines for cancer immunotherapy. We will examine DC biology, lessons learned from preclinical and clinical studies and finally efforts to improve current vaccine formulations.

Upon uptake of antigen in the context of danger signals, the DC begins the process of maturation. This is a multistep, temporally-regulated process that includes several phenotypic and morphologic changes, such as decreased phagocytosis, altered expression of

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chemokine receptors and upregulation of costimulatory molecules, cytokines and chemokines. The maturing DC actively migrates from the periphery through the afferent lymphatics to the lymph nodes [1,2], where it presents phagocytosed and processed antigens on MHC molecules in the context of costimulatory molecules, for the activation and expansion of naive or memory antigen-specific T cells [3,4].

Although tolerance to self antigens is established during lymphocyte development, many tumors aberrantly express antigens that are normally expressed only in the fetus or in immune-privileged sites like testes, to which tolerance has not been established. Other tumors express mutated antigens like p53 or novel proteins created by chromosomal rearrangements, such as bcr-abl [5]. The activation of effector T cells specific for these tumor-associated antigens (TAAs) is facilitated in tumors that alert the innate immune response by their abnormal growth patterns, expression of heat-shock proteins or downregulation of MHC class I molecules. Such immunogenic tumors, however, use a range of mechanisms to inhibit the effector functions of TAA-specific T cells. Many secrete soluble inhibitory factors, such as IL-10, TGF- β or VEG-F, which inhibit T cells both directly and indirectly by inhibiting DC maturation. If tumors do not themselves produce inhibitory molecules, they may recruit other cell types, such as T regulatory cells (Tregs) and myeloid-derived suppressor cells (MSCs) that do. Tumors that express no danger signals may not alert the innate immune system, so that tumor antigen-specific T cells remain 'ignorant'. Other tumors may present antigens in the absence of costimulation or in conjunction with inhibitory ligands, such as programmed death ligand (PD-L)1 and caecam1, resulting in T-cell anergy or death [6]. Therefore, a major purpose of DC vaccination is to activate naive T cells or break specific tolerance to TAAs by providing a potent stimulus that elicits a Th1 immune response characterized by antigen-specific helper and cytotoxic T lymphocytes (CTLs) that will seek out and eliminate the tumor. However, owing to the suppressive nature of most tumors, T cells activated by vaccination may subsequently be inactivated at the tumor site. Therefore, multiple doses of vaccine may be required to maintain T-cell numbers and function over sufficient time to eliminate tumors and ensure that T cells enter the memory compartment to act as rapid responders in case of tumor relapse.

The ideal dendritic cell vaccine

Preclinical and clinical studies have demonstrated that for optimal induction of tumor-specific T cells, DC vaccines should exhibit three major qualities: first, an ability to migrate to lymph nodes, which provide an optimal environment for T-cell activation. Second, longevity; the DC must maintain its mature phenotype in the lymph node for sufficient time to activate and expand a tumor-specific T-cell response that is able to eliminate the tumor. Finally, the DC must stably present TAAs; this may be easier to achieve with vector-transduced or transfected DCs than with protein or peptide-loaded DCs.

Generation of dendritic cell vaccines

Since the first trial of DC vaccines for cancer was published in 1996, over 100 clinical studies have been reported, but there is little consensus as to the optimal manufacturing strategy [7,8]. While some groups have used CD34⁺ hematopoietic progenitor cells differentiated into DCs by culture with Flt3-L and other cytokines [9,10], peripheral blood mononuclear cells (PBMCs) are the most common source of cells for vaccine preparation [11]. Since DCs circulate in peripheral blood with low frequency, they are usually differentiated from monocyte precursors that comprise up to 10% of PBMCs. PBMCs are usually obtained by leukapheresis and enriched for monocytes by adherence to plastic or CD14 selection. Differentiation of monocytes into immature DCs is then achieved by culture

with cytokines, most often granulocyte/macrophage colony stimulating factor (GM-CSF) and IL-4. Alternative cytokine cocktails, including GM-CSF/IL-15 [12] and IFN- β /IL-3 [13], have also been used to optimize vaccine preparation, although these are not as well-studied in the clinic. There is no standard protocol for antigen loading, maturation, dosing or route of administration of DC vaccines and no consensus as to the best formulation has emerged. DCs may receive maturation signals from cytokine cocktails, such as GM-CSF, IL-4, IL-1 β , IL-6, TNF- α and prostaglandin (PGE)-2 [14], or from Toll-like receptor (TLR) ligands (pathogen-associated structures that are recognized by receptors of the innate immune response) such as dsRNA or unmethylated cytosine-phosphate-guanosine (CpG) motifs. They are then introduced to the patient in a variety of doses intradermally, intramuscularly, intravenously or intratumorally [11,15]. Most trials have injected multiple doses of DCs ranging from 1×10^5 to 1×10^{10} cells per dose, given at intervals ranging from days to months. Possibly the only undisputed characteristic of DC vaccines is their impeccable safety record; over 1000 patients have received DC vaccines with no reported fatalities.

Loading the dendritic cell: sources of antigen

An effective DC vaccine for malignancy must provide cognate T cells with three 'signals':

- Antigen presented as peptide epitopes on MHC molecules;
- Membrane-bound costimulatory molecules such as CD80 and CD86;
- Soluble costimulatory molecules such as the proinflammatory cytokine IL-12.

Costimulation and cytokine requirements will be discussed below, but to provide the first signal, DCs are loaded with TAA materials as listed in **Table 1**. Antigens may be supplied by pulsing with single peptides or tumor lysates [11,16–19], or by genetic modification with RNA [20,21], DNA [22] or viral vectors expressing tumor antigens [9,23,24]; the choice depends on the knowledge of antigens expressed by the tumor and the availability of tumor tissues or other clinical grade sources of antigen. Malignant melanoma, prostate carcinoma, colorectal carcinoma, breast cancer and multiple myeloma are the most commonly treated malignancies [15], and many TAAs including gp100, tyrosinase, Melan-A/melanoma antigen recognized by T cells (MART)-1 and the melanoma antigen (MAGE) family first identified in melanoma are now being targeted in several types of cancer. Peptides representing immunodominant epitopes of tumor antigens are the most common sources of antigen and are used to pulse DCs before infusion. However, this strategy is limited by the availability of known peptide epitopes and their restriction to individuals with a given HLA type, commonly HLA-A2 or A1 [17,18,25,26]. Furthermore, knowledge of class II peptides has been limited and in the absence of MHC class II-restricted CD4⁺ T cells, the persistence of CD8⁺ effector T cells may be curtailed. Expression of tumor antigens is frequently inconsistent between and within tumors and so loading DCs with epitopes derived from multiple antigenic proteins offers a better chance of recognition and less chance of escape by epitope mutation; Banchereau and colleagues showed an association between the induction of an immune response to two or more epitopes and patient survival in a melanoma trial in which DCs were loaded with multiple tumor-associated peptides [10,16,25]. An alternative strategy to studying individual peptides is to use overlapping peptide libraries (15 mer overlapping by 11 or 20 mer overlapping by 15) that cover entire tumor antigens, which, by including all possible epitopes, removes the problem of HLA restriction and epitope escape [27].

The use of whole antigen can overcome some problems associated with the use of peptide epitopes; for example, DCs loaded with proteins or transfected with RNA or DNA encoding tumor antigens are able to present multiple epitopes without HLA restriction and may be

more likely to induce high-affinity CD4⁺ and CD8⁺ T cells. Clinical and immunological responses to DCs loaded with idiotypic proteins have been observed in B-cell malignancies, such as multiple myeloma and lymphoma; however, tumor antigens must be customized to each patient and purification of idiotypic proteins from each individual patient is labor-intensive [7,28,29]. Use of mRNA to express transgenes in DCs is becoming increasingly common owing to high transfection efficiencies and reduced safety concerns compared with viral transduction methods. A Phase I clinical study in which 13 patients with hormone-refractory, metastatic prostate cancer were vaccinated with prostate-specific antigen mRNA-transfected autologous DCs showed induction of antigen-specific T-cell responses in all patients and complete but transient clearance of circulating tumor cells in three patients [30].

When specific tumor antigens are unknown, DCs loaded with tumor lysates, tumor RNA or whole tumor cells may be an option if tumor cells are available in sufficient quantity [19,31–33]. This approach potentially elicits a polyclonal T-cell response to numerous epitopes in multiple tumor antigens, limiting the chances of immunologic escape. For example, in a Phase I clinical trial, patients with advanced renal cell carcinoma were treated with autologous tumor mRNA-transfected DCs and had enhanced survival and polyclonal tumor-specific CTL responses [21,34]. Tumor cells are typically treated with ultraviolet (UV), heat shock or γ -irradiation to induce apoptosis or necrosis; such stress stimuli promote efficient presentation of tumor antigens on MHC class I by DCs [32,33]. A recent clinical trial in which 18 patients with relapsed non-Hodgkin lymphoma were vaccinated with autologous tumor-loaded DCs achieved significant clinical responses (three complete response, three partial response and eight stable disease) associated with a reduced frequency of Tregs and increased frequencies of cytotoxic natural killer (NK) cells and IFN- γ secreting, tumor-specific CTLs [32]. Another strategy to facilitate presentation of the entire repertoire of tumor antigens is fusion of DCs with autologous tumor cells by chemical or electrical means. Treatment of stage IV renal cell carcinoma patients with a DC–tumor fusion vaccine resulted in two partial clinical responses and eight disease stabilizations among 20 evaluable patients [35]; several clinical trials are ongoing [201,202]. If tumor material is limiting, autologous tumor-derived RNA can be amplified from a small amount of primary tumor sample for DC transfection.

A potential problem with protein loading is the half-life with which antigens are presented on the DC surface; for example, the half-life of MHC class I peptides on the surface of mature DCs ranges from approximately 9 to 119 h [36]. Therefore, antigens have been expressed from DNA plasmids and viral vectors that, by continuous gene expression, can maintain antigen expression for the life of the DC. Gene delivery into DCs can be accomplished using a range of vectors including fowlpox, vaccinia, lentivirus and adenovirus [9,23,24,37–40], which may also provide potent DC maturation signals via TLRs. There are safety concerns associated with the use of viral vectors including genomic integration (lentivirus) and replication competence (vaccinia), as well as immunogenicity, particularly in the presence of pre-existing immunity (vaccinia and adenovirus) that may result in immune elimination of the DCs or competition for HLA molecules by strong viral antigens. However, these problems may be outweighed by data indicating that viral vectors can express their product in DCs for as long as 3 weeks [24]; furthermore, viral vectors offer the advantages of codon optimization or proteasome targeting for more efficient protein expression [41]. All these strategies utilize the unparalleled ability of DCs to phagocytose, process and present antigens on both MHC class II for CD4⁺ T-cell help and MHC class I for priming of CD8⁺ T cells.

Maturation, migration & longevity

Maturation

Dendritic cell maturation, characterized phenotypically by high expression of membrane-bound costimulatory molecules such as CD80, CD86, CD83 and MHC class II molecules, as well as by extended half-lives of peptide-MHC complexes and secretion of T-cell stimulating chemokines and cytokines [36], is a complex process that occurs in response to proinflammatory cytokines and pathogen-associated molecular patterns (PAMPs) associated with microbial structures or cellular stress. PAMPs are recognized by pattern recognition receptors (PRRs) including the TLRs; differential activation of these receptors on DCs determines the pattern of cytokines secreted, which in turn dictates the class of T-cell response produced [42]. For example, Th2-polarized responses are driven by IL-4- and IL-10-producing DCs, while inducible Treg responses, which suppress T-cell immunity, are driven by DCs expressing IL-10 and TGF- β . Conversely, stimuli such as TNF- α or bacterial lipopolysaccharide lead DCs to direct a Th1-polarized response driven by IL-12 production and dominated by IFN- γ -producing CD8⁺ CTLs [43,44], which recognize and kill aberrant cells in an antigen-specific manner. Hence, DC activity may induce tolerance rather than immunity if the DC is not appropriately matured. Immature DCs are highly phagocytic and are retained in tissues in a relative resting state in which, presenting only healthy 'self' proteins, they are tolerogenic. Subcutaneous injection of immature DCs was found to elicit antigen-specific suppressive responses [45]; therefore, it is critical that the DC vaccine is appropriately stimulated to produce membrane-bound and soluble costimulatory molecules.

Migration

Upon maturation with stimuli such as TLR ligands or cytokine cocktails, DCs rapidly down-regulate expression of chemokine receptors such as CCR2 and CCR6, which direct them to sites of inflammation, and upregulate expression of CCR7 [2,46], which mediates migration of mature DCs into the lymph nodes via its ligands CCL19 and CCL21 [47]. Here, mature, antigen-presenting DCs encounter resting naive and memory T cells that also express CCR7 and circulate through lymph nodes, where they colocalize with CCL19-expressing mature DCs. These interactions result in antigen-specific T-cell proliferation and initiation of the adaptive immune response. Cellular migration is critical to the establishment of immunity, as indicated by mouse models in which DC or T-cell migration is perturbed [1,48]. Both preclinical and clinical studies have shown that the magnitude of the T-cell response correlates with the ability of DCs to migrate to lymph nodes and thus with their maturation state [49–51]. In agreement with this observation, melanoma patients with higher numbers of mature lysosomal associated membrane protein (LAMP)-positive DCs in their lymph nodes as measured by immunohistochemical analysis exhibited increased survival [52]. Recently, a novel strategy was described in which DCs expressing the chemokine CCL21 from an adenovirus vector created functioning, ectopic, lymph node-like structures that enhanced host antitumor immunity and vaccine efficiency [53].

The route of administration may be critical to the success of DC vaccination, although there is no consensus as to which route is most effective. Clinical trials have evaluated intranodal, intradermal, subcutaneous, intravenous and intratumoral administration; some of these studies used multiple routes of administration in sequential or comparative formats [29,54]. It was shown in a recent clinical trial that less than 5% of ¹¹¹In-labeled DCs administered intradermally migrated to the draining lymph nodes, while large numbers remained at the injection site and were taken up by CD163⁺ macrophages, effectively reducing the vaccine dose by over one log [50]. However, this study also indicated that even low numbers of DCs (<5 × 10⁵) in the lymph nodes were able to potentially induce a specific immune response.

Longevity

While many studies give detailed information regarding the *in vitro* characteristics of vaccines, including maturation, phenotype and cytokine profile, the *in vivo* phenotype and function of the DCs after infusion are rarely known, and so the surrogate readout of vaccine efficacy is post-vaccine T-cell activity (enzyme-linked immunospot [ELISPOT], delayed-type hypersensitivity response and cytotoxicity, among others) and clinical responses. The persistence of DCs in a mature state after infusion cannot be assumed, since in the absence of maturation stimuli at the injection site, DCs may dedifferentiate and/or die, and if robustly stimulated *in vitro* they may rapidly become exhausted [55]. Analysis of the location and number of viable DCs at various time points following vaccination would aid the design of more effective vaccines, but as yet good imaging strategies for DC migration in humans are lacking.

Genetic modification to improve dendritic cell vaccines

While T-cell responses to DC vaccines have been reported in approximately half of the published clinical trials, objective tumor response rates remain low. Increased efficacy has come with increased understanding of the complexities associated with the balance between immunity and tolerance and the characteristics required by effective DCs. However, the development of strategies to ensure these characteristics are retained after infusion remains challenging. Studies in mice have demonstrated that even after antigen loading and maturation, auto logous wild-type DCs are ineffective at inducing a T-cell response strong enough to completely eliminate established tumors [56]. To combat these issues, investigators have genetically modified DCs in multiple ways to enhance their function and efficacy (**Figure 1**). These strategies fall broadly into two categories: overexpression of positive regulators and inhibition of negative regulators.

Overexpression of surface stimulatory molecules

Multiple signaling pathways are critical to the induction of a mature, stimulatory DC and an optimal T-cell response. Membrane-bound costimulatory molecules are some of the most important products of mature DCs and have been overexpressed using a variety of viral and nonviral vectors in both preclinical and clinical trials [24,39]. For example, DCs expressing the tumor antigen carcinoembryonic antigen (CEA) together with the three costimulatory molecules CD80, CD54 and CD58 from a fowlpox vector were used to vaccinate 14 cancer patients, leading to increased CEA-specific T cells in ten patients and one partial remission [24]. Ligation of CD40 on DCs with CD40L on antigen-specific Th cells is known to enhance DC expression of costimulatory molecules, cytokines and chemokines. To mimic this interaction, DCs have been modified by adenoviral transduction [40] or mRNA electroporation [57] to express CD40L. In order to better control the CD40 pathway, Spencer and colleagues engineered a CD40 construct whose activity is induced upon addition of chemical inducer of dimerization (CID) AP1903 [58]. Inducible CD40-expressing DCs activated with TLR4 ligands effectively primed antigen-specific Th1 antitumor responses and, after addition of the CID, exhibited enhanced migration both *in vitro* and *in vivo* [58]. This strategy is currently in clinical trials using prostate-specific antigen as an antigen in patients with prostate cancer.

Other costimulatory molecules that have interested researchers include GITR-L, 4-1BBL, CD70 and OX40L [57,59–62]. Vaccination with a combination of DCs expressing tumor antigen RNA and secreting anti-GITR-mAbs enhanced tumor-specific T-cell immunity and resulted in sustained long-term memory T-cell responses in mice [59]. Transgenic expression of CD70 on murine DCs was enough to break CD8⁺ tolerance and establish protective immunity to tumor challenge or virus infection [60,61]. Electroporation with

mRNAs encoding a combination of CD70, CD40L and a constitutively active TLR4 generated mature, cytokine and chemokine-producing human DCs endowed with enhanced T-cell stimulatory capacity [57,63]. Thus, sustained expression of one or multiple costimulatory molecules can enhance DC stimulation and the resulting T-cell response.

Overexpression of soluble stimulatory molecules

Enforced expression of soluble mediators such as cytokines and chemokines also increases the efficacy of DC vaccination. Local production of cytokines such as IL-12 by DCs may avoid the toxicity associated with systemic administration of inflammatory cytokines [64]. Overexpression of IL-12 in DCs enhanced migration, maturation and antitumor activity in a variety of preclinical models [65]. Intratumoral vaccination of mice with DCs engineered to express IL-12 and IL-18 from an adenovirus vector increased clearance of both local and distant established tumors, compared with Ad-null-transduced DCs [66], and in combination with stimulatory molecules such as CD80 or GM-CSF, IL-12 enhanced the efficacy of DC vaccines for murine prostate and colon cancer [67]. As discussed above, DCs modified to express chemokines that attract inflammatory cells, including T cells and endogenous DCs, produced ectopic lymphoid structures [53], as well as significant antitumor effects in preclinical models [53,68,69]. Other investigators have coexpressed activating receptor–ligand pairs, such as receptor activator for NF- κ B (RANK)–RANK ligand (RANKL) [70] and IL-15–IL-15R α [71] in DCs for autocrine signaling. Thus, increasing the potency of DCs by expression of costimulatory molecules or secretion of immunostimulatory cytokines can increase the potency of T-cell responses and enhance the elimination of model tumors, but these results have yet to be translated into the clinic.

Enhancement of dendritic cell longevity

Dendritic cell longevity is crucial to the efficacy of the vaccine; therefore, genetic modifications that upregulate antiapoptotic signaling or reduce proapoptotic signaling have been investigated. The PI3K/Akt1 pathway regulates multiple critical cellular activities including growth, survival and cytokine secretion. To enhance the survival of DCs for tumor immunotherapy in preclinical models, Akt was genetically modified for constitutive signaling and further enhanced by targeting to lipid rafts [72]. Activation of DCs containing lipid raft-targeted constitutive Akt1 promoted Bcl-2-dependent DC survival, which in turn enhanced efficacy in multiple therapeutic tumor models [72]. Bcl-2 family members, known to control DC survival [73,74], have also been directly modulated to improve vaccine efficacy. Small interfering RNA (siRNA) targeting of the proapoptotic molecules Bax, Bak, and Bim was shown to enhance DC survival and antitumor effects in murine models [75,76].

‘Inhibiting the inhibitor’ for enhanced vaccine efficacy

While enhancement of positive regulators represents a promising approach to enhancing vaccine activity, DCs remain susceptible to endogenous inhibitors which, under normal circumstances, serve as checkpoints to help maintain tolerance and prevent autoimmunity. Since a major goal of DC vaccines for cancer is to break self-tolerance to tumor antigens, these negative regulators are a key obstacle. Several types of inhibitors exist including those expressed in the cytoplasm (e.g., suppressor of cytokine signaling [SOCS]-1 and A20), those expressed on the cell surface (inhibitory receptors, e.g., PD-L1) and those secreted into extracellular spaces (soluble inhibitors, e.g., indoleamine-2,3-dioxygenase [IDO]).

Cytoplasmic inhibitors

We and others have shown that downregulation of negative regulators of DC maturation can enhance and prolong their immunostimulatory characteristics, resulting in enhanced T-cell

immunity and tumor elimination. SOCS1 functions as a negative regulator of cytokine secretion through inhibition of the JAK/STAT pathway. Silencing SOCS1 in mouse bone marrow-derived DCs (BMDCs) led to enhanced antigen-specific T-cell responses and regression of established murine tumors; these effects were mediated by enhanced IL-12 production [77,78]. In agreement with the murine studies, human monocyte-derived DCs in which SOCS1 was silenced exhibited enhanced priming of antigen-specific T-cell responses *in vitro* and in humanized mouse models [79]. The zinc-finger protein A20 (TNFAIP3) is a ubiquitin-modifying protein expressed at high levels in lymphoid organs, including the thymus and spleen. It functions as a negative regulator downstream of TLR and TNF receptor (TNFR) signaling pathways and hence is an inhibitor of NF- κ B signaling. siRNA targeting of A20 in mouse BMDCs led to increased production of proinflammatory cytokines such as TNF- α , IL-12p40 and IL-6, inhibition of regulatory T cells and enhanced T-cell-mediated antigen-specific antitumor activity [56]. Knockdown of A20 in human monocyte-derived DCs similarly enhanced levels of NF- κ B and activator protein (AP)-1 transcription factors, skewed naive CD4⁺ T cells toward a Th1 phenotype and more potently activated tumor antigen-specific CD8⁺ T cells [80].

In an effort to enhance multiple critical functions of DCs, we targeted IL-1 receptor-associated kinase M (IRAK-M), a negative regulator of TLR signaling upstream of both the NF- κ B and mitogen-activated protein kinase pathways that control cytokine secretion, longevity and migration in DCs. IRAK-M exhibits highly restricted expression in monocytes and macrophages [81,82] and its loss is associated with hyperactivation of the innate immune system. Altered expression of IRAK-M is associated with conditions such as sepsis and cirrhosis [83–85]. We have recently shown that BMDCs derived from *IRAK-M*^{-/-} mice exhibit enhanced migration to lymph nodes, Th1-skewed cytokine secretion and increased longevity; furthermore, vaccination of tumor-bearing mice with antigen-pulsed, lipopolysaccharide-matured *IRAK-M*^{-/-} DCs led to a significant reduction in tumor growth and enhanced survival compared with control vaccination with wild-type DCs [86]. However, these modifications are also untested in clinical trials.

Inhibitory receptors

Inhibitory receptors are another potential target for enhancement of DC vaccine efficacy. DCs express a wide range of receptors such as scavenger receptors, C-type lectin receptors (CLRs) and immunoglobulin superfamily members, some of which function as inhibitors. Scavenger receptors mediate binding, phagocytosis and clearance of foreign particles, such as bacteria and inhaled allergens [87,88]. CLRs are a diverse family of proteins recognizing defined carbohydrate structures; ligands vary widely from extracellular matrix proteins to pathogen-associated structures [89,90]. The immunoglobulin superfamily members recognize ubiquitously expressed endogenous molecules such as inter-cellular adhesion molecules or MHC I that may be involved in activation or tolerance.

Inhibitory receptors are often involved in the development of tolerance, maintenance of homeostasis and downregulation of the immune response. Inhibition by these receptors is generally mediated through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic regions, which function by recruitment of inhibitory protein tyrosine phosphatases, such as SHP-1 and SHP-2. There are more ITIM-containing co-inhibitors than coactivators in the immunoglobulin super-family, while the C-type lectin family includes just three inhibitory receptors [89–91].

These inhibitory receptors may be useful targets for therapeutic intervention; some are listed in **Table 2**. For example, several clinical trials evaluating the safety and efficacy of PD-1 blockade are ongoing; some of these trials combine blocking monoclonal antibodies with

DC vaccination [203–205]. In addition, the newly-defined macrophage receptor with collagenous structure (MARCO) is a class A scavenger receptor expressed on certain subsets of macrophages and DCs whose deficiency was recently shown to mediate enhanced migration of pulmonary DCs in an ovalbumin (OVA)-asthma model [86]. MARCO-silenced DCs exhibited enhanced p38-dependent migration *in vitro* and *in vivo* with no apparent alteration of cytokine secretion; furthermore, therapeutic vaccination with MARCO-silenced DCs controlled the growth of established B16 tumors [92].

Alternative methods to exploit DCs for cancer therapy

In vivo targeting

The production of sufficient DCs for vaccine preparation from cancer patients can be challenging; furthermore, physiologic DC maturation *in vivo* is a kinetic process involving a temporally and spatially regulated series of steps that may not be recapitulated *in vitro* where experimental manipulation may lead to inappropriate maturation, decreased longevity and unwanted polarization. Targeting DCs *in vivo* with antigens and adjuvants may simplify vaccination and produce more physiological DC maturation. Because of their capacity for antigen uptake and because their expression is restricted to DCs, CLRs are being exploited to target DCs *in vivo*. Triggering CLRs on DCs using CLR antibodies linked to antigen leads to rapid internalization followed by entry of the payload into the antigen processing pathway; such targeting enhances antigen presentation several hundredfold and allows generation of antigen-specific DCs *in vivo* [93–95]. CLR-specific antibodies covalently coupled to antigens, such as DEC-205–OVA, have been shown to mediate enhanced specific immune responses and antitumor immunity when administered with adjuvants in murine models [96,97]. In a similar strategy, lentiviral vectors expressing antigen and targeted to DC-specific intercellular adhesion molecule-3-grabbing nonintegrin efficiently transduced (SIGN) DCs *in vitro* and *in vivo* injection of the vector induced antigen-specific T cells and tumor regressions [37]. Thus, *in vivo* targeting of DCs may enhance potency while simplifying vaccine preparation.

Combination therapies

As described previously, multiple strategies exist for enhancing positive regulatory pathways, attenuating inhibitors and *in vivo* targeting to improve the immunostimulatory power of DC vaccines; however, clinical studies to date suggest that even the most potent DC may be unable to eradicate human tumors. The solution may lie in combining genetically modified DCs with additional treatments, such as antibodies, immune modulating drugs, chemo/radiation therapy or adoptive transfer of T cells [20,98–104]; a few of these combinations are discussed below.

Many vaccines induce powerful T-cell responses, but a major block in vaccine efficacy may lie downstream of T-cell activation. Multiple factors produced in the immunosuppressive tumor microenvironment or by DCs themselves can directly inhibit effector T-cell activity or promote Treg responses. Examples include enzymes such as COX-2 and IDO, and cytokines such as TGF- β and IL-10 [101–103,105]. COX-2 directly suppresses DC function by decreasing IL-12 and increasing IL-10 production as well as by enhancing tumor metastasis and resistance to apoptosis; these effects are reversed when COX-2 is inhibited [103]. Inhibition of COX-2 augmented the effects of a TAA-expressing adenoviral vaccine, producing increased tumor infiltration by antigen-specific CD8⁺ T cells, reduced serum and tumor levels of PGE-2, and enhanced survival of mice bearing large, established tumors [101]. The tryptophan-modifying enzyme IDO is expressed by tumor cells and IDO-competent DCs, and its expression leads to conversion and enhancement of local Treg populations [105]. These Tregs, in turn, produce inhibitory cytokines and directly inhibit

immunity, compounding the immunosuppressive environment of the tumor and tumor-draining lymph nodes. It was recently shown that blocking IDO promoted reprogramming of Tregs towards proinflammatory Th17-like cells in an IL-6-dependent manner; combination of IDO blockade with a tumor vaccine led to enhanced conversion of Tregs *in vivo* and decreased growth of established tumors [102]. Blocking soluble factors produced by the tumor and regulatory cells, therefore, can enhance vaccine efficacy and promote antitumor immunity.

Cytotoxic T lymphocyte-associated 4 (CTLA-4) is an inhibitory receptor expressed by T cells that competes with the costimulatory molecules CD80/86 on activated antigen-presenting cells, resulting in decreased IL-2 production and cell cycle arrest in G1 [106]. CTLA-4 blockade with a monoclonal antibody (ipilimumab) has been used to treat several types of tumors, resulting in significant autoimmunity but also durable, objective clinical responses [107,108]; furthermore, preclinical studies have indicated that therapeutic vaccination can synergize with CTLA-4 blockade [109,110]. Similarly, blockade of the inhibitory PD-L1–PD-1 pathway combined with DC vaccination and adoptive transfer of antigen-specific T cells achieved durable B16 tumor regressions in mice [98]; in addition, PD-1 inhibition in conjunction with DC immunization is being explored in current clinical trials [203,204].

Blockade of suppressive factors produced by Tregs may be a useful approach; alternatively, Tregs have been directly depleted by various strategies including the use of denileukin diftotox (DAB₃₈₉IL-2 or Ontak®, Eisai Pharmaceuticals, NJ, USA). This targeted immuno toxin is a fusion between the active domain of diphtheria toxin and IL-2; therefore, it binds selectively to cells highly expressing the IL-2 receptor CD25 subunit and mediates their death. Selective depletion of Tregs (marked by CD25^{high}) without toxicity towards other subsets has been shown *in vitro* and *in vivo*; furthermore, treatment of patients with denileukin diftotox enhanced the efficacy of DC vaccination when compared with vaccination alone [20,104]. Effects of these combination treatments appear to be highly dose- and time-sensitive, but depletion of CD4⁺CD25^{high} Foxp3⁺ Tregs and concurrent increases in tumor antigen-specific T-cell activity following immunization have been reported.

Conclusion & future perspective

Why do human clinical trials fail to reproduce the efficacy found in murine models? Most animal models of vaccine therapy depend on transplantable tumors. By contrast, spontaneous human tumors may develop in the host for months or years prior to detection, during which time they build a complex network of immunosuppressive stroma and infiltrating cells. The B16 melanoma model is considered a rigorous model for murine vaccine studies, since it is aggressive, expresses weak tumor antigens derived from endogenous proteins and uses a range of immuno suppressive mechanisms. However, in this model, even the best vaccines are rarely effective if administered after 5–7 days, likely preceding the establishment of inhibitory cell types. Spontaneous animal models are more difficult to study owing to variable penetrance, time to progression and continued development of new tumors, but these may be more representative of the human condition [111,112]. Investigators must realize the practical limitations of animal models that provide a useful tool, but will not replace rigorous clinical testing as, ultimately, only studies in humans will provide information on clinical efficacy.

While most clinical trials achieve T-cell immune responses to tumor antigens, these rarely produce significant tumor regression. The presence of TAA-specific T cells in the peripheral blood does not necessarily reflect their presence, frequency or function within the tumor,

since T cells may fail to migrate to tumor sites and are often inhibited in the tumor microenvironment. The many possible modifications that have proved beneficial in murine models require testing in clinical trials. Since the immunologic and clinical efficacy of vaccines can be evaluated in small numbers of patients if response rates are high, these modifications should be tested in small academically funded trials that do not require the massive infusion of money required for industry initiated trials.

Patient selection has emerged as a crucial determinant of vaccine efficacy: the majority of patients in DC vaccine trials have had late-stage malignancies; often patients with a large tumor burden who have received multiple rounds of chemotherapy, radiation and surgery. In a result ant immuno suppressed state, patients may be unable to mount an effective response following vaccination [113]. Immunotherapy may be more successful when applied to patients with a low disease burden who have an active immune system; in agreement, some clinical trials have observed more complete responses and partial responses in patients with the smallest tumor burden [32,33]. Successful immuno-therapy should prevent disease metastasis and late recurrence.

The US FDA announced in 2009 that concepts of clinical trials of anticancer vaccines and conventional cytotoxic agents may differ [206]. For Phase I clinical trials, safety is the primary end point; this is especially important for administration of nonspecific cytotoxic chemotherapeutic agents that can produce severe short- and long-term toxicities and greatly decreased quality of life for patients. DC vaccines, by contrast, have proven safe in over 100 clinical studies with a maximum tolerated dose not yet reached. Therefore, standard trial design may be inappropriate for DC therapies and alternative end points must be established. Immune responses are good secondary end points, but emerging results suggest that many patients who do not achieve complete remission may have increased long-term survival with disease [114], an outcome that is not predicted by tumor responses at earlier time points. Furthermore, the enhanced quality of life associated with immunotherapies should be given more weight in comparisons with c hemotherapies, which frequently prolong a miserable existence.

We speculate that in the future, DC vaccines will not be a sole mode of therapy, but a weapon in a complementary arsenal. Effective therapies may require combinations of genetically modified DCs with additional treatments such as modulation of angiogenic pathways, depletion of soluble inhibitors, blockade of regulatory cells or adoptive transfer of T cells, themselves genetically modified to resist T-cell-directed tumor immune evasion strategies [115].

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Executive summary

The ideal vaccine

- The ideal dendritic cell (DC) vaccine should be mature, migratory, long-lived and be able to stably present tumor-associated antigen(s) in order to initiate and maintain an effective adaptive immune response.

Generation of the vaccine

- DCs differentiated from patient peripheral blood mononuclear cells are most commonly used in the clinic, but there is no standard protocol for antigen loading, maturation, dosing or administration of DC vaccines. Investigators should strive to develop a common method for vaccine generation so trials can be more comparative.
- DC vaccines have proved to be safe and nontoxic in over 1000 patients.

Loading the dendritic cells: single peptides to whole-tumor cells

- The more effective vaccines are those that elicit CD4 and CD8 T-cell responses to multiple antigens.
- The goals in loading DC vaccines should be enhanced antigen stability and broadened repertoire T-cell response.

Maturation, migration & longevity

- CCR7-dependent DC migration is necessary for initiation of the adaptive immune response. Since only mature DCs express CCR7, this implies that DCs used for vaccination purposes should be mature.
- Enhanced DC longevity leads to increased T-cell responses, so a long-lived, mature DC is critical. ■ Few studies that directly address the *in vivo* fate of the vaccine product following infusion have been performed; surrogate readouts, such as postvaccine T-cell activity (by enzyme-linked immunospot, delayed-type hypersensitivity response and cytotoxicity) and clinical responses are used. Analyses including location and number of viable DCs at various time points following vaccination would be informative to the design and administration of more effective vaccines, but imaging strategies are lacking.

Genetic modification to improve dendritic cell vaccines

- Genetic modification to improve DC vaccines falls broadly into two categories: overexpression of positive regulators and inhibition of negative regulators.
- Overexpression of cytokines, chemokines and costimulatory molecules have enhanced DC vaccine efficacy in preclinical and clinical studies.
- Removal of negative regulators such as cytoplasmic inhibitors (e.g., suppressor of cytokine signaling-1 and A20), inhibitory receptors (programmed death ligand 1) and those secreted into extracellular spaces can also improve DC vaccines.

Alternative methods to exploit dendritic cells for cancer therapy

- *In vivo* targeting of DCs may simplify vaccine production by generating antigen-specific DCs under more physiological conditions without isolating the cells.

Conclusion

- DCs are the most potent antigen-presenting cells in the body and their use as cancer vaccines is greatly increasing.

■ DC vaccines have elicited tumor-specific immunity in many patients as indicated by immunological measures of T-cell specificity and function; however, objective clinical response rates remain low.

■ In order to truly exploit the strengths of genetically modified DCs, vaccines should be combined with other strategies including inhibition of regulatory T cells, modulation of inflammatory pathways or adoptive transfer of T cells.

Figure 1. Genetic modification of dendritic cells can lead to the enhancement of immunogenicity, migration, longevity, cytokine secretion or a combination of these, thus yielding a more effective vaccine.

Table 1

Possible antigen sources for dendritic cell vaccination: their advantages and disadvantages.

Antigen source	Advantages	Disadvantages	Ref.
Tumor-associated peptide(s)	Fast, simple and inexpensive	HLA-restricted, limited by knowledge of epitopes, produce limited T-cell repertoire, short-term expression	[10,17,18,26,54]
Whole antigens from DNA or RNA	Not HLA-restricted, broader antigen repertoire, expansion of both CD8 ⁺ and CD4 ⁺ T cells, longer lasting presentation of antigen(s), small amount of material can be easily amplified (PCR), possibility of targeting antigen to MHC class I and class II	Toxicity to APCs associated with transfection	[21,22,30,34,57]
Whole antigen–protein	Not HLA-restricted, broader antigen repertoire, expansion of both CD8 ⁺ and CD4 ⁺ T cells	Toxicity to APCs associated with transfection, short-term expression	[116–118]
Idiotypic protein	Not HLA-restricted, broader antigen repertoire, expansion of both CD8 ⁺ and CD4 ⁺ T cells	Labor-intensive, requires access to tumor material and patient-specific antigen generation, limited to B-cell malignancies	[28,29]
Tumor lysate/whole tumor cells	Not HLA-restricted, broad antigen repertoire, expansion of both CD8 ⁺ and CD4 ⁺ T cells	Presentation of unknown (possibly self) antigens, may require access to tumor material and patient-specific antigen generation	[19,31,32,33,35]
Viral vector-delivered whole antigen	Not HLA-restricted, broad antigen repertoire, expansion of both CD8 ⁺ and CD4 ⁺ T cells, longest-lasting presentation of antigen(s), possibility of targeting antigen to MHC class I and class II	Possible immunity to viral vector/residual viral gene products, safety/regulatory limitations	[9,23,24,37–40]

APC: Antigen-presenting cell.

Table 2

Known and putative targets for enhancement of dendritic cell vaccine efficacy: receptors acting as inhibitors in dendritic cells.

Receptor (dendritic cells)	Ligand (T cells)	Effects on dendritic cells and dendritic cell vaccines	ref.
Immunoglobulin superfamily			
DIgR2	Unknown	DIgR2-silenced mBMDs: enhanced Ag-specific CD4 ⁺ and CD8 ⁺ T-cell responses <i>in vitro</i> and <i>in vivo</i> , protected tumor-bearing mice from challenge in prophylactic EG7-OVA model	[119]
ILT-3	Class I MHC antigens	ILT-3-silenced DCs: ↑ cytokines (IL-1α, IL-1β, IL-6), ↑ chemokines (CXCL10, CXCL11), increased T-cell proliferation and NF-κB/MAPK activity;	[105,120,121]
ILT-4	HLA-A, B, C and G	ILT-3/ILT-4 expression induces differentiation of CD8 ⁺ CD28 ⁻ T(s) cells and T-cell anergy, both highly expressed on tolerogenic, Treg-inducing DCs	[105,120]
PIR-B	MHC class I	Regulates CTL activity by blocking CD8–MHC class I binding; <i>Pirb</i> ^{-/-} DCs: increased T-cell activity, graft rejection, tumor suppression and chemotaxis and decreased maturation	[122,123]
PD-L1	PD-1	Suppresses T-cell proliferation and cytokine production; blocking PD-L1 in human or mouse DCs leads to enhanced Ag-specific T-cell activity and antitumor activity	[124–127]
SIRP-1α/SHPS-1	CD47	Inhibits migration of LCs <i>in vitro</i> and <i>in vivo</i> , no effect on survival; SIRP-1α ligation on DCs inhibits maturation and IL-12 production; CD47 ligation on T cells inhibits IL-12/Th1 responsiveness and promotes anergy	[128,129]
C-type lectins			
BDCA2	Ags?	Defining marker for pDCs; BDCA2 ligation inhibits production of type-I IFNs, IL-6 and TNF-α	[89,91]
DCIR	Lectins	<i>Dcir</i> ^{-/-} mouse: significant DC expansion and susceptibility to autoimmunity (collagen-induced arthritis and enthesitis); DCIR triggering in human pDCs inhibited TLR9-mediated IFN-α production	[90,130]
DCAL-2	Endogenous ligand(s)?	Suppresses TLR-matured DC cytokine production and T-cell stimulation but enhances CD40-matured DCs. May function differently depending on DC stimulation	[131,132]
Scavenger/other receptors			
MARCO	Bacterial proteins	Enhanced migration <i>in vitro</i> and <i>in vivo</i> , no apparent alteration of cytokine secretion, therapeutic vaccination with MARCO-silenced DCs controlled growth of established B16 tumors	[87,92]
CR3	iC3b	CR3-ligated BMDCs or hDCs: decreased inflammatory cytokines (IL-1β, IL-6, IL-12 and TNF-α), decreased ability to prime Th1 or Th2 responses	[133,134]

↑ : Upregulates; Ag: Antigen; BMDC: Bone marrow-derived dendritic cells; CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; DCAL: Dendritic cell-associated C-type lectin; DCIR: Dendritic cell immunoreceptor; hDC: Human dendritic cell; HLA: Human leukocyte antigen; IFN: Interferon; ILT: Immunoglobulin-like transcript; LC: Langerhans cell; MARCO: Macrophage receptor with collagenous structure; MAPK: Mitogen-activated protein kinase; OVA: Ovalbumin; PIR-B: Paired immunoglobulin-like receptor B; pDC: Plasmacytoid dendritic cell; PD-L: Programmed death ligand; SHPS: SH2-domain bearing protein tyrosine phosphatase substrate-1; SIRP: Signal regulatory protein; Th: Helper T cell; TLR: Toll-like receptor; TNF: Tumor necrosis factor; Treg: Regulatory T cell.